

SOME CONTRIBUTIONS TO THE BACTERIOLOGY

AND EPIDEMIOLOGY OF

ENTERIC AND BRUCELLA INFECTIONS

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(The sections indicated * include the results
of personal observations)

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One of the most frequent duties of the bacteriologist is to assist the clinician in the diagnosis of febrile conditions of obscure origin. In this country two of the fevers uppermost in his mind are those due to the organisms of the enteric group and undulant fever due to Brucella abortus. When the diagnosis has been made, two questions must arise: how the patient acquired his infection and what might have been done to prevent it. The public health bacteriologist, whose interests now tend increasingly to extend beyond the laboratory to the wider fields of epidemiology and preventive medicine, must play an important rôle, as an equal partner with the medical officer of health, in finding the answer to these questions. This partnership was fostered by the war and is now well established. It has been strengthened by the organization of the Public Health Laboratory Service which provided the opportunities for many of the present observations, first in an extensive rural area and later in a reference laboratory for brucella infections.

In the first part of this thesis the advances that have been made in the investigation of enteric fever during the last ten or fifteen years are reviewed and the results of some personal inquiries are described. The second part comprises a discussion of some aspects of the epidemiology of undulant fever, based on the examination of about 400 strains of brucella recently isolated in this country and elsewhere.

I THE ENTERIC FEVERS

A. THE ORGANISM

It is proposed to confine attention to Salm. typhi and Salm.paratyphi B, the two organisms responsible for enteric fever in this country.

1. Salmonella typhi

During the period between the beginning of the century and the outbreak of the 1939-45 war, knowledge of the characters of the typhoid bacillus had become established, it was thought, so far as practical diagnostic procedures were concerned. Improvements in immunization technique were hoped for, but the lines of advance were not yet defined. The typhoid bacillus was known as a flagellated Gram-negative bacillus with a fairly characteristic appearance on certain differential culture media, which could be primarily identified by its ability to ferment a range of carbohydrates. Final identification was made by serological means. The significance of the earlier discovery of the flagellar (H) and somatic (O) antigens, and of the importance of distinguishing them, and the antibodies to which they give rise, was appreciated in diagnostic and other work.

It soon became apparent that there was no good reason for classifying the typhoid bacillus in a different genus from the other salmonellae; it

happened to be one of the few species that did not produce gas in the sugars it fermented, but its general characters and antigenic constitution were closely similar. The name Salmonella typhi began to replace the more generally used terms such as Bacterium typhosum in this country (more slowly perhaps than in America) and the typhoid bacillus figured largely in the extensive researches which revealed a vast amount of information about the antigens of the salmonellae and the relations between the different members of the group. This work is of course associated with the names of Bruce White in this country and Kauffmann in Copenhagen. It resulted in the recognition of the schema bearing the names of these two workers, in which the now very large number of salmonellae are divided on the basis of their heat-stable somatic antigens into nine main O groups, and are further characterized by their heat-labile flagellar antigens. It is not proposed at this time to discuss in detail this fundamental and now well-known work, which included the development of the researches of Andrewes (1925) and of Kauffmann and Mitsui (1930) who showed that certain of the salmonellae have diphasic H antigens; that is, the individual bacteria may have one or other of two alternate forms of antigenic make-up, constituting Phases 1 and 2. The fact that the antigens of one of these phases (usually Phase 2) occur in

common throughout many of the diphasic species accounts for some of the serological cross-reactions between these organisms, a relationship which was puzzling before the details were discovered.

The typhoid bacillus is a monophasic organism whose antigenic structure at the time referred to was usually given as IX. XII : d :- . Loss, on prolonged subculture or in other ways, of the smooth somatic antigen exposes the rough (R) antigen, a change associated with instability of suspensions in saline, with loss of serological specificity, change in colonial morphology and loss of virulence. Further loss of somatic antigens exposes, as Bruce White showed, other antigens $p_1 p_2$, and T - but it is not believed that these are of practical significance.

Later Knowledge

1. Antigens

Two observations of relatively little importance may first be mentioned. Kauffmann (1941), who had previously described variants in which somatic antigen I was lost, discovered that antigen XII consists of three components, XII, XII₁ and XII₃, and that antigen XII₂ was liable to be lost in certain strains, a loss that would only be detectable by means of an absorbed "pure" XII₂ anti-serum. One importance of this finding is the possibility that this serological loss would indicate

immunological deficiency and preclude the use of such strains in vaccine preparation. Another point has been made by Hayes (1947) who found in India that there were stable antigenic types possessing different combinations of these XII antigens, and that the use in agglutination tests of suspensions not carefully selected for their content of these antigens might give vague end-points, since only those organisms possessing the antigen corresponding to the antibody in the patient's serum would be agglutinated. Further, the response of man and the rabbit to antigen XII₂ is different and human sera should be used for selecting strains with which to prepare suspensions.

The other minor observation was also made by Kauffmann (1936c) who was experimenting with the technique of exposing hidden antigenic phases of salmonellae by growth in the presence of anti-serum designed to suppress the existing phase. In this way Kauffmann suppressed the H antigen of the typhoid bacillus by growth in münchen antiserum and brought to light a hitherto unsuspected antigen j, a finding the interest of which is that it is part of the evidence accumulating in support of Bruce White's suggestion that the salmonella species are genetically descended from a common ancestor and derived by a process of antigenic loss.

By far the most significant observations on the typhoid bacillus in recent times are concerned with the discovery of the Vi antigen by Felix and Pitt (1934 a and b) and the developments which followed this discovery. These workers described smooth, recently isolated strains of Salm.typhi which were not agglutinable by O antiserum; these strains were of exceptional mouse-virulence compared with O-agglutinable strains. Treatment with heat (boiling for 5 minutes, or 60°C. for half an hour) rendered the organisms O-agglutinable. The insensitivity of the organisms to O antibodies was found to be due to the presence of an additional antigen, named the Vi antigen. Rabbits immunized with living organisms produced Vi antibodies which agglutinated the virulent living bacteria. Pure Vi sera could be obtained by absorption of the O antibodies by an O strain. The rabbit's serum was capable of conferring passive protection in mice against virulent (Vi) strains, whereas O serum was ineffective against these strains but was able to protect against the toxic effect of O strains. Vi antigen was present in maximum amount in bacilli grown for 4-5 hours and might not be detected in 18-hour cultures (Craigie and Brandon 1936). Felix, Bhatnagar and Pitt (1934) showed that Vi antigen developed at a relatively narrow range of temperature of incubation around 37°C. Maintenance of strains

on egg medium in sealed bottles is effective in preserving Vi antigen (Bensted 1940).

Further knowledge of the properties and stability of the Vi antigen accumulated. Owing to its heat-lability, agglutination tests must be made at 37°C. In the light of later work it appeared that heat, although it has a damaging effect on the Vi-antibody-producing power of the typhoid bacillus, does not actually destroy the Vi antigen. A series of experiments by Peluffo (1941) showed that Vi-agglutinability was lost and O-agglutinability restored at 50°C. after 4 hours in saline, 2 hours in 0.3 per cent. formol-saline and 15 minutes in 0.5 per cent. phenol-saline. However, Vi bacilli were still Vi-agglutinable after 3 hours at 75°C. in absolute alcohol, and such organisms absorbed agglutinins from a Vi antiserum, and on inoculation into rabbits produced a high titre of Vi antibodies with the same protective properties as a serum prepared with living bacilli. The question of the thermostability of the Vi antigen is a complex one, as is further suggested by the work of Stuart and Kennedy (1948) who consider that its apparent destruction may be due to release of the antigen into the medium; free antigen was found in the supernatant fluid of heated suspensions, and it was relatively thermostable. However, the method of testing for Vi antigen, whether by precipitation tests, agglutinogenic property or otherwise, was important.

Certain workers (Ando and Nakamura 1950) consider, from experiments with typhoid bacilli treated in various ways and with antigenic fractions, that the Vi antigen is a complex consisting of a labile part responsible for Vi-agglutinability, inhibition by O antiserum and immunizing power in mice, and a heat-stable part ("T") detectable by precipitation tests and with feeble protective properties.

The effect of certain chemicals on the Vi antigen is curious. The early view was that it was destroyed by phenol, but the inactivation is in fact due to a reversible reaction between phenol and the antigen; when the phenol is removed and replaced by saline, the organisms are capable of stimulating Vi antibodies in the rabbit. Organisms treated with formalin still agglutinate with Vi antiserum and give rise to high titres of Vi agglutinins in rabbits, but the rabbit antibodies are deficient in opsonizing activity and in protective power in mice challenged with Vi bacilli (Felix and Bhatnagar 1935). Organisms treated with alcohol however stimulate in the rabbit, the horse and man Vi antibodies of full functional efficacy, in the same way as do living bacilli. This form of treatment will be discussed in a later section.

It has become customary to refer to typhoid strains as follows: V strains, fully Vi-agglutinable

and O-inagglutinable: VW strains, partially Vi-agglutinable and O-agglutinable: W strains, Vi-inagglutinable and fully O-agglutinable. Bhatnagar, Speechly and Singh (1938) have described a strain of Salm.typhi, the Vi I strain, which contains no H antigen, only a trace of O antigen and a large amount of Vi antigen. It is a smooth strain and its constitution makes it specially valuable for titrating the Vi antibody content of sera, from which it would otherwise be necessary to absorb the H and O agglutinins.

Mention must be made of a fundamental difference of opinion that exists on the nature and function of the Vi antigen. Certain workers, particularly Ørskov and Kauffmann (1936) contend that it acts as another toxin (in much the same way as the O antigen) and that mouse-virulence tests actually measure toxicity. Batson and his colleagues (1950) of the Biological Products Division of the United States Army Medical Service have also stated that the Vi antigen plays no part in virulence or in active or passive immunity as tested in the mouse. However, Felix and Pitt (1951) have recently collected the experimental evidence in favour of the opposite view. They contend that the workers referred to have failed to study certain details of technique, for example the accurate estimation of the Vi and O content of the strains they used. This can be done

by measuring the ability of the organisms to absorb the corresponding antibody from Vi and O antisera. Vi antigen can also be estimated by determining the titre the strain gives in an agglutination test with a Vi antiserum - the greater the Vi content, the lower the titre (due probably to the neutralization of considerable antibody by the greater amount of Vi antigen). Felix records, against a maximum unitage of 100, the Vi and O content of some of the well-known typhoid strains:-

	Ty 2	Watson	Ty 6S	O 901	Ty 2 Rough	Vi I
Vi	100	40	100	0	0	40
O	100	100	0	100	0	trace

This knowledge made it possible to compare the virulence and toxicity of these strains in mice - the virulence by the injection of graded numbers of living bacilli, and the toxicity by injection of much larger numbers of bacilli killed by heat. The only strains of high virulence were Ty 2 and Watson, showing that adequate amounts of both Vi and O antigens are necessary for high pathogenicity. The strains with high O content (Ty 2, Watson and O 901) were the only ones with high toxicity. These, and further tests in which mixtures of different strains were injected, led to the conclusion that the most virulent strains are those possessing Vi and O antigens in maximum amount - Vi or O antigen alone

confers only low virulence, but Vi-containing strains with even a small amount of O are of considerable virulence. In the living cell, Vi antigen is less toxic than O antigen, and its main function in virulence is to protect the O antigen from the action of natural or immune antibody.

It will be necessary to discuss this question further in the section dealing with vaccines, but in the meantime the writer would express his view that Felix has produced by far the stronger evidence in favour of his contention.

It has been shown by Nicolle, Jude and Le Minor (1950) and by Landy (1950) that V and W colonies of Salm.typhi may be distinguished under a dissecting microscope, with the use of oblique translumination. V forms are opaque and have a golden-yellow to reddish-copper colour. W forms are more translucent and have a greyish-green irridescence.

ii. Studies on Flagella: the Pijper Controversy

A brief account may be given of a controversy initiated by the observations of Adrianus Pijper of Pretoria, which has stimulated much discussion and a considerable amount of published work. It bears on the question as to whether typhoid bacilli have flagella at all, in the usually accepted sense of definite thread-like structures endowed with the function of propelling the organism. Pijper had developed a technique for studying and photographing

living organisms by dark-ground illumination, with the use of the brilliant South African sun as illuminant. His beautiful cinematograph films of motile typhoid bacilli and of the H, O and Vi types of agglutination were well-known. In 1938 Pijper came to the conclusion that typhoid bacilli had only two flagella which became entwined at one end of the organism into a single "tail" responsible for motility, and that the usual peritrichous arrangement was an artefact due to disintegration and drying. On the basis of later work Pijper (1946, 1947, 1949) now asserts that bacilli are helical in shape, that they move by their own undulating and gyratory motions and that the polysaccharide slime on the surface becomes twisted off to form the trailing threads or "mucous twirls" which have been mistaken for active flagella. He supports his contention by observations that the slime and flagella stain similarly, that motile bacteria can pass readily through tangles of other organisms and "reverse" in a way they could not do if equipped with peritrichous flagella, and that a culture of organisms showing "tails" was still motile when the tails had been removed by vigorous shaking.

This challenge to the orthodox view invoked a brisk response. Conn and Elrod (1947) working, as Pijper had done, with bacilli whose movement was slowed-up by suspension in methyl cellulose, failed

to confirm that the rods were flexible. They observed motility in bacilli too short to be capable of "undulating motion", and indeed in certain cocci also. Particularly impressive were their electron micrographs clearly showing flagella as definite structures traversing the cell wall and attached to the body of the organism; in one instance a flagellum had torn away and ruptured the cell wall. Many of the same points were made by Boltjes (1948) who drew an analogy between the flagella of organisms such as Salm.typhi and those of higher forms, and noted that the flagella of rigid organisms, stranded and immobile in a preparation were often to be seen in vigorous action. He points out that, since only motile typhoid bacilli have H antigen, Pijper's hypothesis would mean that bacteria encumbered by a capsule are motile and that only those devoid of capsules are non-motile. Fleming (1950), who induced organisms to assume "coiled-spring" forms by growth in penicillin-agar, and found that their circular movement could be stopped and started by interposition of a filter in the illuminating system, noted by phase-contrast microscopy that the flagella could clearly be observed in action before the organism started to move. Mallett, Koffler and Rinker (1951) shook suspensions of bacteria even more violently than did Pijper, and found by electron microscopy that numerous flagella were still attached. They

think that the disappearance of the "tails" in Pijper's work is due to the fact that the tail is a bundle of flagella which breaks on shaking into its constituent elements, less easily visible by dark-ground techniques. It is also relevant to note that the slime layer of the Salmonella, Proteus and other groups is predominantly polysaccharide, whereas the lability of flagellar material during mild treatment by heat, acid and ethanol suggests a protein nature. Weibull (1949) has in fact shown that the flagella of Proteus consist of a fibrous, myosin-like protein.

Pijper does not discuss the antigenic implications of his hypothesis, and his reply to the electron pictures is to the effect that these are necessarily desiccated preparations subject to artefact and that the appearances are not to be accepted as good evidence. The consensus of opinion, which is shared by the writer, is that the available evidence is still in favour of the orthodox view. The controversy has served a useful purpose in throwing light upon some problems of flagellation and motility, and has a practical bearing on certain aspects of bacterial classification which depend on numbers and situation of flagella. The question was fully discussed at a meeting of the Society for General Microbiology (Symposium 1949).

iii Viability of the Enteric Organisms

During the period under review a number of observations respecting the viability of the enteric organisms in different conditions were made. Some of these are of epidemiological interest.

Beard (1940) carried out some experiments in California in which typhoid bacilli were added to various kinds of soils in pots which were kept in the open. Survival was found to depend largely on retention of moisture; for example, the organisms were readily recovered from loam for periods up to 6-7 weeks but disappeared from sand in 4-7 days. Changes of pH over the usual soil ranges of 6.5 - 8 had little effect, but viability was brief (only a day or two) in peat, probably owing to its acidity (pH 3-4). Typhoid bacilli were recovered from a moist soil kept at freezing temperature up to 24 months, an observation which recalls the outbreak at Plymouth, Pennsylvania, in 1885, in which no fewer than 1,004 cases were infected by a water supply contaminated from the faeces of a man with typhoid fever. The faeces had been cast out on a hillside above the stream, and had lain frozen from Christmas until thawed by the warmer weather of March (Stallybrass, 1931).

In Germany, Dold (1943) described an epidemic of paratyphoid B at a wedding feast, due to a salad prepared in a bath-tub in which the dry linen from a

case of enteric fever had previously lain. This led him to test the viability of various pathogens in fluid stools and on linen soiled with faeces and allowed to dry. Tests were made at room temperature in the dark. In the fluid stools and on the linen, the average survival times of typhoid bacilli were 4 and 98 days respectively, and of paratyphoid B bacilli 11.5 and 191 days respectively. In later experiments (Dold 1949), the same worker showed that the organisms survived four to six times as long when dried on filter paper than in stools, an observation perhaps due in part to the fact that bacteriophages and Proteus died out more quickly on the filter paper.

During the war years there were a number of interesting reports from the United States (Schlesinger 1943, Rice 1944) and specially from Canada (Bowman 1942, from Manitoba; Foley and Poisson 1945, from Quebec; Menzies 1944, from Alberta) of outbreaks of typhoid fever, some very extensive, which were spread by cheese. The subject is reviewed by Meyer (1944). Menzies describes a typical outbreak in which fresh green cheddar, prepared from raw milk contaminated by a carrier, was consumed two weeks after it was made. Campbell and Gibbard (1944) were thus led to test the survival time of typhoid bacilli in cheese made from artificially infected milk in an experimental plant of commercial type. The cheese was first held

for 14 days at 58-60°F. for ripening. Thereafter typhoid bacilli were recovered for periods up to at least 10 months from cheeses stored at 58-60°F. There was no correlation between acidity and survival under the experimental conditions, and organisms were recovered in the smooth Vi form. Most authorities recommend that cheeses should be pasteurized or stored for 3 months before release for sale.

iv Bacterial Variants

(a) Isolation of Small Colony Strains

A phenomenon observed by Morris, Sellers and Brown (1941) in Georgia is worthy of brief comment. They isolated from the blood, faeces, urine or sputum of 10 persons, not all connected with one another, typhoid bacilli giving colonies only 0.2 mm. in diameter on ordinary agar in 1-2 days, but attaining normal size when flowers of sulphur, sodium thiosulphate, sodium sulphite or cystine were added. Sometimes a larger colony would develop, and later, the surrounding minute colonies would enlarge and coalesce with it, in a manner resembling satellitism. The small colony variants appeared to behave normally in regard to biochemical, serological and pathogenic characters.

(b) "Bacterium typhi flavum": a supposed variant of Salm.typhi.

In the decade preceding the war a number of Continental workers, particularly in Germany, described an organism which they named Bacterium typhi flavum. It was a motile, Gram-negative rod which produced yellow colonies on agar, and its special interest lay in the claim made by these workers that it is a variant of Salm.typhi and that it can revert to its parent form on repeated sub-culture and presumably under natural conditions. It was originally isolated from the faeces of typhoid contacts, and later from soil, water and other sources. In its pigmented form the organism was supposed to be completely, or only mildly, pathogenic, and its capacity for changing into the fully virulent typhoid bacillus was considered to account for the appearance of isolated cases of enteric fever in widely separated areas, seemingly without contact with previous cases or carriers. Four workers, or groups of workers, claimed to have observed the transformation in vitro of Bact.typhi flavum into Salm.typhi, sometimes from single cell cultures. Numerous other workers failed to confirm these claims and considered it probable that the proponents of the other view had unwittingly commenced their experiments with a mixture of organisms, or introduced the yellow organism in various vegetable juices

used in their culture media; in those instances in which it was isolated from the blood of typhoid patients, it might have been a saprophytic invader from the ulcerated gut. One of these workers in fact drank a large amount of two of the suspected strains without ill-effect. Since the recommendation was actually made that persons excreting Bact.typhi flavum should be treated as typhoid carriers, the problem appeared to have some importance from the public health standpoint, and a study of the organism was therefore undertaken. This work has been published (Cruickshank 1935) and the results are briefly summarized.

Own Observations

Nineteen strains of Bact.typhi flavum were obtained from Bürgers (Königsberg), Grossmann (Göttingen), Dresel (Greifswald) and Seydel (Warsaw), including a number from typhoid patients and some with which transformation into Salm.typhi was said to have occurred. All were aerobic Gram-negative rods, motile at 22°C. but rarely at 37°C. They produced yellow, round, low convex colonies as a rule, but striated, mucoid, rough or "bossed" colonies were not infrequent. Some strains produced colonies with the curious granular biconvex bodies that had been noted, as "Wetzsteinformen", by previous workers. Gelatin was slowly liquefied. All strains

produced acid without gas from glucose, mannitol, sucrose, salicin, rhamnose, arabinose and xylose; some strains fermented maltose; none fermented lactose, inositol or dulcitol. Serologically, the H and O antigens were diverse, the inter-relationships between the H antigens being more widespread than those of the O antigens. There was no significant relation between any of the Bact.typhi flavum strains and Salm.typhi. Many of the strains were repeatedly subcultured over many weeks but none of the variants that appeared suggested a transformation into Salm.typhi. Organisms with precisely the same characters, serologically related to some of the Continental strains, were readily isolated from the air of the writer's laboratory, from the animal house, from oats and from grass in a London suburban garden.

The experimental evidence and the literature were reviewed, and it was concluded that there was no good reason to think that Bact.typhi flavum was in any way related to Salm.typhi or that the latter can arise from it. It was considered that it in fact should be classified with the Chromobacteria, and was of no pathological or hygienic significance.

v Other New Knowledge of Salm.typhi

There are two important lines of research in which recent advances have been made but which have been excluded from detailed discussion in this review.

(a) Studies of the chemistry of the somatic antigens of Salm.typhi have been made by extraction of fractions (i) by the trichloroacetic acid method of Boivin and Mesrobianu (1936, and numerous subsequent papers), (ii) by the tryptic digestion method of Topley, Raistrick and their co-workers (1937), and (iii) by the diethylene glycol method of Morgan and Partridge (1942). The essence of this work is the isolation of the dominant O somatic antigen (and of the Vi antigen) as a phospholipid-polysaccharide complex. This extensive work has recently been summarized briefly by Van Heyningen (1950).

(b) The sensitivity of the enteric bacilli to the new antibiotics has been the subject of numerous studies. The most promising agent up to the present is chloromycetin. Of 97 strains of Salm.typhi tested, all were sensitive to 0.6-12.5 micrograms per ml.; 74 were sensitive to 5 micrograms or less. Of 12 strains of Salm.paratyphi B, all were sensitive to 0.5 to 16 micrograms per ml.; 5 were sensitive to 5 micrograms or less. Levels of 10 to 15 micrograms per ml. are attainable in the blood and higher levels in the urine (quoted in Report, 1950).

2. Salm.paratyphi B

The general characters of Salm.paratyphi B are well-known and need not be repeated here. Strains isolated from cases of enteric fever form a more mucoid type of colony than Salm.typhi and do not ferment d-tartrate. The antigenic formula is [I] , IV, [V] , XII ... b↔ 1, 2... It is thus a diphasic organism, but strains devoid of Phase 2 occur and are known as Salm.paratyphi B var. java. They are of value in preparing pure Phase 1 suspensions and antisera.

According to Felix and Pitt (1936) Salm.paratyphi B also possesses a Vi antigen. This antigen was more sensitive to heat and acid than the other somatic antigens, but it differed from the Vi antigen of the typhoid bacillus in developing at 21°C. as well as at 37°C.; also, Vi strains were fully O-agglutinable, and O antisera appeared to protect mice from virulent strains as well as Vi antisera. Vi strains were more virulent than those not possessing that antigen. Kauffmann (1936a, 1936b, 1947) contends strongly that this antigen, which is not destroyed by alcohol and still retains some agglutinability and agglutinin-absorbing properties after heating for 2 hours at 125°C., is in fact somatic antigen V and has no special relation to virulence. However, Felix states that practically every paratyphoid B strain isolated in this country and examined up to

the present contained the "heat-labile Vi antigen", and bacteriophages active on this antigen are the basis of the typing scheme which will be discussed in a later section.

B. CULTURAL DIAGNOSTIC METHODS

1. (a) Blood Culture

i Technique

Blood culture was used successfully as early as 1906 in the diagnosis of the enteric fevers, and it is established that it is the most valuable method of making a bacteriological diagnosis in the early stages of the disease. The routine procedure, which need not be described in detail, is to take 5-10 ml. of blood into an equal amount of sterile ox bile, or into 50-100 ml. of 0.5 per cent. sodium taurocholate. These substances are anticoagulants and also inhibit the bactericidal action of the blood. A similar substance which was shown by Von Haebler and Miles (1938) and Stuart (1948) to be of value is Liquoid (Hoffmann - La Roche) - sodium polyanethol sulphonate. In a comparative trial in which 5 ml. of blood were taken into 100 ml. of boiled blood broth, and into 1 ml. of 0.3 per cent. liquoid, with plating after 24 hours' incubation, Stuart obtained 12 of 170 positive cultures only in blood broth and 18 only in the liquoid. Degrees of bacteraemia could also be readily estimated by

dispersing measured amounts from the liquid tube into poured agar plates. Penfold, Goldman and Fairbrother (1940), who did not find any advantage with liquid, confirm the view of most workers that the best results in obscure cases of pyrexia will be obtained by using a range of media including saponin agar (best for Strep.viridans), glucose trypsin agar (best for staphylococci), Hartley broth and Robertson's meat broth. A report by Soman (1946) in which 15 of 17 positives were obtained with bile broth, and only 9 with 0.1 per cent. saponin broth in cases of enteric fever, indicates less success with saponin than the previous workers - but two positive cultures were obtained only with saponin broth.

A usual modern technique (Mackie and McCartney 1948) is to inject the blood directly into the medium through the rubber diaphragm in the perforated screw-cap of the container, exposed in a sterile fashion by tearing off the "viskap" covering. The same technique has been used by Weiser and Dye (1950) in instructions to United States Army Medical technicians and by Scott (1951), the procedure being a modification of Castenada's culture method. The medium, in a square 2-ounce bottle, consists of an agar slope with, in addition, a quantity of Brewer's fluid thioglycollate medium. Blood for culture is injected into the bottle, which is tilted so that the agar surface also receives the

inoculum. Growth may occur in the fluid medium, and colonies may appear on the agar surface. The tilting of the bottle may be repeated every few days where growth does not at first occur. As in the more conventional methods, para-aminobenzoic acid (5 mg. per 100 ml.) and penicillinase may be added to the fluid medium to neutralize the inhibitory effects of any sulphonamides or penicillin that may be present in the patient's blood.

It has long been known that, when blood has been submitted in a dry tube, it is well worth while attempting to cultivate the organism from the clot. Such specimens may not have been taken with meticulous aseptic technique and a medium, which would inhibit staphylococcal contaminants and permit small numbers of typhoid bacilli to grow, would be valuable. Grubb (1943) experimented with p-bromo-cinnamic acid which has this selective action but requires careful adjustment according to the pH of the medium and the volume of blood added. He found the answer in a simple broth medium containing 0.1 per cent. of sodium deoxycholate adjusted to pH 7.0. It inhibited staphylococci, grew typhoid bacilli from small inocula and needed no critical adjustment. Seidenstucker (1949) made a direct comparison of culture from blood and from blood clot in 71 cases of enteric fever: positive results were obtained in 83.1 and 53.5 per cent. of cases respectively.

TABLE 1

Percentage of Positive Blood Cultures in
Successive Weeks

	1	2	3	4	Later
1. Coleman and Buxton	89	73	60	38	26
2. Mann, Rainsford and Warren	80	62	50	36	-
3. Gay	73	80	53	40	-
4. Stuart and Pullen	81	62	32	12	-
5. Chatterjee	55	70	75	55	-
6. Batty Shaw and Mackay	80	90	84	-	-

Per cent Positive

- = No observations

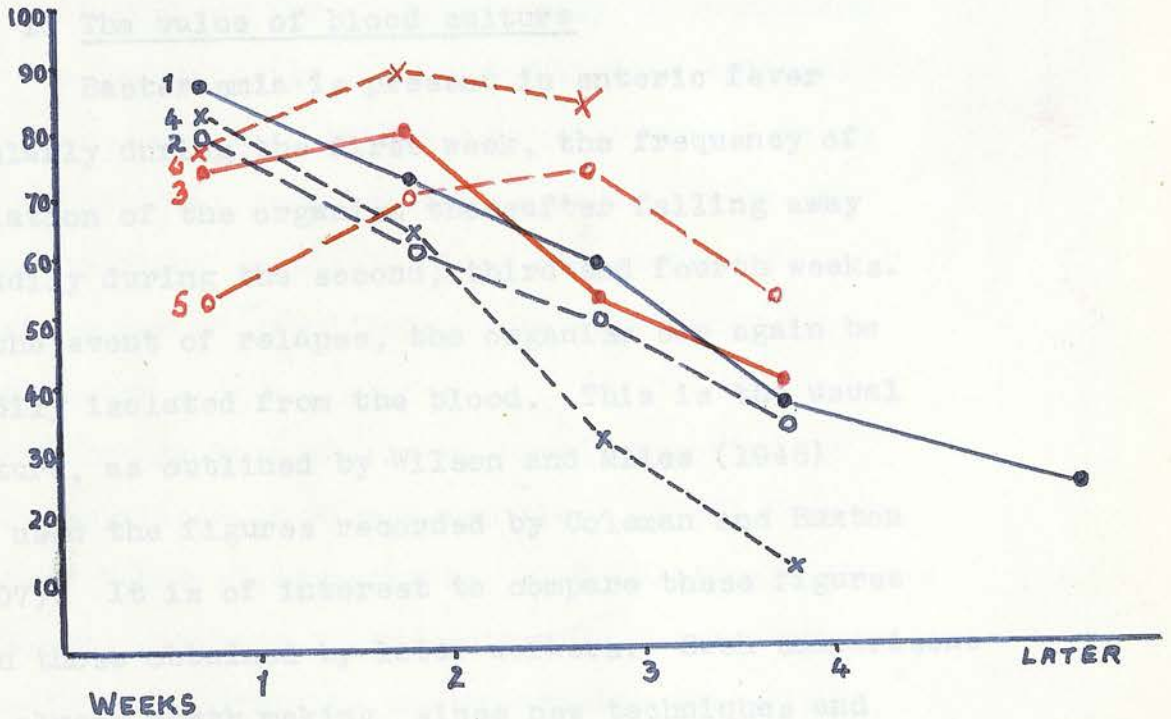


Fig. 1

It is therefore clear that clot-culture is a valuable procedure but that the results do not equal those obtained by blood culture.

Rappaport and Glikin (1950) have recently devised a microtechnique in the belief that positive cultures might sometimes be more readily obtained from capillary blood. The skin of the finger-tip or heel is disinfected with a special mixture of glycerin, formalin, soap and spirit ("Septoform") and punctured with a lancet; blood is taken up in a 1 ml. syringe for transfer to suitable media. Good results are claimed.

ii The value of blood culture

Bacteraemia is present in enteric fever regularly during the first week, the frequency of isolation of the organism thereafter falling away steadily during the second, third and fourth weeks. In the event of relapse, the organism can again be readily isolated from the blood. This is the usual picture, as outlined by Wilson and Miles (1946) who used the figures recorded by Coleman and Buxton (1907). It is of interest to compare these figures with those obtained by later workers. Such comparisons are always worth making, since new techniques and new culture media may be introduced from time to time. In any event, they may provide confirmation of previous observations. Table 1 shows the figures,

and compares in graphic form the results obtained in successive weeks by Mann, Rainsford and Warren (1915), Gray (1918), Chatterjee (1942), Stuart and Pullen (1946) and Batty Shaw and Mackay (1951).

It will be seen that, in no fewer than three of these reports, the percentage of positive cultures obtained in the second week was higher than in the first, and that, in the series recorded by Chatterjee and by Batty Shaw and Mackay, the figure in the third week was even higher than that in the first week. Clearly, then, the important diagnostic procedure of blood culture should not be neglected in the later weeks of illness. Differences in the results obtained by different workers are of course easy to understand - they can be accounted for by the various cultural techniques employed, by the degree of invasiveness of the infecting organism and, especially, by the volume of blood cultured, since the number of organisms per ml. is likely to be well under ten in the later stages.

It has been the usual view, supported by the observations of Hébert and Bloch (1922) from a study of 7,500 blood cultures, that positive cultures are rarely obtained if the patient's temperature is below 101-2°F. This is not invariably true. For instance, Batty Shaw and Mackay (1951) in their recent description of an epidemic of double infection with Salm.typhi and Salm.paratyphi B in Palestine

obtained positive results from all of 65 blood cultures made at body temperatures over 102°F., from 46 of 50 at temperatures from 99-102°F., and from 17 of 23 at temperatures from 97-99°F. Further, little difficulty was experienced by these workers in obtaining positive blood-cultures although all their patients had received T.A.B. inoculation - a contribution to the somewhat discrepant literature on this question. The discrepancy is understandable, since the outbreaks described were of different degrees of severity and were investigated by different workers using various techniques under Service conditions, often in time of war.

2. Culture of Bone-marrow

Bone-marrow is rich in reticulo-endothelial tissue, and it is reasonable to expect that the organisms would be present there earlier and for a longer period than in the blood where their presence is relatively transitory. The technique, which is carried out with novocaine infiltration, causes little discomfort. A shielded needle is inserted with a boring movement into the sternum opposite the third intercostal space, and about 0.5 ml. of marrow fluid is withdrawn. The method may have a special value in obese persons or in children in whom the veins are difficult to find.

Medulloculture has not been much used in this country but a number of reports are available

from abroad, in which cultures from the blood and from the marrow of patients suspected of having enteric fever have been compared (Ott 1938; Franza and Colarusso, 1939; Ling, Taur, Hsueh and Young, 1940; Itskovitch, 1940; Piaggio Blanco, Paseyro and Sanguinetti, 1942; Seidenstucker, 1949; Hirsowitz and Cassel, 1951). The figures have been arranged in tabular form (Table 2). Although, for obvious reasons, the percentage of positive isolations varies considerably from one worker to another, it will be noted that, in every series, the percentage of positive results obtained with marrow-culture was very substantially higher than with blood-culture. Other points made by these observers are that a high percentage of cases gave positive cultures in the stage of defervescence so that the advantage of marrow-culture over blood-culture became greater in the later weeks of illness; also, the great majority of cultures from the marrow were detectable within 24 hours' incubation, which is not always so with blood-cultures. The same points have recently been emphasized by Ling, Lin and Chen (1948), who regard a positive marrow-culture with a negative blood-culture as of good prognostic significance, and a positive culture in convalescence as indicating that the danger of relapse is not over.

TABLE 2

Comparison of Blood Culture and Marrow Culture in Typhoid Fever

	No. of cases	Percentage Positive	
		Blood Culture	Marrow Culture
Ott (1938)	7	28.5	100
Franza <u>et al</u> (1939)	200	33.1	48.8
Itskovitch (1940)	56*	49.0	90.2
Ling <u>et al</u> (1940)	110	30.0	68.2
Piagga Blanco <u>et al</u> (1942)	45	60.0	93.3
Seidenstucker (1949)	56	44.6	58.9
Hirsowitz and Cassel (1951)	40†	52.5 (21/40)	64.3 (18/28)

* 5 had marrow-culture only.

† Only 28 had marrow-culture: the positive blood cultures were obtained at some stage of illness, not necessarily simultaneously. In no case was blood culture positive alone.

3. The Bacteriological Examination of Faeces and Urine

It is well-known that the infecting organism can be isolated from the faeces in most cases during the acute stages of illness and that, as the stage of defervescence passes into convalescence, the organism is present in steadily decreasing numbers. One of the greatest advances during the years under review was the development of much more efficient and selective culture media, and the stringencies of war also led to a number of useful improvisations of methods and media. The literature of the subject is large and of varied quality. It is proposed to discuss the more important solid and fluid media that have been described, and to refer to comparisons of their efficiency that have been reported and to the various routine procedures recommended by different workers.

i Culture Media

(a) Solid media

Wilson and Blair agar, which contains bismuth sulphite, glucose and brilliant green, has retained its popularity for the isolation of Salm.typhi and Salm.paratyphi B. First described in 1931, it has been modified several times by Wilson. The principle of the medium is that the enteric bacilli reduce bismuth sulphite to sulphide in the presence

of a certain excess of sodium sulphite, bismuth sulphite inhibits the growth of Bact.coli and most other unwanted bacilli. The medium is therefore highly selective and produces readily recognizable colonies. It has, however, several disadvantages. It is rather complicated to prepare, it does not keep well at room temperature or in the refrigerator, colonies of Salm.typhi can often not be seen until the plates have been incubated for 36-48 hours, and some strains may not show the typical black colonies. Further, it may vary from batch to batch in selectivity. The temperature at which the various ingredients are mixed and the thickness of the plates may also produce variable results (Adler 1949).

It is not surprising, therefore, to find that a large number of modifications have been reported. In those described by Tabet (1938), De Loureiro (1942) and Hobbs, King and Allison (1945) the concentrations of the ingredients have varied considerably:- bismuth ammonio-citrate (0.3 - 0.6%), glucose (0.32 - 0.52%), Na_2HPO_4 (0.35 - 0.5%), ferrous sulphate (0.04 - 0.08%), brilliant green (0.005 - 0.0025%). Tabet (1949) later described another modification which he found necessary to obtain good results with Salm.typhi and Salm. paratyphi B in Egypt; paratyphoid A bacilli would

not grow on it. De Loureiro's modification was an effort to overcome instability due to oxidation, by the use of three separate clear solutions which were mixed when required. Tyageraja (1940) even omitted the glucose, ferrous sulphate and sodium phosphate, and claimed that his medium was not only simpler to prepare but kept better and gave good results. It is clear that Wilson and Blair's medium is a notorious example of the way in which culture media have been devised and modified empirically, without any exact knowledge of the mechanism by which the numerous inconsistencies are produced. The most extensive and serious attempt at a rational approach is described in a monograph by Lovrekovich (1941). An interesting comparative trial was made by Hobbs, King and Allison (1945) who examined some hundreds of specimens of faeces from enteric carriers. Their results with the Tabet and De Loureiro media, with a dried Difco product and a made-up powder of their own showed that there was little difference between them, the percentage of the known positives detected by the different media being respectively 76.5, 82.0, 77.3 and 78.9 per cent. for Salm.typhi; with Salm.paratyphi B there was also very little difference between any of the media. A noteworthy point (Report, 1943) is that the addition of 0.015 per cent. of ferrous sulphate to that contained in

the original formula enhances the blackening of typhoid colonies which would otherwise be difficult to recognize, without interfering with selectivity.

Of the other solid media, deoxycholate citrate agar is probably the most useful. It contains sodium deoxycholate, lactose, ferric sodium citrate, sodium citrate and neutral red as indicator. It has the great advantage for general work that it also supports the growth of dysentery bacilli. Other solid media have had their adherents; their virtue lies mainly in some combination of dyes. Jones (1936) claimed for brilliant green eosin agar that it was better than MacConkey agar and equal to Wilson and Blair agar, but was easier to make and gave quicker results. Taft and Daly (1947) considered that eosin-methylene blue agar adjusted to pH 8.0 was the best single medium for isolation of all Gram-negative bacilli. Jeter and Wynne (1949), who point out that the ideal differential medium should contain an acid and a basic dye which do not combine when mixed, make strong claims for acid fuchsin - methylene blue agar; it supports the growth of delicate enteric species and gives distinct red colonies of lactose-fermenters and blue colonies of non-lactose-fermenters. Hoyle (1943) prefers brilliant green-acid fuchsin agar, which does not require the precise titration of dyes necessary for brilliant green-eosin agar; he found it satisfactory

in colonial differentiation, selectivity, keeping quality and constancy of results. Further (Hoyle 1949) he discovered that it was not subject to a disadvantage shown by deoxycholate agar, namely, the failure of the pathogens to grow from the excreta of patients receiving sulphonamide treatment.

Of the more exotic media, mention may be made of the D.E.C. plate of Panja and Ghosh (1943) which gave good results in India in the isolation of dysentery and enteric bacilli and also of the cholera vibrio, and, as an example of a wartime improvisation, the so-called Ti-Cello plate of Kanz (1948). The latter, devised in a period of shortage of agar, consists of a pad of filter paper soaked in lactose-fuchsin broth and overlaid with a disc of Cellophane rendered opaque by treatment with titanium. The surface of the Cellophane receives the inoculum; colonies of the pathogen are porcelain-white, those of Bact.coli are red; the swarming of Proteus is inhibited.

ii Fluid Enrichment Media

In the years just preceding the war, brilliant green broth, for suppressing the growth of Bact.coli and thus favouring the growth of typhoid and paratyphoid bacilli, was beginning to give way to one or other of the formulae for tetrathionate

broth described by Müller, Kauffmann and others. These media contained, in addition to the basal broth, thiosulphate, tetrathionate, iodide and chalk. Experience soon showed that good results were much more readily obtained in the isolation of paratyphoid B bacilli than of typhoid bacilli, and that the results of experimental work with pure cultures were often not entirely reproduced in practice. The problem was approached scientifically by Knox, Gell and Pollock (1943). Tetrathionate is formed by the interaction of iodine with excess of thio-sulphate. Sodium thiosulphate itself is not a satisfactory enriching agent, because the typhoid bacillus is sensitive to it and Morgan's bacillus is not. Any excess of iodine is inhibitory to all species. A "balanced" tetrathionate was therefore devised, in which the iodine and thiosulphate were mixed in the exact proportions indicated by the chemical equation. At a concentration of 0.03 M this supported good growth of typhoid bacilli and salmonellae and inhibited Proteus and most non-pathogenic bacteria. Morgan's bacillus sometimes grew. Certain observations, such as the appearance of acidity and opacity in uninoculated media, and the apparent inhibition of H₂S production by some organisms, could be explained. Tetrathionate is unstable in high concentrations and it reacts with H₂S. Also, reduction of tetrathionate to thiosulphate

can occur in the presence of hydrogen, the tetrathionate acting as a hydrogen-acceptor; this reduction can be effected by Salm.paratyphi B, other salmonellae, Proteus and, to a lesser extent, by Salm.typhi. Acid is thus produced, necessitating the presence in the medium of a buffer such as chalk or phosphate - but these substances may then favour the growth of Proteus.

For the best results it would seem that two formulae are necessary when this nature of the pathogen is not known. Rolfe (1946) has given two such formulae for tetrathionate broth; one allows the growth of salmonellae and most strains of Salm.typhi, and is not very inhibitory to Proteus; the other allows the growth of few organisms other than salmonellae. These media contain the minimum of free thiosulphate.

The value of tetrathionate enrichment media has been made amply clear, especially for Salm.paratyphi B. A typical finding is that of Bohls and Mattmann (1950) who examined 740 faecal specimens for pathogens by direct culture on eosin-methylene blue and S.S. (Shigella-salmonella) agar, and on S.S. medium after enrichment in tetrathionate; of 111 positive findings, 23 would have been missed without tetrathionate. Preuss (1949a) considers that potassium tetrathionate is better than the sodium salt since it is more chemically stable; for Salm.

typhi he adjusts the medium to pH 6.5 and omits the chalk in order to suppress Proteus. In one trial (which also included direct plating) he obtained a total of 47 isolations of Salm.typhi, of which 38 were detected by enrichment in his medium, against 16 with Kauffmann's tetrathionate. The finding of Tabet (1949 b) in Egypt may be noted, that tetrathionate prepared according to three of the most popular formulae inhibited or even killed Salm.paratyphi A. Tetrathionate agar has also been used by Preuss (1949b) and by Knox (loc.cit.) but it appears to have no advantage over the more usual media.

Another highly successful selective medium, selenite-F, was described by Leifson (1936), and again by Gohar (1943) who used sodium selenite in a strength of 1/500 in broth or peptone water.

Littmann (1943) pointed out that broth containing 0.4 per cent. of sodium acid selenite is toxic to Gram-negative bacilli but that, in the presence of 1 per cent. sodium phosphate it is detoxified sufficiently to grow Salm.typhi while inhibiting Bact.coli at least for the first 8-12 hours' incubation. Hobbs and Allison (1945a) replaced the lactose in Leifson's formula by mannitol and found that, of 38 faecal specimens positive for typhoid bacilli, 32 isolations were made from both a lactose and a mannitol medium, 5 only from the

mannitol and 1 only from the lactose. Of four liquid enrichment media (Wilson and Blair, tetrathionate, deoxycholate-citrate and selenite) selenite was the most effective for Salm.typhi, and was as selective as tetrathionate for the isolation of Salm.paratyphi B. The medium appeared to keep for an indefinite period without loss of selectivity. Anderson (1948) considers that the optimum time for subculture is after 18 hours' incubation.

Good results have been reported with two less familiar enrichment media. Knothe (1949) used 0.03 per cent. potassium chromate broth at a critical pH of 6.8. Of 19 specimens positive for Salm.typhi, 16 isolations were made through tetrathionate and 16 through chromate; of 121 specimens of which 119 were positive for Salm.paratyphi B and 3 for Salm.enteritidis, 89 were detected by tetrathionate and 111 by chromate. As is usual with such comparisons both media had to be used to obtain the maximum number of positive results. Another interesting enrichment medium is cacotheline- or hydroquinone-brilliant green broth which Jones and Handley (1945) found of special value for the isolation of pathogens from high contaminated material.

The enrichment procedure, culture in tetrathionate broth followed by plating on Wilson and Blair agar appeared to be the most

ii Comparison of various culture media for
isolation of enteric pathogens

It is of interest to analyse the figures obtained in comparative trials of various media used for the isolation of enteric bacilli from faeces. The results recorded in the more important trials made in recent years are set out in Tables 3 and 4, those of Hobbs and Allison (1945 a and b) being tabulated separately because this careful trial consisted of a series of separate comparisons which cannot be properly summated. The figures for Salm. typhi and Salm. paratyphi B have been separately recorded. The most successful solid medium or enrichment procedure in each series has been underlined in green.

Certain facts emerge very clearly from these findings. For the isolation of Salm. typhi, the most efficient solid medium for direct plating is Wilson and Blair agar. In every trial in which it was compared with other solid media it achieved the highest percentage of positives, and in most instances it detected over 89 per cent. of the total number of positive specimens. Next in order of efficiency comes deoxycholate-citrate agar. The superiority of these newer media over MacConkey and Endo agar is again confirmed. Of the enrichment procedures, culture in tetrathionate broth followed by plating on Wilson and Blair agar appeared to be the most

TABLE 3
Percentage of Positive Results obtained with individual solid media and various enrichment procedures

TYPHOID	SPECIMENS		SOLID MEDIA						ENRICHMENT MEDIA										NOTES							
	TOTAL	POSITIVE	WB	DC	MacC	SS	ENDO	Other	TETRATHIONATE					SELENITE				BG		RUYS		TET				
									WB	DC	McC	ENDO	BGAF	WB	DC	SS	LLA	WB		McC	WB	McC	Muller	Kauf		
Gunther and Tuft, 1938	1146	215	96.3	.	.	.	60	44.6	Platings from glycerin suspension.
Mayfield and Gober, 1940	-	724	83	82	.	.	74	60.0	195 positives appeared on one medium only.	
Ruys, 1940 (1)	-	222	89.2	.	.	.	43.3	51.0	48.9	
" (2)	-	96	89.6	.	.	.	36.4	
Hynes, 1942	140	88	85.2	47.7	21.6	.	.	.	67.1	.	13.7	60.3	9.1	37.3	5.7	
Pot, 1943	135	27	66.6	.	.	51.0	9.0	
Cruickshank, 1942	60	59	91.5	88.1	79.7	WB+DC detected all.
Mollov et al., 1943 †	501	40	.	.	42.5	85.0	67.5	
Černozubov et al., 1944	-	2621	81.0	65.3	.	.	36.6	
Peluffo, 1947	-	85	91.8	56.5	.	44.7	.	LLA 29.4	68.6	62.9	52.9	68.6	
Kröger & Danneberg, 1950	-	84	.	38.1	.	.	34.5	BGPR 27.4	Many more positives by enrichment methods.
Jones, 1951 (1)	-	116	.	19.0	34.6	49.1	
" (2)	-	308	31.8	96.4	
Cook et al., 1951	-	27	70.4	40.7	59.3	44.4	.	.	.	74.1	70.5	

PARATYPHOID B, etc.

Ruys, 1940	-	93	18.3	86.0	76.3	47.3	Plating medium after enrichment not stated. (presumably Endo).				
Hynes, 1942	187	?	72.5	58.5	27.5	.	.	.	91.5	.	74.5	89.5	55.0	83.0	56.0	.	.				
Cooper, 1942	409	136	73.5	72.8	
Hoyle, 1943 *	-	31	.	61.3	.	.	.	BGAF 61.3	.	93.5	.	.	100.0	BGAF inhibits Proteus: both media needed for maximum positives.
Černozubov et al., 1944 *	-	384	44.5	66.0	.	75.0	Enrichment gave many more positives.
Kröger & Danneberg, 1950	-	212	.	40.1	.	.	34.4	BGPR 42.4	
Cook et al., 1951 *	-	539	58.1	59.4	75.3	81.1	.	.	.	78.1	85.7	

† includes some dysentery specimens.
* " " salmonella " "
‡: 73 Para B, 27 Typhi, rest Salmonellae.

WB = Wilson and Blair
DC = Deoxycholate citrate
McC = MacConkey
SS = Salmonella-Shigella Agar
LLA = Litmus lactose agar
BGAF = Brilliant green acid fuchsin
BGPR = " " phenol red
Ruys = " " + Esbach reagent (picric + citric acids)

TABLE 4

Analysis of the Figures of Hobbs and Allison (1945, b)

TYPHOID

Percentage Positive

SOLID MEDIA ENRICHMENT MEDIA

POSITIVE SPECIMENS	WB	DC	M _{NC} C	WB	DC	TET.	SEL.
387	<u>77.2</u>	72.1					
59	<u>91.5</u>	74.6	35.6				
112				<u>62.5</u>	51.8	59.0	
58				75.8		46.5	<u>95.4</u>
131						62.6	<u>95.4</u>
	<u>WB+DC</u>						
310		86.5					<u>91.9</u>
196	<u>93.4</u>			70.9			
160	<u>95.6</u>				52.5		
360	<u>94.2</u>					61.1	

But 99% of these were revealed by direct plating.
 20.7% revealed only by selenite
 Selenite alone gave higher percentage than direct plating

PARATYPHOID B

51	<u>86.3</u>	82.4				
23				52.2	79.3	<u>91.3</u>
17						<u>100.0</u>

Selenite is at least as good as tetrathionate

Other figures showed that WB was the best medium for plating from enrichment media, but that it is better to use both WB and DC.

Abbreviations as on Table 3.

effective, until selenite enrichment came under test. From the later trials it is clear that selenite is the most efficient enrichment medium for Salm.typhi and that platings from it should be made, not on MacConkey agar, but on the selective media; Hobbs and Allison (loc.cit.) found Wilson and Blair agar best for this purpose and Jones (1951) detected 99.1 per cent. of 116 positive typhoid stools by the selenite-deoxycholate-citrate plate procedure. To detect the maximum number of positives both media should be used.

For the isolation of Salm.paratyphi B, Wilson and Blair agar is again the most effective solid medium but its superiority over deoxycholate-citrate agar is not so great as it is for Salm.typhi. Tetrathionate enrichment gives better results for Salm.paratyphi B than it does for Salm.typhi and the plating can effectively be made on Wilson and Blair or deoxycholate agar (though Hoyle's small series suggests that brilliant-green-acid-fuchsin agar is worthy of consideration). However, the later trials again show that selenite enrichment is at least as reliable as tetrathionate.

Own Observations

During the course of two investigations, the writer has made observations relating to the efficiency of culture media for the isolation of organisms of

the enteric group.

(1) During an enquiry into the effect of treatment with mepacrine on the typhoid carrier state (see later), faecal specimens were taken from a number of chronic carriers in mental hospitals. Of 59 positive stools 54 isolations were made by direct plating on Wilson and Blair agar, 52 on deoxycholate citrate agar and 47 on MacConkey agar; a combination of the first two media would have detected all but one of the positive specimens. There were no differences in the results obtained by the use of three different formulae for tetrathionate broth - Wright's modification of Muller's formula, Schafer's medium and Kauffmann's modification. Only one specimen was positive through tetrathionate and not on direct plating. The superiority of the newer media over MacConkey was greater than the figures would suggest, because it was usual to find a profuse growth of Salm.typhi on these media when only an occasional colony was with difficulty detected on MacConkey agar.

(2) In a later investigation the writer, with Williams Smith (1949) examined faeces or rectal swabs from 500 dogs, 500 cats and 133 pigeons for organisms of the Salmonella group. Salmonellae were isolated from 5 (1 per cent.) of the dogs, 7 (1.4 per cent.) of the cats and 3 (2.25 per cent.) of the pigeons. Salm.paratyphi B was isolated from a cat. It is relevant to the present discussion that by far

the most successful of the procedures tried was enrichment in selenite broth for 18 hours followed by plating on deoxycholate citrate agar. On only one occasion was there failure to detect a positive specimen by this routine, and tetrathionate enrichment gave relatively poor results. The observation that the direct deoxycholate plates were often obscured by a heavy growth of Proteus and of non-pathogenic non-lactose fermenters of paracolon type led to the discovery of great differences in the constitution of the faecal flora of the various animal species, which may affect the choice of media to be employed for certain enquiries.

iii Screening procedures

In the routine diagnosis of enteric disease bacteriologists have constantly tried to develop methods of saving time and materials by rapidly excluding non-pathogenic bacteria from further examination. The various double- and triple-sugar media, used more in the United States than in this country, are examples of efforts to obtain a quick preliminary orientation. Lately, increasing use has been made of methods of detecting rapidly those organisms which have urease activity or produce indole. In this way it is hoped that Proteus and most types of paracolon bacilli can be rejected at an early stage, a procedure of special importance

in view of the fact that these organisms may grow in tetrathionate broth and in deoxycholate agar.

Christensen (1946) used a medium containing 0.1 per cent. peptone, 0.1 per cent. glucose and 2 per cent. urea in slopes with phenol red as indicator to detect urea-splitting bacteria; the small amount of peptone does not cause false positives and the fermentation of glucose produces enough acidity to counteract the alkalinity due to peptone decomposition. Cook (1948), working with the Proteus group, found that this medium gave positive reactions with all strains in $1\frac{1}{2}$ to 3 hours, considerably earlier than the buffered yeast extract medium of Rustigian and Stuart (1941) that was tested in parallel. By adding 0.3 per cent. of tryptophan to a fluid urea-phenol red solution, Roland and Bourbon (1949) were able to detect urea-splitting in four hours and, next day, to test the same tube for indole-formation. Elek (1948) found that a more sensitive test for urease activity was to use Nessler reagent for the detection of ammonia, and this idea was incorporated in the rapid procedure of Williams Smith and Chave (1949) who used 0.1 per cent. urea and 0.1 per cent. tryptophan in a phosphate buffer at pH 7.2. After 3-4 hours' incubation, a drop of Nessler reagent is added as a test for urease activity, and the indole test performed by the usual Ehrlich procedure. This test proved valuable in saving labour and culture-media

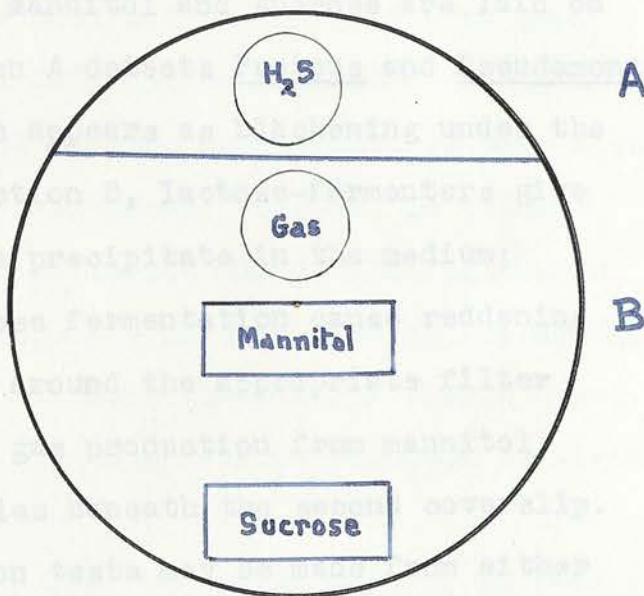
in the survey of the frequency of Salmonella infection in domestic animals by the writer and Williams Smith, referred to above. Very recently Hoyt (1951) has carried simplification for routine purposes a stage further by devising "complete-formula" urea and tryptophan tablets that are ready for use when dissolved in 1 ml. of distilled water.

With the same objects, a series of quick microtechniques for the rapid identification or discard of cultures has been developed by Weaver and his colleagues. The principle of their method is to ensure rapid growth by placing a heavy inoculum of the organisms in a very small quantity of optimal medium preheated to incubation temperature. Their series of papers includes one on indole-production (Arnold and Weaver 1948) and one describing sugar fermentation tests that can be read in four hours or earlier (Hannan and Weaver 1948). Identical results were obtained by these microtechniques and by the conventional methods.

An interesting "screening plate" was devised by Knox (1949), with the object of detecting rapidly any contaminant which might be picked from the selective primary plate along with the suspected pathogen, and of obtaining the maximum information about the pathogen. The plate (Fig. 2) contains two media, A and B. Medium A, a tryptic digest broth agar with sodium thiosulphate and lead acetate, is

FIG. 2

Knox's Screening Plate.



A = Lead acetate - thiosulphate agar

B = Deoxycholate - lactose agar

The small circles are cover-slips.

The rectangles are strips of filter-paper impregnated with the "sugars."

poured into inclined dishes so that it sets as a wedge at one side of the dish. Medium B, an agar with lactose, sodium deoxycholate and neutral red, is poured in the usual way so as to form a flat layer in the remainder of the plate. The colony under test is inoculated heavily in the centre of the plate across sections A and B, spreading towards the margins. A sterile round coverslip is placed on each section, as indicated in the figure, and pieces of filter paper impregnated respectively with 50 per cent. mannitol and sucrose are laid on section B. Section A detects Proteus and Pseudomonas, and H₂S production appears as blackening under the coverslip. On section B, lactose-fermenters give red colonies and a precipitate in the medium; mannitol and sucrose fermentation cause reddening and precipitation around the appropriate filter paper strip, with gas production from mannitol appearing as bubbles beneath the second coverslip. Slide-agglutination tests may be made from either part of the plate. With the use of this screening plate, and the rapid urease and indole-detecting methods already referred to, Cook and Knox (1949) found that they were able to eliminate the great majority of the 1,077 organisms in one series, picked as suspected pathogens, leaving only 94 to be submitted to full fermentation and serological tests. Spaur and Wynne (1951) further developed the same idea by devising an economical plate method for

testing fermentation reactions. Strips of filter paper impregnated with the carbohydrates were "sunk" in melted deoxycholate agar containing phenol red as indicator, and the cultures to be tested were inoculated in linear fashion across the strips after the medium had set. Identical results were obtained in tests made by this method and the ordinary tube technique.

iv. Recommended routine methods for faecal cultures

The methods most likely to give the best results in routine work can be inferred with some certainty from the information collected above. The procedure to be adopted will of course vary with the particular circumstances, whether, on the one hand it is desired to detect any of the full range of intestinal pathogens - enteric bacilli, salmonellae, dysentery bacilli and even the cholera vibrio - or, on the other hand, the responsible pathogen is already known.

It is clear that MacConkey agar is so inferior to the newer media that it need no longer be used except for cases in which it is necessary to detect Bact.coli (as in urine) or paracolon bacilli. Amongst the selective media, there is some room for personal preference, and media such as brilliant green-acid fuchsin agar and eosin-brilliant green-methylene blue agar will no doubt retain their adherents. For Salm.typhi, Salm.paratyphi B and

the other salmonellae one would suggest that the best routine is as follows: direct plating of the specimen on Wilson and Blair and on deoxycholate citrate agar, and enrichment in selenite broth: 18 hours later, if the plates are negative, they are re-incubated for another day and the selenite broth is plated on to both Wilson and Blair and deoxycholate citrate agar. With this route Hobbs and Allison (loc.cit.) detected all but one of 310 positive typhoid specimens. For known paratyphoid B specimens, tetrathionate enrichment with plating on one of the selective media will probably miss few positives. For typhoid bacilli, direct plating on Wilson and Blair agar is necessary, and selenite enrichment is better than tetrathionate.

It is of interest to note the routine used by Galton, Hardy and Mitchell (1950), experts with a wide experience in the isolation and identification of intestinal pathogens in the United States. Faeces, submitted in glycerol-saline suspension, are plated on S.S. (Shigella-Salmonella) and on Wilson and Blair plates: for typhoid bacilli a deep Wilson and Blair poured plate is added: enrichment is made in brilliant green-tetrathionate from which platings are made on brilliant green and S.S. agar plates. Suspected colonies are picked to Kligler's iron agar, and, according to the results, tested by the rapid urease and indole tests, and in Simmon's

citrate. The subsequent routine consists of the usual tests for motility, fermentative powers and agglutination with specific antisera.

v Culture of the enteric organisms from urine

In the past, attempts to cultivate enteric bacilli from the urine have usually consisted in direct plating of centrifuged deposits on solid media, or plating after preliminary culture in broth. Since Bact.coli is not likely to be present in gross excess, selective enrichment is less strongly indicated than some satisfactory method of concentration of the bacteria. Studies on the relative value of such methods have been made by Archer and Ritchie (1950). Culture of Seitz pads through which urine had been filtered gave good results, specially if the pad had been pre-treated with dead coliform bacilli, but the method is hardly practicable when large numbers of specimens have to be examined. Two other methods have given good results and are being further studied by these authors: (1) precipitation with 2 per cent. neutral sodium oxalate (10 ml. to 100 ml. of urine), in a "medical flat" bottle inclined at an appropriate angle and held overnight in the refrigerator: (2) preliminary culture in fluid MacConkey broth, with mannitol in place of lactose, followed by plating on deoxycholate citrate and on Wilson and Blair agars.

vi Recent Information on the Duration of Excretion
of the Pathogen in Paratyphoid B

In view of the development of more effective culture media, it is of interest to review some recent figures recording the duration of infectivity of patients suffering from paratyphoid B. These figures have been summarized in the form of curves (Fig. 3) showing the percentage of patients still excreting the organism in the faeces in successive weeks after onset. To some extent the different series are not strictly comparable. For instance, in some outbreaks specimens from patients in whom the diagnosis had been established were not repeated until the later "clearance" specimens were demanded. Also, in Jameson's series (1951) it is assumed for recording purposes that cases which had once fulfilled the criteria of cure remained bacteriologically negative.

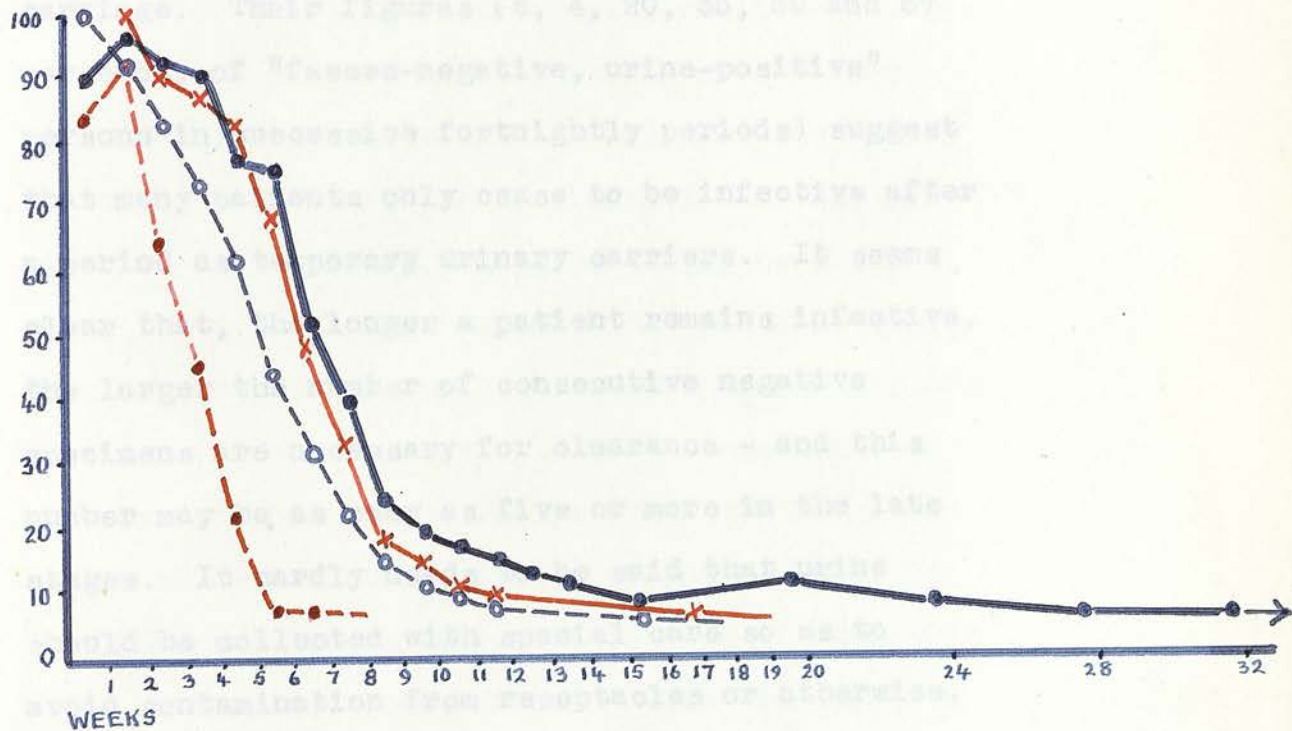
In general, the curves are similar. There is a slow rate of clearing in the first 4 or 5 weeks, succeeded by a much more rapid rate for the next 3-4 weeks, after which the remaining cases clear up even more slowly. About half the cases are still positive at the 7th week, and the frequency does not fall below 10 per cent. until about the 11th week. Clearance was much quicker in the Bristol series of Davies et al (1940) than in that of Holt et al (1942) in Liverpool; the earlier Liverpool series of Glass and Wright (1937) was even slower. There

Fig. 3

Duration of excretion of Salm. paratyphi B

Percentage of infected persons excreting the organism at various periods of illness and convalescence.

Per cent.



- Jameson, Eastbourne, 1951.
- - -○ Holt et al., Liverpool, 1942.
- - -● Davies et al., Bristol, 1940.
- ×—× Glass and Wright, Liverpool, 1937.

is a little evidence that these differences may be associated with differences in the severity of the outbreaks, as indicated by their relative case-fatality.

All workers are agreed that clearance is slowest in females over 20 years of age. Gell and Knox (1942), who made a careful study of the duration of infectivity in 129 cases adequately followed, stress the importance of urinary carriage. Their figures (6, 4, 20, 35, 50 and 57 per cent. of "faeces-negative, urine-positive" persons in successive fortnightly periods) suggest that many patients only cease to be infective after a period as temporary urinary carriers. It seems clear that, the longer a patient remains infective, the larger the number of consecutive negative specimens are necessary for clearance - and this number may be as many as five or more in the late stages. It hardly needs to be said that urine should be collected with special care so as to avoid contamination from receptacles or otherwise.

C. SEROLOGY

i. The Position up to 1939

By 1939 it had been established for many years that the titre of H and O antibodies in the serum of suspected cases of enteric fever should be separately estimated with the use of H suspensions,

which were formolized broth cultures of carefully selected strains, and O suspensions, which were either formolized broth cultures of permanent non-motile variants or emulsions of organisms grown on agar, treated with alcohol, washed and resuspended in formol-saline. Serum-suspension mixtures were usually incubated at 50-52°C. in a waterbath and readings of H agglutination made at 2 hours and of O agglutination at 20 to 24 hours. It could only be expected that identical results would be obtained by different workers if suspensions of standard agglutinability were used. In Britain, the Oxford Standards Laboratory for Serological Reagents was instituted to prepare and issue these suspensions, and a "Standard Agglutination Tube" was also available for comparison, so as to ensure uniformity of reading of the end-point. In 1935 a committee of experts convened by the Health Organization of the League of Nations arranged a comparative trial in four European laboratories in the hope of achieving wider uniformity. Some 60 or 70 sera from typhoid (and a few other) cases were tested by a standard method with the use of Oxford suspensions, and by the routine method of the particular laboratory. The results, reported by Felix and Gardner (1937), showed that the standard method gave the most consistent results and that the H titres, which were easier to read, were in greater agreement than the O.



The results however were disappointingly variable and it was obvious that further research on stable reagents was necessary.

2. Later Observations

1 Standardization of Reagents

One cause of inconsistency in O agglutination tests was discovered. The slow formation of small floccules in these tests necessitates long incubation. As Felix and Olitzki (1929) had pointed out, O agglutinins are thermolabile; it was to avoid damage to them that an incubation temperature of 50-52°C. was recommended instead of 55°C. Further work by Bensted (1940) and by Felix, Rainsford and Stokes (1941) has shown that even this temperature is too high and that enteric O agglutination tests should be incubated at 37°C. for 2 hours, and read after a further 22 hours in the refrigerator at 4°C. Otherwise, the O agglutinins are liable to be significantly reduced. A further point is that O agglutination tests should be done in round-bottomed tubes and read in the manner of the Vi agglutination test described below.

Experience has shown that antigens are not sufficiently stable to be adopted as standards, but it is feasible for agglutinable suspensions to be prepared and issued, provided they are based on corresponding standard agglutinating sera, which

can be dried and preserved in the same way as standard therapeutic antisera (Felix 1950). The Central Enteric Reference Laboratory and the Standards Laboratory for Serological Reagents (Central Public Health Laboratory, Colindale, London) have now prepared eight horse antisera: Salm.typhi H, O and Vi; Salm.paratyphi A H and O; Salm.paratyphi B H (Phase 1), H (Phase 2) and O. These sera are to be examined in six countries on behalf of the Expert Committee on Biological Standards of W.H.O. for their suitability as international standard preparations. If it is agreed that they are satisfactory, they may be used in any country to standardize locally prepared agglutinable suspensions for issue.

ii. The δ antigen

A new antigen of some importance in serological work was described by Stamp and Stone (1944). During an outbreak of paratyphoid B these workers found that their diagnostic salmonella sera were producing agglutination of certain non-lactose-fermenting and lactose-fermenting bacteria which ultimately proved to be strains of Morgan's bacillus, paracolon or other coliform bacilli. Investigation showed that agglutination was caused by a natural antibody in some normal rabbit sera, corresponding to an antigen, which they named the δ antigen, of

common occurrence in many strains of coliform bacilli. Such strains are frequently found in human, rabbit and guineapig faeces, and stock rabbits often possess α agglutinins. The type of flocculation produced is granular or semi-floccular, and it appears rather more slowly than typical H agglutination.

It is obvious that, if sera for diagnostic work contain α antibody either naturally or by injection of organisms containing α antigen, misleading results may be obtained. The remedy is to select for serum production only rabbits free from α agglutination and, if necessary, to remove these antibodies from diagnostic sera by absorption with a suitable α -containing paracolon strain. It is now believed (Bensted, pers.comm.) that there is a range of different α agglutinogens. They are not related to any of the known somatic, flagellar or rough antigens.

iii The "X" antigen

This antigen, which had been described by Topley and Ayrton (1924), was re-investigated by the writer (Cruickshank 1939).

Own Observations

The antigen is liable to appear in broth cultures of many Salmonella which have been incubated for some days at 37°C. The X antigen is not specific

to any one type, so that there are obvious possibilities for cross-agglutination to occur where suspensions contain the antigen and sera contain the X agglutinin. The antigen is thermostable, survives steaming for $\frac{1}{2}$ hour and is unaffected by 0.25 per cent. of formalin. The agglutinates are slightly coarser than the usual somatic type, they form in 2-4 hours but extend to a few higher dilutions of the serum on overnight incubation. That this antigen might be a potential cause of trouble was revealed in the examination of serum from a colleague suffering from an obscure pyrexia, and it was shown that X antigen might be present even in 24-hour broth cultures and in suspensions (even alcoholized) from agar plates. This patient, whose illness was later diagnosed as a Brucella infection, had X agglutinins to a titre of 1/5,120 against a Salm.typhi-murium X suspension.

Of 200 Wassermann sera tested, 50 (25 per cent.) contained X agglutinins at a titre of 1/20, 30 (15 per cent.) at 1/40, 5 (2.5 per cent.) at 1/80 and 2 (1 per cent.) at 1/160. The frequency of X agglutinins was no greater in 63 sera from cases of typhoid and paratyphoid B fever or in 31 sera from cases of tuberculosis. Of 15 laboratory assistants, 3 had low-titre X agglutinins before receiving two doses of TAB vaccine, and 8 showed X agglutinins 14

days after the second inoculation. It would seem that the organisms in the vaccine contained a small amount of X antigen.

It is clear that, since X agglutinins are not uncommon in normal sera, Salmonella suspensions for diagnostic work should not contain X antigen. Eight H and 30 Standard Agglutinable Suspensions (Oxford) were tested and found to be free from X antigen, but it was present in two out of three Salm.typhi O suspensions from another laboratory. Details of the preparation of X antiserum for testing suspensions are to be found in the original paper.

iv Significance of Titres obtained in Widal Tests

(a) Even if full attention is paid to the preparation of bacterial suspensions, and to their standardization against Standard Sera, the assessment of the significance of titres obtained in Widal tests can only be made in the light of knowledge, not only of the duration of illness and the inoculation state of the patient, but also of the frequency of natural agglutinins to the enteric organisms in the general population. A considerable amount of information was available before the war and it is of interest to consider a number of similar reports issued during the war. The more important figures from these reports have been collected in Tables 5 and 6.

TABLE 5

Frequency of agglutinins to Salm.typhi and Salm.paratyphi B
in normal sera

		Percentage agglutinating at or over these titres													
(a) <u>H agglutinins</u>	No. of Sera	<u>Salm.typhi H</u>					<u>Salm.paratyphi B H</u>					Place			
		1/20.	1/40.	1/80.	1/160.	1/320.	1/20.	1/40.	1/80.	1/160.	1/320.				
Atkinson (1941)	400	9.5	6.0	2.5	1.5	0.3	5.8	1.8	0.5	0.3	0.0		South Australia		
Floch <u>et al.</u> (1946)	1747	-	6.4	-	-	-	-	1.0	-	-	-		French Guiana		
Singh (1951)	251	10.3	3.5	0.4	0.0	0.0	12.3	8.3	3.0	0.0	0.0		Jamshedpur, India		
Schwabacher <u>et al.</u> (1943)	100						† (1)	16.0	12.0	5.0	4.0	0.0		England	
							(2)	22.0	15.0	6.0	2.0	0.0		"	
(b) <u>O agglutinins</u>		1/20.	1/25.	1/40.	1/50.	1/80.	1/100	1/20.	1/25.	1/40.	1/50.	1/80.	1/100		
Cruickshank (1939)	200	15.5	-	4.5	-	0.5	-	11.5	-	3.5	-	0.0	-	England	
Atkinson (1941)	400	11.0	-	5.3	-	1.0	-	2.0	-	0.5	-	0.5	-	South Australia	
Schwabacher <u>et al.</u> (1943)	100 [†]	(1)	2.0	-	0.0	-	0.0	-	7.0	-	2.0	-	0.0	-	England
		(2)	9.0	-	2.0	-	0.0	-	22.0	-	8.0	-	1.0	-	"
Wilson (1945)		-	-	19.0	-	8.8	-	-	-	-	-	-	-	India	
Floch <u>et al.</u> (1946)	1747	-	-	-	9.4	-	-	-	-	-	1.8	-	-	French Guiana	
Singh (1951)	251	-	40.4	-	18.1	-	3.8	-	9.5	-	3.0	-	1.5	India	

- = Not tested

† = Independent observations by two teams on the same 100 sera. H agglutinins were tested against a Salmonella Group suspension.

TABLE 6

Frequency of agglutinins to Salm.typhi and Salm.paratyphi B
in the sera of inoculated persons

		Percentage agglutinating at or over these titres										
(a) <u>H agglutinins</u>	No. of Sera	Time since Inoculation	<u>Salm.typhi H</u>					<u>Salm.paratyphi B H</u>				
			1/100	1/200	1/400	1/800	1/1000	1/100	1/200	1/400	1/800	1/1000
Melnotte <u>et al.</u> (1948)	146	3-4 months	64.0	-	-	-	-	42.0	-	-	-	-
Report (1942)	{	1-3 years	58.9	27.9	14.1	10.3	0.0	82.6	62.0	48.2	17.2	6.9
Army Vaccine		70	3-12 months	100.0	62.9	37.2	17.2	4.3	100.0	90.0	80.0	58.6

		Percentage agglutinating at or over these titres												
(b) <u>O agglutinins</u>	No. of Sera	Time since Inoculation	<u>Salm.typhi O</u>					<u>Salm.paratyphi B O</u>						
			1/20	1/25	1/40	1/50	1/80	1/100	1/20	1/25	1/40	1/50	1/80	1/100
Wilson, 1945)	154	12 months	-	-	16.8	-	5.1	1.2	-	-	-	-	-	-
Melnotte <u>et al.</u> (1948)	146	3-4 months	-	-	-	-	-	3.4	-	-	-	-	-	0.0
Report (1942)	{	1-3 years	0.0	-	-	-	-	-	-	-	-	-	-	-
Army Vaccine		70	3-12 months	-	-	-	-	-	2.9	-	-	-	-	-

- = Not tested

A feature of these tables is the great difference between the results obtained by different workers. Some of these differences confirm previous observations that a high frequency of agglutinins, specially O agglutinins, is to be expected in the general population of districts in which enteric disease is endemic. The high figures for typhoid O agglutinins recorded by Singh in India are an example; also the higher frequency of typhoid than paratyphoid B O agglutinins, recorded by him and also by Atkinson in South Australia, reflect the relative incidence of these diseases in these areas. However, there are some discrepancies which cannot be explained in this way, for example the difference in the frequency of O agglutinins in inoculated subjects recorded by Wilson (1945) and in a Report (1942) from the Public Health Laboratory Service. Even more striking are the different results recorded in one series, in which the same sera were examined, using the same suspensions, in two different laboratories (Schwabacher et al. 1943). These discrepancies can only be explained by variations in technique and in the reading of tests, and are a strong argument in favour of standardization of reagents and methods, with the use of a control serum of known titre.

Some points emerge from these figures, however, mostly confirming previous knowledge. Inoculation with TAB vaccine produces high titres of

H agglutinins, which persist for years. Most observers agree that O agglutinins appearing in response to inoculation usually cease to be detectable after a year, but some workers, on the basis of experience in well-inoculated service communities during the war, affirm that O agglutinins at titres of 1/40, 1/80 or higher are not infrequent (Wilson 1945) and agree with the opinion expressed by Mole (1948) that estimation of H or O antibodies gives little or no assistance in the diagnosis of enteric fever in inoculated persons.

So far as this country is concerned, it seems correct to say that H titres to Salm.typhi or Salm.paratyphi B at titres over 1/50, and O titres over 1/80 or 1/100 will rarely be found in normal sera, so that these titres in patients with obscure pyrexia must be regarded as suggestive of enteric infection. In inoculated persons, H agglutinins are unlikely to be of much assistance, but O titres over 1/80 or 1/100 are likely to be significant. In case of doubt, a further specimen should be examined a few days later to determine whether the titre is rising.

(b) Cross-relation of somatic antigens. The possession by Salm.typhi and Salm.paratyphi B (and other salmonellae) of certain common somatic antigens causes a considerable degree of cross-agglutination

between these organisms. This is well-known and the opinion is sometimes held that the use of O suspensions of both organisms is unnecessary in the investigation of pyrexial illness of unknown origin. Precise enquiries were considered desirable.

Own Observations

(i) Sera were obtained from 58 cases of typhoid fever from two epidemics, and from 25 cases of paratyphoid B. These sera were tested against standard O suspensions of Salm.typhi, Salm.paratyphi A and B. The results are set out in Table 7. Of the 38 typhoid sera, 29 (76.3 per cent.) agglutinated Salm.typhi O, 32 (84.2 per cent.) Salm.paratyphi O and 22 (57.9 per cent.) Salm.paratyphi A O. Considering only the first two suspensions, in 22 cases the titre to the infecting organism was higher, while in 7 cases it was lower; in 9 cases the titres were equal or agglutinins to both organisms were absent. Of the 25 paratyphoid B sera, 22 (88 per cent.) agglutinated Salm.paratyphi B O, 12 (48 per cent.) Salm.typhi O and 2 (8 per cent.) Salm.paratyphi A O. The titre for the infecting organism was higher than for Salm.typhi in 21 cases, lower in one case, and in three the agglutinins were equal or absent. In 11 cases the use of a typhoid O suspension would have failed to detect agglutinins that would have been detected by the

TABLE 7

Cross-agglutination between Salm.typhi
and Salm.paratyphi B

Titres of sera from cases of enteric fever, tested
 against O suspensions of both organisms
 (Titres expressed as reciprocals)

Typhoid Fever						Paratyphoid B Fever		
Sera from Somerset outbreak			Sera mainly from Croydon outbreak			Sera from Liverpool and Southampton		
Case	Ty O	B O	Case	Ty O	B O	Case	B O	Ty O
1	1280	160	21	640	80	1	0	0
2	320	160	22	640	320	2	80	80
3	0	80	23	40	40	3	160	40
4	0	0	24	640	640	4	640	320
5	0	0	25	1280	180	5	80	0
6	320	320	26	160	80	6	160	20
7	160	320	27	640	160	7	320	80
8	80	40	28	320	80	8	40	20
9	20	0	29	320	320	9	160	0
10	160	80	30	20	40	10	160	80
11	0	0	31	640	320	11	80	0
12	320	160	32	640	320	12	160	20
13	640	0	33	320	160	13	80	0
14	320	320	34	320	40	14	0	0
15	0	0	35	1280	640	15	0	20
16	640	320	36	0	40	16	80	0
17	160	80	37	640	80	17	160	0
18	0	20	38	640	160	18	40	0
19	0	0				19	20	0
20	0	40				20	80	0
						21	160	0
						22	160	0
						23	320	40
						24	640	80
						25	320	40

homologous paratyphoid B O suspensions, the titres in 9 cases being over 1/80. It would thus seem advisable to use both O suspensions in the investigation of suspected cases of enteric fever in this country.

(2) Cross-agglutination between salmonellae is due to possession of common major antigens or to minor antigens some of which are only indicated by dots... in the schema. An interesting case occurred in the writer's experience in which puzzling findings were clarified by a knowledge of antigenic relations. A patient with obscure pyrexia showed high-titre agglutinins to Salm.typhi O but not to Salm.typhi H. One possibility was that he might have been infected with a non-motile strain of the typhoid bacillus. It was recollected that Salm.enteritidis has the same O antigen as the typhoid bacillus [IX (XII)], but an unrelated H antigen. Tests of the patient's serum revealed that he had in fact H agglutinins to high titre to Salm.enteritidis, and the diagnosis was later confirmed by the isolation of that organism from the pus from an empyema.

v New Ultra-Sensitive Serological Tests

(a) The polysaccharide lysis test was devised by Thomas and Mennie (1950) to overcome the difficulty that their typhoid cases, in Natal, gave much lower titres to the usual O 901 Salm.typhi suspension than to the infecting strain. The test is based on the observation by workers in Melbourne that red blood cells coated with specific bacterial polysaccharides were agglutinated by the corresponding agglutinins, and lysed if complement was present. Thomas and Mennie coated sheep red cells with a Boivin extract of Salm.typhi. Sera to be tested were absorbed with uncoated red cells to remove heterophile antibodies, and set up in a series of dilutions with treated cells and complement. The end point of complete haemolysis is the "polysaccharide lysis titre." These titres were much higher than the agglutinin titres to O 901 and usually somewhat higher than the titre to the homologous strain.

(b) The anti-globulin technique of Coombs has been used by Stewart and McKeever (1950) to amplify the titres of Salm.typhi O antibodies. The principle is to expose the bacteria to the patient's serum, then to determine their agglutinability by rabbit antiserum containing antibody to human serum (or globulin). The "indirect titres" thus obtained showed an average amplification of about 4, 6 and

41 times respectively in the sera of normal persons, persons inoculated with TAB vaccine and persons presently or lately infected with typhoid bacilli. It is suggested that the test might be used as a screening test for carriers (in whom titres were consistently high) or as an exclusion test in diagnosis of typhoid fever.

vi Vi Agglutination of Salm.typhi

The discovery of the Vi antigen of the typhoid bacillus was naturally followed by enquiries into the possibility that the presence of Vi antibodies in the patient's serum might be of diagnostic value. The technique was less straightforward than the usual agglutination test, because the necessary bacterial suspension was prepared from a typhoid strain possessing Vi antigen but also sensitive to the action of H and O agglutinins. It was therefore necessary, as a preliminary to the test proper, to absorb the patient's serum with a suspension of a typhoid strain (for example H 901) which possesses H and O, but no Vi, antigen. This was a cumbersome procedure in routine practice and might also have the effect of nonspecifically reducing the titre of any Vi antibodies present. The discovery by Bhatnagar of the Vi I strain, which is sensitive to Vi agglutinins but is unaffected by H or O agglutinins, was therefore particularly welcome. It was at first

used in the living state but a suspension preserved with formol is now usually used, in a density of 2,000 million organisms per ml.

Strict attention to technique is essential for consistent results. The test should be made in tubes 2 inches long and half an inch in external diameter, with the lower ends rounded. The sequence of serum dilutions commencing at 1/5, should consist of 1 ml. volumes, to each of which is added one drop (0.05 ml.) of Vi suspension. In this country a standard agglutinable suspension is issued by the Standards Laboratory and also a standard agglutinating Vi serum, which must be set up with each series of tests. The Vi titre of the serum under test is the dilution giving agglutination equal to that produced by the stated dilution (usually 1/1,200) of the standard serum. Additional controls required are a Vi-negative serum, and saline controls containing 0.85 per cent., 2.5 per cent. and 5.0 per cent. NaCl, the purpose of which is to give early warning of deterioration of the suspension (usually in the direction of excessive sensitivity, first apparent in the higher salt concentrations). All tubes are shaken, placed for 2 hours in the 37°C. incubator and left in the cold room for a further 22 hours. The control tubes (and the negative serum) should show a round "button" of deposited organisms

with a clean margin. Agglutination is indicated by greater or lesser degrees of "scatter" of the organism with loss of definition of the central button; in tubes showing "total" agglutination the button has disappeared, the base of the tube is covered with clumped and deposited organisms and the supernatant fluid is clear. "Trace" readings in the 1/5 and 1/10 dilutions may occur in some normal sera and are not to be regarded as significant.

As with all serological tests, the significance of Vi titres can only be assessed in relation to the frequency of Vi agglutinins in normal persons. Klein (1943) reported Vi agglutinins at a titre of 1/8 in 3 per cent. of 300 Wassermann sera. Soman (1945) using a living Vi I suspension and a preserved Vi suspension recorded that 8.3 per cent. of 300 Wassermann sera were positive at 1/5 and 2.65 per cent. at 1/10. The most important series is that of Mackenzie and Taylor (1945) who examined 1,040 employees of the Metropolitan Water Board by the standard technique and found that 3.7 per cent. had Vi agglutinins at a titre of 1/5 or over; searching tests failed to reveal typhoid infection in any of these persons. Much higher frequencies were recorded by some other workers, including Davis (1940) in Rhodesia and Morgan and Drysdale (1940) in the Sudan; for example, 55 of 200 "normal" Sudanese were stated to have Vi agglutinins at 1/5, and 37 at 1/10. There

is, however, considerable evidence that the technique and reading of results of the last two series were not made in the recommended way.

With regard to clinical cases of typhoid fever, Bhatnagar (1938) in an examination of 134 cases found that Vi agglutinins were usually produced at some stage, rising to a maximum of 1/50 or 1/100 by the 6th to 10th day and falling to about 1/10 by the 18th day. In a later report Bhatnagar (1944) states that an early rise in Vi titre is only to be expected in mild cases and that a high and sustained titre is of good prognostic significance; in certain types of typhoid fever Vi titres were low or absent. Seshadrinathan and Pail (1940) found Vi agglutinins at 1/10 or over in 79.2 per cent. of 255 cases. Soman (1945) found Vi antibodies in 61 per cent. of 75 cases at titres of 1/10 or over, and Bensted (1940) in 76.3 per cent. of 80 cases, mostly at titres of 1/40 to 1/100. The consensus would seem to be that Vi agglutinins are present in most, but not all, typhoid cases at some stage, that they may be transient and of low titre, and that they may have some prognostic significance. They may therefore be of some value in clinical diagnosis, though the test is in fact rarely done as a routine for this purpose.

It is however as an aid to the detection of the chronic typhoid carrier that the Vi agglutination test is of greatest value, since, as was first pointed out by Felix, Krikorian and Reitler (1935), Vi antibodies are present in the serum of most carriers. This was an important discovery, because the possession of H or O agglutinins has not been found to be a reliable guide, and the examination of large numbers of specimens of faeces and urine is laborious and may be fraught with occasional doubt as to the authenticity of the specimens; further the serological method is independent of the intermittency of excretion of the organisms. For example, Fry (1944) found only 22 of 80 specimens of faeces from one carrier positive over a period of 16 months but the Vi agglutination test was consistently positive at a titre of 1/20. There are numerous reports testifying to the value of the method. Positive results were obtained in South Africa by Pijper and Crocker (1937, 1943) with all of 13 carriers; in the United States by Eliot (1942) with 43 of 45 carriers, by Coleman (1942) with 62 of 82 carriers and by Klein (1943) with 37 out of 41; in India, by Bensted (1940) with 6 out of 7; and by Felix (1938) in Britain and elsewhere in Europe, with 49 out of 52 chronic carriers. It thus appears that about 90 per cent. of carriers have Vi agglutinins in their

blood. According to Felix, they may be absent in persons who have been carriers for very long periods (30 years or more), the assumption being that the Vi-antibody-producing apparatus has become exhausted. Some interesting cases have also been cited in which persons with high Vi titres have not been found to be excreting typhoid bacilli but have proved to be harbouring the organism in some closed focus, such as a typhoid osteitis.

The value of the Vi agglutination test as a "screening" test in the search for chronic typhoid carriers is obvious. It has been noted above that the antibodies are likely to be found in 3-4 per cent. of normal persons in this country, not an unduly high number. A striking example of its usefulness came within the writer's experience.

Own Observations

During a period of three years two groups, each of three cases, of typhoid fever had occurred in the Public Assistance Institution at Tavistock, Devon. The victims were mostly nurses and one death had occurred. Specimens of faeces from persons who might possibly have caused the cases had been examined from time to time with negative results. It was decided to try to find a carrier by means of the Vi agglutination test. On a single morning blood was

taken from the 64 inmates, mostly old people long resident in the institution, and from some of the staff. The results, available next day, showed that eleven persons had agglutinins to Salm.typhi H or O or both at a titre of 1/25 or over, but that only one had Vi agglutinins. This was a bedridden old lady who had been an inmate during the entire period. Her Vi titre was 1/100, and typhoid bacilli were isolated from her stools. She could give no history of typhoid fever. She was removed to an isolation hospital and proved to be a chronic carrier. After her return to the institution she was carefully nursed as a known carrier and no further cases have occurred in the years that have elapsed. This is a good example of the way in which an anxious situation was cleared up in 48 hours by means of the test. It may be stated that these observations were made in the early experimental days of the test, and that three specimens of faeces and urine from all the inmates were later examined at leisure in case some other carrier had been missed. This laborious procedure revealed no other carrier.

The proper use of the Vi test is to act as a pointer to a person whom it will be advisable to investigate very fully from the bacteriological point of view. In Pretoria, the very severe view is taken

(Pijper and Crocker 1943; Nelson 1947) that a person showing a Vi titre of 1/5 is to be regarded as a carrier (perhaps excreting such small numbers of typhoid bacilli that they cannot be detected by present cultural methods) and legal powers are available to segregate these persons or to give them some form of employment in which they cannot endanger the community. These workers use a modified method in which the tubes are centrifuged and read after the 2 hours' incubation at 37°C. to avoid the possibility of bacterial growth which might occur with longer incubation in the hot climate (Pijper, Crocker and Todd 1943).

A further important use of the test is to act as an additional safeguard in ensuring that typhoid convalescents are free from infection, on discharge from hospital. The usual "three consecutive negative specimens of faeces and urine" cannot guarantee bacteriological cure, but a patient likely to become a carrier will usually show Vi agglutinins in the blood. The existence of a steady or rising titre on examination at intervals of a few months warrants suspicion and indicates careful examination of faeces, urine and duodenal juice. This use of the Vi test is now recommended by the Ministry of Health (1945).

Other techniques for the Vi test have been

used. Brower (1944) compared four methods, including that of Detre, who used a living suspension of Ty 2 passaged in O antiserum till it is fully O-in-agglutinable, then deprived of its flagella by washing. None of the methods appeared to be so reliable as that described above, but the Detre method, with which Almon and Stovall (1940) had found 20 out of 26 carriers positive at titres of 1/40 or over, was the best. Saint-Martin and Desranleau (1951) describe a slide-agglutination test, with the use of a suspension of the Vi I strain, alcoholized, washed and resuspended in 50 per cent. glycerol-saline. Serum-suspension mixtures are shaken for 4 minutes and read after half an hour with a binocular microscope. Positive tests were given by 1.2 per cent. of 9,192 "normal" sera, 1.8 per cent. of 1,164 sera from inoculated persons, 15 per cent. of 530 sera from clinical cases of typhoid fever and by all of 26 sera from chronic carriers; 64 per cent. of the latter reacted in a dilution of 1/20 or higher.

vii The Vi Agglutination Test for Salm.paratyphi B

A test analogous to that used for the detection of typhoid carriers can be applied to chronic carriers of paratyphoid B bacilli. There is however no known strain of Salm.paratyphi B that is sensitive to Vi agglutinins alone, as is Bhatnagar's Vi I strain of

Salm.typhi. The absorption of H and O agglutinins from the serum under test by means of a Vi-negative strain is thus necessary. The test is therefore less suitable for routine purposes, and is also less valuable because chronic paratyphoid B carriers do not show Vi agglutinins with the same high frequency as typhoid carriers. Felix however has used the test successfully to help in deciding whether a paratyphoid B excreter is in fact a chronic carrier likely to have been the cause of an outbreak or a temporary excreter merely infected in the course of the epidemic. The chronic carrier will show a steady or rising titre of Vi (and possibly also O) antibodies on repeated tests made at intervals of 5 or 6 weeks (Wallace and Mackenzie 1947).

D. EPIDEMIOLOGY

1. Chronic Carriers

(a) A chronic carrier of enteric bacilli is usually considered to be a person who has harboured the organism for at least a year, without showing the usual evidence of infection. It is generally thought that 2-5 per cent. of typhoid patients become permanent carriers, the frequency being rather lower for paratyphoid B. Recent reports have confirmed these opinions. For example, Gray (1938) in Mississippi calculated that 3.27 per

cent. of typhoid patients become carriers, and Vogelsang and Bøe (1948) in Norway found that 3.3 per cent. of 360 typhoid patients and 2.0 per cent. of 1,027 paratyphoid B patients became carriers. The Norwegian workers consider that, if a person continues to excrete the organism three months after cessation of illness, the chances of spontaneous cure are small. Their series also presented a good opportunity to confirm the greater liability of females to develop the carrier state - 3 out of 4 of the typhoid carriers and 9 out of 10 of the paratyphoid carriers were women. A similar observation was made by Littmann, Vaichulis, Ivy, Kaplan and Baer (1948) in a series that was specially significant because the sex incidence of the acute cases from which the carriers were derived was equal. Vogelsang (1950) in his recent excellent monograph discusses the subject. In his Norwegian series as a whole 84 per cent. of 74 faecal typhoid carriers and 94 per cent. of 52 paratyphoid B carriers were females, and he wonders whether the sex ratio is due to the fact that women, as milk- and food-handlers, are most likely to be involved in the search for the source of outbreaks. However, his figures for typhoid patients who became carriers are very similar and he concludes that this is not so.

Some workers have made their observations the basis of interesting calculations on the incidence of typhoid carriers in the population at large. Gray (loc.cit.) calculated that there were 6,000 in the State of Mississippi (1/350 of the population) and Morzycki (1949) that 0.2 per cent. of the males and 0.4 per cent. of the females of two districts of Poland (population about 20,000) were carriers. Weinstein (1946) states that there are 500 registered carriers in New York City and estimates that there are 4,500 more who are not known. In such communities extensive outbreaks due to known carriers do not now occur and the fact that the unsupervised carriers (responsible in all for an average of 17 cases each year) pass on the infection quite infrequently, is a tribute to the success of modern methods of sanitary control.

Most chronic carriers are faecal excreters of the organism, but urinary excreters are specially important because urine is passed more frequently and, at least in the country, more casually. Pijper has mentioned the danger of such carriers employed as house-boys in South Africa, and several workers (Walton 1949, Neva 1949) have drawn attention to the problem in Egypt where a high frequency of urinary carriers (many of them temporary but no less dangerous) was found. Walton, in a group of 270 male food-handlers

at Ismailia, isolated Salm.typhi from 8 and Salm. paratyphi C from 6 men. Neva found 9 of 76 acute cases of enteric fever excreting the organism in convalescence. This high incidence is probably due to the damage to the urinary tract caused by schistosomiasis, a frequent condition in the Nile Delta.

The question of the existence of intestinal as opposed to gall-bladder carriers is important in relation to treatment by cholecystectomy. The bile carrier state can be determined by duodenal intubation. Some of the difficulties of the technique are evident from the work of Saphir, Baer and Plotke (1942) who isolated typhoid bacilli from the bile of 68 per cent. of 95 faecal carriers but in some cases only at the sixth or seventh attempt. Serious attention must obviously be paid to the views of Vogelsang (loc.cit.) who isolated the organism from the bile of all of 64 carriers; 62 were positive at the first test, the other 2 at the second attempt. Vogelsang doubts the existence of pure chronic intestinal carriers and finds little good evidence in the literature that a focus in the lower intestine or the appendix could be held responsible for the carrier state. It is true, however, that cholecystectomy fails to cure the faecal carriers.

2. Cure of the Carrier State

Only brief reference will be made to the considerable amount of published work on attempts

to cure carriers by treatment with various drugs and antibiotics. Many of these reports are of trials made on one or two cases. These are of limited value, depending on the care with which the existence of a true chronic carrier state was established, and of the stringency of the criteria of cure, which should include the examination of many specimens of faeces over at least a year, terminal examination of bile and perhaps observation of decrease or disappearance of Vi agglutinins. Even if cure is established the possibility remains that it might have occurred spontaneously, so that a single successful case can at best be regarded as a pointer to a more extended and equally strict trial.

Before the discovery of antibiotics a long list of drugs including soluble iodophthalein and the various sulphonamide preparations were tried with variable results. The writer made a small contribution during this period (Cruickshank 1942).

Own Observations

Seven chronic typhoid carriers in a mental hospital in Devon, who had been under observation for a year or more, were given mepacrine orally in a total dosage of 1.4 - 1.5 gm. in divided doses thrice daily over 4 or 5 days. Salm.typhi was readily and repeatedly isolated from all the carriers

during the fortnight after treatment was completed. Mepacrine in the dosage given therefore failed to influence the carrier state.

The arrival of the antibiotics held out more promise of success, for penicillin was found by Evans (1946) who examined 66 strains, to inhibit Salm.typhi in concentrations of 10 units per ml. in most instances, and sulphathiazole was found to act synergically with it (Bigger 1946; Stewart 1947; Thomas and Hayes 1947). It is usually possible to maintain a penicillin concentration of 2 units per ml. and a sulphathiazole concentration of 1-5 mgm. per 100 ml. in the blood, levels which should theoretically inhibit most strains of Salm.typhi. Of the various trials that have been made of penicillin-sulphathiazole for the treatment of chronic carriers two are outstanding. Fry, Jones, Moore, Parker and Thomson (1948) treated 17 carriers, first with continuous treatment then with the intermittent course suggested by Bigger for the elimination of "persisters." In spite of the very large amounts of penicillin and sulphathiazole given there were only three apparent successes. Bigger and Daly (1949) gave ten carriers a continuous course, in which they received 52 gm. of sulphathiazole and 30,000,000 units of penicillin in $6\frac{1}{2}$ days; one carrier was apparently cured. Four of the others were now given intermittent

treatment - four 3-day courses with rests, covering 21 days during which 100 gm. of sulphathiazole and 57,600,000 units of penicillin were given. Two cases passed the very stringent tests of cure. The results are thus more promising than any hitherto reported.

Streptomycin, aureomycin and chloromycetin have been tried but none have proved effective, in spite of the efficacy of the last-named drug in the treatment of clinical cases of typhoid fever. Recently Vaichulis and his co-workers (1950) claim to have cured 19 of 25 chronic typhoid carriers by feeding with suspensions of B. subtilis (probably the Marburg strain) which was shown to be antagonistic in vitro; they also claim further success with "synergistic therapy" - mass dosage of penicillin in combination with carinamide, three sulphonamides and iodophenolphthalein.

It would appear that cholecystectomy is still the only effective and fully tested method of curing typhoid carriers. This is the view of Feemster and Smith (1945) based on a careful study of the question, and of their own results which show a rate of cure of about 90 per cent. in persons definitely proved to be gall-bladder carriers. Vogelsang (1950) has collected 11 series of reports, dated 1929-45. When a recent series reported by Littmann, Vaichulis and Ivy (1949) is added to these, the complete figures are as follows:- total number

operated upon, 359: cured, 270 (72.9 per cent.): failed, 53: uncertain, 26: deaths, 20. The percentage of cures is probably in fact a little higher since some of those who did not complete the tests for cure and are recorded as "uncertain" were likely to have been cured. It is to be noted that the total fatality rate of the operation was 5.4 per cent. and that considerably higher rates are on record, especially in the elderly people who are often concerned.

3. Typing of Salm.typhi

(i) Fermentative Types

In 1901 Jensen had classified typhoid bacilli into three types: Type I fermented xylose but not arabinose, Type II did not ferment either "sugar", Type III (rarely found) fermented both. Numerous workers made a further study of the method, especially Kristensen (1938) who showed that the types were stable, and were constant for the same person and for persons infected from the same source. The method thus had epidemiological value and, although largely superceded by the Vi-phage typing technique, it is still sometimes used. For example, Olitzki and Shelubsky (1949) found it of value in Israel, where all strains typable by the Vi phage method were shown to be either A or C; by testing strains also for their ability to utilize citrate and d-tartrate, they classified their typhoid strains into six groups.

(ii) Vi-bacteriophage Typing

The typing of typhoid bacilli by specific bacteriophages is without doubt the most striking advance that has been made in the investigation of enteric disease. Craigie and Yen (1938 a and b) working in Canada had, along with Brandon, already described phages specific for Vi strains of Salm. typhi. These were of four types - Types I, II, III, IV - differing in serology, particle size, thermal death point and range of lytic activity. Phages of Type II, when propagated on given Vi strains, were now found to develop selectivity (due probably to the selective favouring of mutants) so that it was possible to obtain preparations which, when used in a "critical test dilution", permitted Vi strains of Salm.typhi to be divided into 11 different types, labelled A, B₁, B₂, C etc. The types were stable, in the sense that there was no change from one type to another, but loss of Vi antigen (V→W change) entailed loss of specific phage sensitivity. The value of Vi-phage typing in epidemiological work was quickly established. Cases infected from the same source carry the same type, so that any given epidemic is due to a single type and the source is to be sought in the person of a carrier or other individual excreting that type. The differences between the types cannot be demonstrated by any of the usual serological methods.

It may be mentioned that later work by Felix and Anderson (1951 a) indicates that phage-sensitivity is associated with lysogenicity of the bacteria, i.e. that phage type-specificity is determined by a resistance pattern evolved in strains of the organism as a result of contact with phages. Experimentally, by treating strains with phages obtained from lysogenic bacteria, type transformation has been carried out in the laboratory, but it seems unlikely that this happens often in nature or that it could influence the value of the method in epidemiological work.

Experience soon showed that typhoid Vi-phage typing was a powerful weapon for the epidemiologist and that it provided almost the only means by which a medical officer could link sporadic cases and trace their source of infection. The number of types rose from 18 in 1941 to 24 in 1944, and since that time 5 further types, making 29 in all, have been provisionally recognized pending further investigation. The types are designated A, B₁, B₂, B₃, C, D₁, D₂, D₄, D₅, D₆, E₁, E₂, F₁, F₂, G, H, J, K, L₁, L₂, M, N, O, T; and (the provisional types) 25, 26, 27, 28 and 29. Later research suggests that Type A, which is sensitive to all the typing phages, should be regarded as a variant of some of the other Vi types, but epidemiological observations warrant its retention in the scheme.

The full schema giving details of the reactions of these Vi-type strains to routine test concentrations of typing phages has recently been published by Felix and Anderson (1951 b). In any survey there will probably be found strains possessing Vi antigen but resistant to all the typing phages; these are known as "Untypable Vi strains". There also occur "Degraded Vi strains" which give overlapping cross-reactions with all or some of the typing phages. Vi-negative strains do not possess Vi antigen and are not susceptible to Vi phages.

Craigie and Felix (1947) have given an authoritative account of the present position of Vi-phage typing of typhoid bacilli. Only phages derived from the original Vi-phage II and issued from the Central Enteric Reference Laboratory should be used for typing. They should not be propagated locally to build up a new stock because of the danger of contamination by airborne phages or by phages carried on the bacteria used for propagation. Uncontaminated concentrated phage preparations retain potency for years at 4°C. and test dilutions keep for many months. The details of testing are important. A synthetic standard culture medium is not yet practicable, but "Bacto" agar or any good tryptic meat digest agar may be used, provided it is found to give satisfactory results with strains N, O and T against the full range of phages; these

strains support only small-size phage clearings.

Tests should be incubated at 37-38°C. (not lower)

and readings are best made at 8 and 24 hours.

Cultures submitted for typing should consist of at least six smooth colonies picked to a single Dorset egg slope, to minimize the chance of submitting a Vi-negative culture. Organisms are best maintained on the same medium at 4°C.

The Central Enteric Reference Laboratory in London now acts as the International Reference Laboratory from which Standard Vi-phage preparations (shown to be of the necessary stability) and the corresponding Vi-type strains are distributed to the various national laboratories in the 24 countries that have so far joined the scheme. This Laboratory also acts as a Bureau in which information about cases and carriers of enteric fever in Britain is collected. This information, specially the phage type of strains carried by carriers, has proved of great value in the elucidation of outbreaks.

Records are now available of the frequency of the different Vi-phage types of typhoid bacilli in various parts of the world, but, as Felix has pointed out, the frequencies have almost invariably been based on the number of cultures or patients examined. A much more correct idea of the true frequency is obtained by recording foci of infection,

so that a group of cases infected from a common source would count as one. Calculations made in this way (Felix 1951) show that the commonest types in the years 1942-49 in Great Britain are A (27.3 per cent.), E₁ (24.0), C (11.3) and D₁ (5.8). Types D₅, D₆, G, H and J are not indigenous to this country, and Types B₁, B₃, E₂, K, L₁ and M have not been recorded here. The other established types have all been isolated in Britain.

Personal Observations on the Epidemiology of
Typhoid Fever in a Rural Area

It is appropriate at this stage to give a short account of attempts made by the writer while in charge of Devon County Laboratory during 1941-44 to elucidate the sources and modes of spread of typhoid fever occurring mostly as scattered sporadic cases in this large rural area. With the co-operation of the various medical officers of health it was possible to isolate and find the phage-type of the infecting organism in all the clinical cases of typhoid fever that occurred during this period. Field inquiries were made and in many cases chronic carriers were located. The details are summarized in Table 8. There were 83 clinical cases in all, with 7 deaths (8.4 per cent.); 4 of the patients became chronic carriers (4.8 per cent.). of the 16 carriers located, 14 were women and 2 men.

TABLE 8

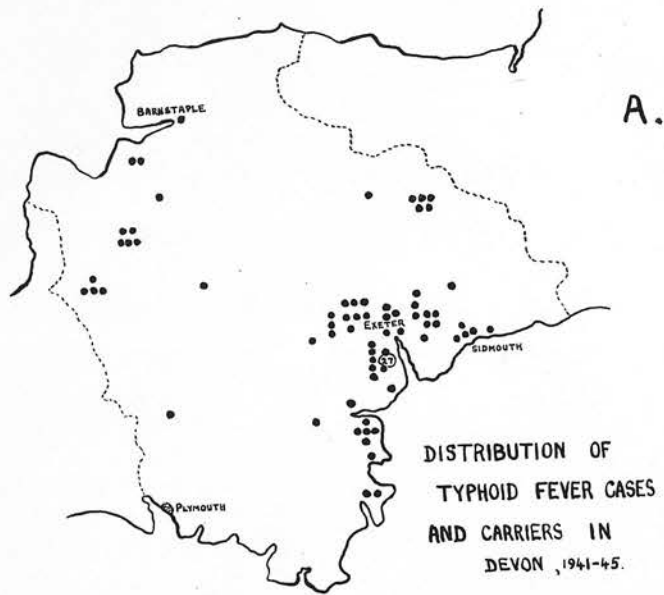
Vi-Phage Types of Typhoid Cases and Carriers
in Devon, 1941-44

Vi-phage type	Clinical Cases			Chronic Carriers (excluding cases which became carriers)	
	Total	Deaths	Number who became chronic carriers	Total	Number in Mental Hospitals
A ..	5	1	0	2	1
C ..	29	1	0	1	1
D1 ..	6	1	0	2	0
E1 ..	13	2	2	3	2
F1 ..	4	0	0	3	3
FO ..	22	2	2	1	0
New type Richmond	1	0	0	0	0
Untypeable Vi-forms	2	0	0	0	0
O form ..	1	0	0	0	0
Total ..	83	7	4	12	7

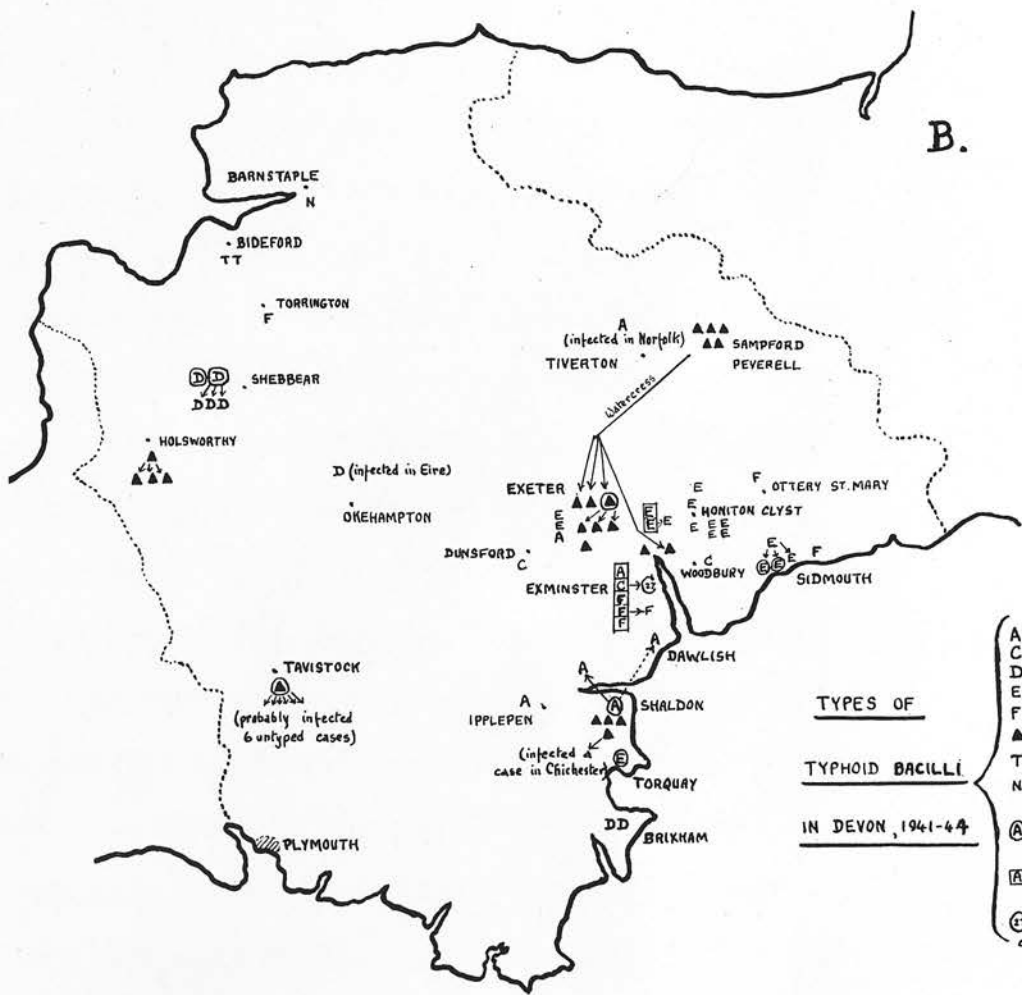
* Formerly known as "Type 91"

The results of the epidemiological investigations, illustrating the assistance given by Vi-phage typing, are shown in the two maps (Plate 1). Map A shows the location of typhoid cases and carriers each indicated by a spot in the only way possible before the advent of typing. Map B shows the much fuller information given by typing, and in numerous instances the links that it was then possible to establish between scattered cases and their source of infection. A few examples of different types of outbreak are given.

(a) Domestic groups. These investigations are relatively straightforward. For example, three young children in one household in the village of Holsworthy got typhoid fever, notable for its mildness - they might have eluded diagnosis if blood culture had not been done. The infecting strains were all Type O and the source of infection proved to be the lodger - a missed case, also of Type O - who travelled the country as a lorry driver. A similar group of cases (wife, daughter and house-keeper) were infected in Sidmouth by an elderly man in whom early diagnosis of the relatively mild illness was missed - he was a urinary excreter who unfortunately also had prostatic enlargement; he used a catheter, which he washed after use at the kitchen sink. The organisms all belonged to Type E₁.



DISTRIBUTION OF
TYPHOID FEVER CASES
AND CARRIERS IN
DEVON, 1941-45.



TYPES OF
TYPHOID BACILLI
IN DEVON, 1941-44

- A
- C
- D = D1
- E = E1
- F = F1
- ▲ = Type 91
- T = Imperfect Vi
- N = New Type ('Carlisle')
- Ⓐ = Carrier, type A
- Ⓐ = Carrier in Mental Hospital
- Ⓢ = Type C outbreak of 27 cases

PLATE 1.

A curiosity of this group was that two of the women became chronic carriers.

(b) Institutional infections. Typhoid carriers are fairly common among the inmates of mental hospitals and it is useful to have a record of their phage-type. In one instance this was of value in indicating which of five known carriers was responsible for infection in a nurse. In the same hospital an outbreak, described by Allan (1943), occurred in 27 nurses. They had obviously been infected at a meal in their dining-hall. All the cases were due to Type C bacilli. This finding excluded the responsibility of four known carriers in the institution (1 Type A, 3 Type F₁), and in due course an unsuspected Type C carrier was located - this was a mental patient sometimes employed to carry food in the kitchen. She had no history of typhoid fever and the evidence pointed to her as source of the infection.

(c) Scattered Cases. Cases "dropping" at intervals of a few weeks or months in widely scattered places present the most serious problem to the epidemiologist. In these circumstances phage-typing has a particular value in determining whether the cases have a single origin. If they belong to a number of types, a single source can be excluded (except perhaps when the contamination is from sewage, which might possibly contain more than one type of bacillus).

An instance occurred in the present investigations. Over a period of 23 months, cases (11 in all) appeared in a rural district lying to the east of Exeter and especially in service and civilian personnel employed at a large R.A.F. establishment. The cases were infected with Type E₁ and, as the station was an operational one, there was considerable anxiety lest a more serious outbreak should arise. At one stage, a fighter pilot billeted in a tavern near the aerodrome got typhoid fever. In the absence of phage-typing he would inevitably have been regarded as another case of the same group. However, his organism proved to be Type 0, not Type E₁. From our records the probable origin of the local Type 0 cases was known. This type had previously caused several cases in and around Exeter and had been traced to watercress from infected beds at the village of Sampford Peverell, 20 miles away. The false trail was therefore quickly avoided, and inquiries established that the victim had in fact eaten at the relevant time watercress obtained by the innkeeper from these same beds. The Type E₁ cases were later found to have been possibly conveyed by milk.

(d) Elucidation of the Isolated Case. The investigation of the apparently isolated case is probably the most difficult problem of all. It must comprise careful history-taking, and bacteriological

examinations of possible contacts (serological tests, particularly for Vi-agglutinins, and cultural tests of faeces and urine), the net being cast in ever-widening circles in the hope of disclosing a carrier. A fatal case (Type D₁) in a boy at a residential school at Shebbear was thus investigated and only when inquiries were pursued beyond the school precincts was a clue obtained. An old man, a gardener who visited the school daily, was found to be a Type D₁ carrier, and this led to the discovery that his wife was also a chronic carrier of the same type (with no history of typhoid fever). The old lady was probably responsible for the boy's infection, as she came in from time to time to assist at school tea-parties.

Finally, a striking example may be given of the elucidation of two isolated cases which would undoubtedly have remained unsolved in the absence of phage-typing. A boy living in a very isolated bungalow in the Newton Abbot district developed typhoid fever due to bacilli of Type A, a type rarely found hitherto in the county. After much inquiry the source of his infection was found in an occasional visitor to the bungalow, Mr. X, a member of the Observer Corps stationed on a neighbouring hill. Mr. X, unknown to himself, was a chronic typhoid carrier, and his bacilli were also

(b) Typing

of Type A. He was advised to move to a place where there was water-carriage disposal of sewage. Some months later another Type A case occurred in a maid in an institution at Dawlish, many miles away. The usual investigations provided no clue to the source of her infection. However, our records reminded us of Mr. X, the Type A carrier, who was soon located in the small seaside town of Shaldon. The girl's father was found to be a labourer working on the sewer outfall discharging from this town on to the shore, and the final link was a bag of winkles collected by him from the rocks nearby and eaten by the girl at the relevant time.

Typing of Salm.paratyphi B

(a) Fermentative Types

Attempts to classify strains of Salm.para-
typhi B according to their power of fermenting
rhamnose and inositol have found less favour here
than on the Continent, where Kristensen and others
claimed that the method was of epidemiological
value. It also appears that strains causing enteric
fever usually fail to ferment d-tartrate; they
produce a "slime-wall" around their colonies.
Strains causing gastro-enteritis ferment d-tartrate,
do not produce a "slime-wall" and are more pathogenic
to mice.

(b) Vi-phage Typing

Paratyphoid B bacilli possess a thermo-labile Vi antigen and a method of typing by specific bacteriophages, on the same principle as for typhoid bacilli, has been developed. By 1947 five types and subtypes were recognized, and this number has now increased to ten, some of which are commoner abroad than in this country. The types are: 1, 2, 3a, 3a₁, 3b, Beccles, Taunton, Dundee, BAOR and Newburn. The extended schema showing the details of the reactions produced by the typing phages has recently been published (Felix and Callow 1951). Type I strains are lysed by all the phages in a manner analogous to Type A typhoid strains. The typing phages were all derived from the original "Type I, Aberdeen" phage, with the exception of Beccles, which was derived from another Type I phage, and BAOR which was a phage carried by a Type I bacillus. This indicates that the enteric typing authorities have relaxed one of their rules, that all typing phages in any scheme should be derived from a single phage. Another relaxation of rule is that identification of strains should be by their sensitivity to single phages and not their pattern of reaction to a number of phages (as is the case with staphylococcal phage-typing). In fact, varieties of pattern within types have been observed, associated with geographical areas and perhaps

possessing epidemiological significance. The typing phages fall into three serological groups. There is considerable evidence that there is a close association between the type of a strain and the presence or absence of certain "natural" phages that it may carry. This connection between type and lysogenicity has been studied in detail by Nicolle, Hamon and Edlinger (1951) and Hamon and Nicolle (1951), who were able, by infecting organisms with phages extracted from other strains, to change bacilli from one type to another. It is not believed that these changes induced artificially in the laboratory happen in nature often enough to limit the usefulness of the typing method in epidemiology.

The value of Vi phage-typing of paratyphoid B bacilli has been amply confirmed since the first report of Martin (1947) at Ipswich. The frequency of the different types varies much more from year to year than does that of the typhoid types. The commonest types in Britain are 1, 3a and Taunton. The percentage of untypable Vi strains during the eight years 1942-49 varied from 8 to 17 per cent. It would probably have risen about 1945-46 with the return of soldiers and tourists to this country, but the discovery of the new typing phages about that time kept the figure to its previous level. Vi-negative strains of Salm.paratyphi B are very rarely encountered.

Some workers (Sholtens 1950, Boyd 1950) have suggested another method of typing paratyphoid strains, by identifying the phages that they carry. This method is unlikely to be practicable because, as Felix has shown, these natural phages are probably too great in number and variety.

5. Tracing of Carriers by Sewage Examination

It was shown many years ago by Wilson in Belfast, Gray in Edinburgh and others that it was possible to isolate enteric bacilli from the sewage of towns. The high efficiency and selectivity of the new culture media promised to make this a less difficult task and by their means King (1944) isolated paratyphoid B bacilli from the sewage-effluent of an ordnance factory and succeeded in tracking down a chronic carrier. In a more complex investigation Gell, Hobbs and Allison (1945) isolated typhoid bacilli from a stream, believed to have been the source of infection in a boy, and traced the infection back through a sewage effluent to an institution housing some 2,000 inmates and staff. Examination of sewage from separate blocks, then of pooled faecal specimens from groups of persons, ultimately led to the identification of a number of hitherto unsuspected carriers and to the elucidation of several typhoid infections of obscure origin. Typing by the Vi-phage method is of course an essential part of such investigations.

Clark, Geldreich, Jeter and Kabler (1951) in the United States have obtained successful results by filtering large volumes of water through a cellulose disc (the "membrane filter" of Zsigmondy) which is then laid on an absorbent pad soaked in double strength Wilson and Blair fluid medium in a sterile Petri dish. Typical colonies developed on the surface of the disc, and, in laboratory experiments, about half the typhoid bacilli added to the water sample were recovered.

The latest development is that described by Moore (1948) who devised a method of isolating paratyphoid B bacilli from flowing sewage. A pad of gauze, 4 ft. by 6 inches folded into 8 thicknesses, was secured by string and lowered through the manholes so that it was immersed in the sewage. After 48 hours the swab was withdrawn, sent to the laboratory in a suitable jar and cultured in fluid selenite medium from which platings were made on to a modified deoxycholate citrate agar. By this method Moore traced infection back from the sewer outfall of a seaside town through the sewage tributaries to the house of an ice-cream vendor whose wife was a chronic carrier and had almost certainly been the source of numerous cases. In a survey of a larger town the method disclosed carriers in almost embarrassing abundance. Later, Moore (1950) adapted the method

for the detection of typhoid carriers. For Salm.
typhi the gauze swab was washed with 20 ml. of
broth and three five-fold dilutions of the washings
plated in 0.1 ml. volumes on well-dried Wilson and
Blair agar plates. Selenite enrichment was also
used. The latest recommendation is to leave the
swab for 5 to 7 days, then to place it in 50 ml. of
double strength selenite from which, on arrival in
the laboratory, three dilutions are made - 1/10,
1/100 and 1/1,000. The relatively large amount of
selenite medium tends to prevent overgrowth by
unwanted organisms and to dilute inhibitory substances
often present in sewage. After overnight incubation
platings are made on deoxynolate citrate and Wilson
and Blair media. By this technique Lendon and
Mackenzie (1951) traced typhoid bacilli of Type E₁
from a contaminated stream through the sewers of a
residential area to a house in which a carrier of
the same type - a 70-year old man with no history
of enteric fever - was discovered.

E. PROPHYLACTIC IMMUNIZATION

Many papers on prophylactic immunization against
the enteric fevers have appeared during the last 12
years. The occurrence of outbreaks of typhoid fever
in some well-inoculated service communities indicates
that there is room for improvement in the vaccines
in present use, and research has mainly been directed

towards the development and assessment of new types of vaccine. Up to the outbreak of war the commonest vaccine was one in which the organisms were killed by heat and preserved with phenol. When the importance of the Vi antigen was understood, other methods in which that antigen was preserved had to be devised. The main factors to be considered are therefore: the selection of bacterial strains, the mode of killing and preservation, methods of laboratory assay of potency, the question of standardization, and the assessment of efficacy in the field. It is of interest to review the work of the two main groups - Felix and the service authorities in the United Kingdom, and Batson, Longfellow, Luippold and others of the United States Army - since this exemplifies the two commonest types of vaccine now in use, and illustrates some of the difficulties in their assessment.

1. The British Alcoholized Vaccine

The bacterial strains used in this vaccine are of maximum mouse virulence, possessing a full quota of O and Vi antigen. The H antigen has no immunizing value. The typhoid, paratyphoid A and B strains are respectively designated Ty2, HA6 and HB3. There is no evidence to support any contention that it is better to use freshly isolated or local strains; nor is there any evidence from laboratory or field

studies to suggest that different Vi-phage types of bacilli should be represented in the vaccine. The important quality of a strain is that it should be able to maintain consistently its production of Vi antigen in the conditions of artificial culture and maintenance. Methods of selection of typhoid strains by assaying their virulence in mice would gain in significance if it was known that there was a correlation between virulence for man and mouse. Interesting evidence that this correlation exists has been produced by Findlay (1951) who found, in comparable tests, that 5 strains from a mild outbreak produced the following ratios of deaths to mice challenged (percentages in brackets): 0/20 (0), 1/20 (5), 2/100 (2), 0/10 (0) and 1/10 (10) - against the following figures for 5 strains from a severe outbreak, inoculated in the same dosage: 8/20 (40), 8/20 (40), 53/100 (53), 10/10 (100) and 7/10 (70).

Preliminary experiments (Felix 1941) showed that phenolized TAB vaccine produced little or no Vi antibody response in rabbits or horses, and that alcohol-killed organisms produced Vi antibodies in these animals and in about half of a group of human subjects. The next step was to find a suitable preservative since alcohol-killed bacteria stored in 0.5 per cent. phenol or 0.35 per cent. tricresol lost the capacity of stimulating Vi antibodies. The

solution was to use alcohol also as preservative; organisms killed with 75 per cent. alcohol and preserved in 22.5 per cent. alcohol showed no deterioration over long periods.

It was necessary to make certain that this concentration of alcohol had antiseptic properties adequate to guard against contamination of the vaccine. With this object a group of workers, including the writer, undertook a series of experiments (Cruickshank, Hobbs, McFarlan and Maier 1942).

Own Observations

Vaccine bottles containing 25 ml. quantities of TABC vaccine preserved with (a) 0.5 per cent. phenol, (b) 22.5 per cent. alcohol and (c) 25 per cent. alcohol were contaminated by the addition of Staph.aureus, Bact.coli, Ps.pyocyanea, B.subtilis (spores) and Cl.sporogenes (spores). The bottles were held at laboratory temperature and bacterial counts were made at intervals. In no instance was there any destructive effect on the sporing organisms. In all the other experiments in which results could be compared, the 25 per cent. alcoholized vaccine became sterile first and the 22.5 per cent. alcoholized vaccine last, with the 0.5 per cent. phenolized vaccine in an intermediate position. However, in spite of the very large inocula - far greater than could arise from an imperfectly sterilized

needle in practice - the contaminating organisms were not always destroyed within 24 hours even with the use of 22.5 per cent. alcohol. The general conclusion was that 22.5 per cent. alcohol was only slightly inferior to 0.5 per cent. phenol in its germicidal potency and that it is capable of destroying within a few hours vegetative bacteria that may gain access to TABC vaccine by contamination.

The recommended dosages of the alcoholized vaccine for adult males are 0.25 ml. and 0.5 ml. at an interval of at least 10 days (0.2 ml. and 0.4 ml. for females). Local and general reactions are milder than those caused by phenolized vaccine (Felix, Rainsford and Stokes 1941). The same workers found in groups of naval recruits that the vaccine produced a Vi antibody response six to seven times greater than heated or phenol-preserved vaccines. It was hoped that the alcoholized vaccine would not stimulate H agglutinins and that the Widal test in inoculated persons would thus gain in usefulness, but this proved not to be so. Confirmation of the mildness of the reactions and the adequacy of the O and Vi antibody response was also obtained by De Amaral and De Lacerda (1947), who observed Vi titres mostly at a level of 1/80 - 1/320 after the usual two inoculations, and a two-fold to ten-fold rise in titre in previously inoculated persons.

A number of methods of laboratory assay of vaccines are available. Some of these are probably a more reliable index of potency than others. The active protection test in mice, in which animals immunized with vaccine are subsequently challenged with living organisms, fails to reveal some types of damage to the Vi antigen produced by sterilization procedures. In this respect passive protection tests with the use of serum from immunized rabbits have proved more satisfactory (Felix 1951c). A complete assay, in Felix's view, should comprise active protection tests in mice (which are relatively easy to do and can be used to reject unsatisfactory vaccines), immunization of rabbits with estimation of Vi and O antibody response and of passive immunizing value of the serum in mice, and tests of Vi and O responses in man. On the basis of good results in these tests, the alcoholized vaccine was adopted by the Royal Air Force in 1943 and the British Army in 1944.

Standardization of the potency of vaccines is desirable, and for this purpose Felix suggested that a dried "Provisional Standard Anti-typhoid Serum" might be used as a basis. A recent advance of some importance is the observation that alcoholized vaccine retains its immunogenic properties unimpaired for at least 10 years if stored at 1-2°C.

(Felix and Anderson 1951b). An analogous observation was made by Henderson, Peacock and Richley (1951) who found no loss of potency in an acetone-dried suspension of Salm.typhi kept for 10 years in a variety of stores and cellars often at hot summer temperatures. Future recommendations for vaccine standardization will therefore probably be as follows: For vaccines properly prepared from reliable strains it will suffice to test occasional samples in rabbits for production of adequate Vi- and O-antibody response, in parallel with a "standard vaccine" of one of the types just mentioned. For official control a complete assay would be required, comprising comparative active immunization tests in mice and rabbits with the vaccine under test and the "standard vaccine", and passive protection tests in mice with the use, in parallel, of the rabbit sera and the "Provisional Standard Anti-typhoid Serum." The components of TABC vaccine other than Salm.typhi would be tested in the same way.

2. The United States Army Vaccine

Since 1936 the United States Army Medical School has used for vaccine-preparation the typhoid strain variously known as Strain 58, Boxhill or the Panama Carrier strain. Since the first World War the policy of inclusion or omission of paratyphoid A and B bacilli in the vaccine has changed several

times, but they are now included. The organisms are killed by heating at 56°C. for 1 hour and are preserved with phenol. Batson (1949) in his review of the subject admits that alcoholized vaccine will stimulate the production of Vi antibodies in a greater proportion of persons than will heat-killed phenol-preserved vaccine, but contends that, although the antibody response to the latter is predominantly of the anti-O type, the most successful experiences in mass immunization have been accomplished with it. He considers however that future research may indicate the advisability of retaining the Vi antigen in an unaltered state or of fortifying the vaccine with Vi-containing extracts obtained perhaps from other coliform bacteria possessing Vi antigen (Luippold 1946).

At least two doses of vaccine are required, and a usual course consists of 3 subcutaneous injections each of 0.5 ml. Intradermal inoculation was found to give a remarkably good antibody response, with a minimum of untoward reactions, to a relatively small dose, and it is recommended for re-stimulation of previously immunized persons. On the basis of studies of sera from normal persons and convalescents, Longfellow and Luippold (1940) concluded that a person whose serum protected mice against 100 minimum lethal doses of typhoid bacilli (as tested by their mucin method) was immune and that immunity

induced by inoculation lasts 1-2 years, or longer in some cases.

For assay of vaccines the usual American practice has been to immunize groups of mice actively with a constant dose of vaccine and determine their resistance to increasing multiples of lethal doses of the organism, suspended in mucin. Luippold (1945) proposed a "Typhoid Immunogenic Unit" based on survival of mice given graded doses of antigen and challenged with a constant dose of bacteria. A detailed description of the current practice of the U.S. Army Medical School has recently been given by Batson, Brown and Oberstein (1951). It consists in comparing the survival rates in groups of mice given graded doses of the unknown and of a reference standard vaccine, and subsequently challenged with a constant dose of Salm.typhi in mucin. Very full details are given in this paper of the method of performing the tests and of calculating relative potencies by the Knudsen-Curtis method. The authors give their reasons for considering it the most satisfactory biological assay available, but it has nevertheless been severely criticized by Felix and Pitt (1951) on the following grounds: They consider that Batson has failed to correlate virulence with possession of Vi antigen because he studied his strains by the "crude and inaccurate" technique of slide-agglutination and that his challenge strain

is of inadequate virulence. They consider that the mucin technique (which inhibits the normal action of the phagocytes and allows the organism to multiply) does not make a strain more virulent or make the test more like a human infection, but only introduces additional unknown factors. They point out that immunization and challenge by the same (intraperitoneal) route produces fallacies due to the induction of non-specific resistance, for which there is considerable evidence. For active immunity tests Felix immunizes subcutaneously and challenges intraperitoneally three weeks later; passive immunization (which he prefers for reasons already stated) is given intramuscularly with intraperitoneal challenge two days later.

3. Comparison of the British and American Vaccines

An interesting comparison of the two types of vaccine has been made recently by Miller, Clark and Dierkhising (1951) in Egypt. The stimulus to this work was the observation that there had been a number of severe epidemics of typhoid fever among British troops in the Suez Canal Zone since 1944, with attack rates of 15-34 per cent. and case fatality rates of 10-11 per cent. Some of these troops had received alcoholized vaccine. Two British alcoholized vaccines, and a British and an American phenolized vaccine were tested for

agglutinogenicity in rabbits and mice, and for ability to confer active protection on mice. The results were consistently in favour of the British vaccines. In the protection tests the alcoholized vaccines were about three times more effective than the British phenolized vaccine, and twenty times as potent as the United States phenolized vaccine. No explanation for the differences is offered. The United States armies appear to have had in recent years much less typhoid fever than the British, but there is of course no evidence that troops immunized with the U.S. vaccine and exposed to the same risk would in fact have had a more favourable experience.

4. Other Types of Vaccines

Numerous other types of vaccine have been described during the last ten or fifteen years. The claims made for them usually are based on their ability to stimulate O and Vi antibodies in man or in laboratory animals. The methods used for assay have been diverse and there are few reports of trials in the field that would enable one to assess their comparative value. It is therefore not proposed to discuss them in detail. They include vaccines sterilized by silver and merthiolate (Rainsford 1939), brilliant green (Ruiz Merino 1946), formalin (Spitznagel and

Trainer 1949), ether (Gohar and Eliam 1942), acetone (Monaci, Riga and Scarpari 1947); adsorbate vaccines, consisting of formolized extracts of cultures adsorbed on aluminium hydroxide (Walters, Fiscoeder and Weidenmuller 1950: Rauss 1942); and antigenic extracts of the bacteria produced by chemical fractionation (Morgan 1945). The report on the adsorbate by Rauss records that, in a period of three years after immunization, the typhoid morbidity rates per 10,000 of a population in Hungary were 8 for a group of 12,813 persons receiving one injection of alum-precipitate, 7 for 10,962 receiving three doses of an ordinary vaccine and 23 for an unimmunized control group of 10,059. The vaccine about which most field data are available is the "endotoxoid" of Grasset (1939, 1945, 1951) developed in South Africa. This is an extract of a Vi strain of Salm. typhi, obtained by freezing and thawing, formolized to abolish its toxicity. It gives relatively mild reactions on injection. Grasset has produced in successive papers figures relating to increasing numbers of persons inoculated with endotoxoid, the last published figure being over three million, including several hundred thousand African labourers in the Rand gold mines. He claims that, as compared with the five-year period before endotoxoid was used, there has been a nineteen-fold reduction in morbidity and an eighteen-fold reduction in mortality from

typhoid fever in the mines. This mode of assessment in different periods of time, during which sanitary and other conditions may have improved, does not commend itself to the statistician in the same way as a properly controlled trial, but the figures are nevertheless striking and are supported by the low incidence (0.24 per 1,000) of typhoid fever in inoculated South African troops in the desert campaigns during the war.

Two further points merit brief comment only. Various workers (Freund and Bonanto 1946, Mann and Spinka 1946) have shown that antibody production in rabbits can be enhanced and prolonged by suspension of the vaccine in lanolin-like substances and oils. Some experimental work has been done in man, but it is not yet possible to say whether it is a worthwhile procedure. Oral administration of vaccines would, if effective, be a valuable method for certain communities. The recent work (Guarnacci 1949, Mukerjee 1944) in rabbits, monkeys and man confirms previous observations that if antibodies are stimulated at all by oral vaccines they are of relatively insignificant titre compared to those that appear after parenteral administration. Some Canadian workers (Dolman 1948) feel so strongly that such vaccines are ineffective and liable to give rise to a false sense of security that they recommend legislation to prevent their manufacture.

5. Anti-Typhoid Inoculation: The Present Position

The figures collected by the Anti-typhoid Committee and analyzed by Greenwood and Yule in 1915 left little doubt that inoculation greatly reduced the incidence of enteric fever. Later, inoculation became universal in most armies and controlled figures are rarely obtainable. It is true that the reduction in morbidity and in death rates in various parts of the world has been striking - in the United States, for example, from 31.3 deaths per 100,000 in 1900 to 0.4 in 1945. It is impossible to estimate the part played by prophylactic immunization in lowering the frequency, but in service communities it has probably been an important, if not the dominant, factor. Of the many observations that have been made, a clear example is that of Malbin (1940) who described a water-borne epidemic of typhoid fever in a hospital during the Spanish Civil War: in the unimmunized the incidence was about 25 per cent. as compared with 6 per cent. in the immunized, with case fatality rates of 10.2 and 4 per cent. respectively. Boyd (1943) reported the very much more favourable experience of prisoners inoculated with a British than with an Italian anti-typhoid vaccine in the North African campaign; both groups had been exposed to the same highly insanitary environment and the evidence suggested strongly that the difference was due to the higher potency of the

British vaccine. From such reports it seems probable that active immunization with a properly prepared vaccine gives effective protection against ordinary risk. There are, however, a number of observations showing that this protection is not absolute and that massive infecting doses can produce a high frequency of cases even in a well-inoculated community. This is exemplified by the outbreaks in troops in the Suez Canal Zone to which reference has been made. A typical epidemic was that described by Anderson and Richards (1948) in a Royal Air Force mess. Of 747 persons at risk, 14.7 per cent. were attacked and the case-fatality was 10 per cent. Felix insists that the alcoholized vaccine must be kept continuously at refrigerator temperature during transit and storage, and that it is otherwise unfair to subject it to adverse criticism. This may account for some failures, but it seems unlikely to account for all of them. The conclusion is that further research and trial are indicated, in the hope that a more effective and reliable immunizing agent may be developed.

Summary and Comment on Part I

During the last two decades new methods of interest and value have been added to the armament of the bacteriologist. In the diagnostic field, the efficacy of blood culture in the early stages of

enteric fever has been confirmed, and a number of reports suggest that this procedure is of more value in the later weeks than is often recognized. Culture of the bone marrow is a slightly more troublesome technique but all the available figures show that more positive results are obtained by it than by blood culture, and it might be worthy of wider use.

An outstanding advance has been the development of new culture media for the isolation of enteric pathogens from faeces and urine. Wilson and Blair agar holds its place as the best solid medium for typhoid bacilli; it gives good results also for paratyphoid B bacilli but for this organism deoxycholate citrate agar is quicker and almost as effective. Of the fluid enrichment media, tetrathionate broth is excellent for paratyphoid B but is less satisfactory for typhoid bacilli unless a special "balanced" formula is used. Many workers now prefer selenite enrichment medium which is easier to prepare and works well for both organisms. Ingenious screening techniques have been devised with a view to saving time and culture medium. A number of rapid methods of eliminating non-pathogens by quick micro-tests for indole-production and urea-splitting have been described. One result of the use of more efficient culture media is that views have had to be revised

about the duration of excretion of Salm.typhi and Salm.paratyphi B in convalescence.

The most important new knowledge about the organisms themselves relates particularly to their antigenic structure, and specially to the discovery of the Vi antigens and their significance. Since the earliest days of serology, bacteriologists have striven towards standardization of reagents and methods. Attainment of this objective has been brought appreciably nearer by the establishment of the fact that the "yardstick" must be (as with diphtheria antitoxin and other biological reagents) a dried specimen of a suitable antiserum, and the necessary eight horse antisera against the important antigenic components of the typhoid-paratyphoid A and B group have been prepared. The necessity for the standardization of agglutination techniques has been recognized and the importance of the temperature of incubation has been emphasized by the discovery that O antibodies are more thermo-labile than was previously supposed.

There have been notable advances in the epidemiological field. The observation that chronic typhoid carriers almost always possess Vi agglutinins has proved of great assistance to the medical officer searching for the source of an outbreak and the intermittent excreter is more readily brought to light.

The Vi-phage method of typing typhoid and paratyphoid bacilli has put a powerful new tool in the hands of the investigator, so that no enquiry into sporadic or epidemic enteric fever is complete without it. Foci of infection have been defined and scattered cases linked and traced to their source. In the writer's investigations in Devon eight Vi-phage types of typhoid bacilli were identified indicating that there was at least that number of separate foci of infection. The reservoirs from which 47 cases acquired typhoid fever were discovered with reasonable certainty, and in 37 of these cases the source of infection was a previously unrecognized chronic carrier. The interesting new methods of tracing infection back through sewerage systems have also helped to clear up a number of epidemiological puzzles. So far, attempts to cure carriers with the newer drugs and antibiotics have not given very promising results, but chloramphenicol has been strikingly successful in the treatment of clinical cases.

In prophylaxis the importance of the Vi antigen is reflected in the development of vaccines in which this antigen is preserved. There has been considerable controversy regarding the necessity for its full preservation because the communities inoculated with Grasset's endotoxoid and with the phenolized vaccines used by the American service

authorities appear to have had a low incidence of enteric fever although the Vi antigen is known to be destroyed or damaged severely in the preparation of these vaccines. One cannot fail to be impressed by the careful work of Felix in demonstrating that Vi antigen is essential for full mouse-virulence of the organism and that vaccines in which it is retained are of high protective value in laboratory experiments. The crucial test must be a comparison of the degree of protection conferred upon persons at risk under field conditions, and for this purpose it is essential that the vaccines should be prepared and stored in the recommended way. It is unfair to criticize adversely, for example, an alcoholized vaccine which has been made from "wild" or otherwise antigenically deficient strains, or has been transported or stored outside the refrigerator. There is evidence that the failure of some vaccines to protect certain service communities may thus be explained. It is probable that the superior results obtained with the British alcoholized vaccine in laboratory tests by a combined British-American team (Miller et al, above) will sway the United States authorities in favour of changing to a more potent strain, such as Ty 2, and a method of preparation which preserves the Vi antigen. Vaccines consisting of organisms treated with acetone and dried also

retain their antigenic potency and, if a safe and convenient method of resuspending them for injection can be devised, they might be widely adopted for use overseas.

knowledge of the clinical features and epidemiology of undulant fever in this country, based chiefly upon records submitted to him from clinicians and pathologists. In 1949 a symposium was held in Bethesda, Maryland, at which the recent advances in the laboratory, veterinary and other aspects of brucella infection were very fully discussed. They were later published ("Brucellosis" 1950) by the American Association for the Advancement of Science. It is therefore proposed to confine the present discussion to an account of some personal investigations made during the past six years.

A Study of 403 Recently Isolated Brucella Strains

The material for this work consisted entirely of strains submitted to the Brucella Reference Laboratory of the Public Health Laboratory Service from regional laboratories of that service. Some strains were referred by pathologists and not the facilities for precise identification. Some specially interested workers submitted all the strains they isolated, others only those strains that seemed in any way unusual. A number were sent by hospital pathologists in this country, and some were received from laboratories in Europe and Africa.

II BRUCELLOSIS

In his Milroy lectures for 1950, Sir Weldon Dalrymple-Champneys made a survey of present knowledge of the clinical features and epidemiology of undulant fever in this country, based chiefly upon records submitted to him from clinicians and pathologists. In 1949 a symposium was held in Bethesda, Maryland, at which the recent advances in the laboratory, veterinary and other aspects of brucella infection were very fully discussed; they were later published ("Brucellosis" 1950) by the American Association for the Advancement of Science. It is therefore proposed to confine the present discussion to an account of some personal investigations made during the past six years.

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The strains were mostly isolated from human cases of brucellosis, or from routine samples of milk, or from milk sampled in the course of epidemiological investigations. The isolations from milk were usually made by guineapig inoculation, and the pathologist sending the strain had already determined by the usual tests that the organism in fact belonged to the Brucella genus.

1. Differentiation of Brucella Species: Methods

Strains were grown on liver agar and checked for purity and general identity by microscopic and serological examination (with the use of a Brucella abortus antiserum). They were then submitted to the following tests:

(a) Atmospheric requirements

The strains were inoculated in duplicate on liver agar slopes, one of which was incubated at 37°C. aerobically and the other in a jar containing 10 per cent. of added CO₂. The degree of growth was compared after 1, 2 and 3 days' incubation, and it was recorded whether CO₂ was necessary or had an enhancing effect on growth. Typical freshly isolated strains of Br.abortus require CO₂.

(b) Production of hydrogen sulphide

Production of H₂S was tested by the insertion of a strip of filter paper impregnated with lead acetate between the glass and the screw cap of the tube of liver agar. The amount of blackening was recorded and a fresh strip inserted each day for the first four days of growth. Fig. 4 shows the typical reactions given by Br.abortus, Br.melitensis and Br.suis (American and Danish types) in this test. Br.abortus and the American type of Br.suis produce H₂S on each of the first few days of growth. Br.melitensis may or may not produce a small amount on the first day only. The Danish types of Br.suis do not produce H₂S. It will be noted that the second day of observation is important - a strain producing H₂S on that day is not Br.melitensis.

(c) Sensitivity to Dyes

This valuable test was at first done by the conventional method described by Huddleson (1929, 1931) in which appropriate concentrations of the four test-dyes are incorporated in liver agar plates. Two concentrations of each dye are used:- thionin 1/30,000 and 1/60,000, basic fuchsin 1/50,000 and 1/100,000, methyl violet 1/50,000 and 1/100,000, pyronin 1/100,000 and 1/200,000. The plates are ruled so that nine strains can be tested on each plate, and on a liver agar plate without dye, as a

H₂S Production by Brucella Types.

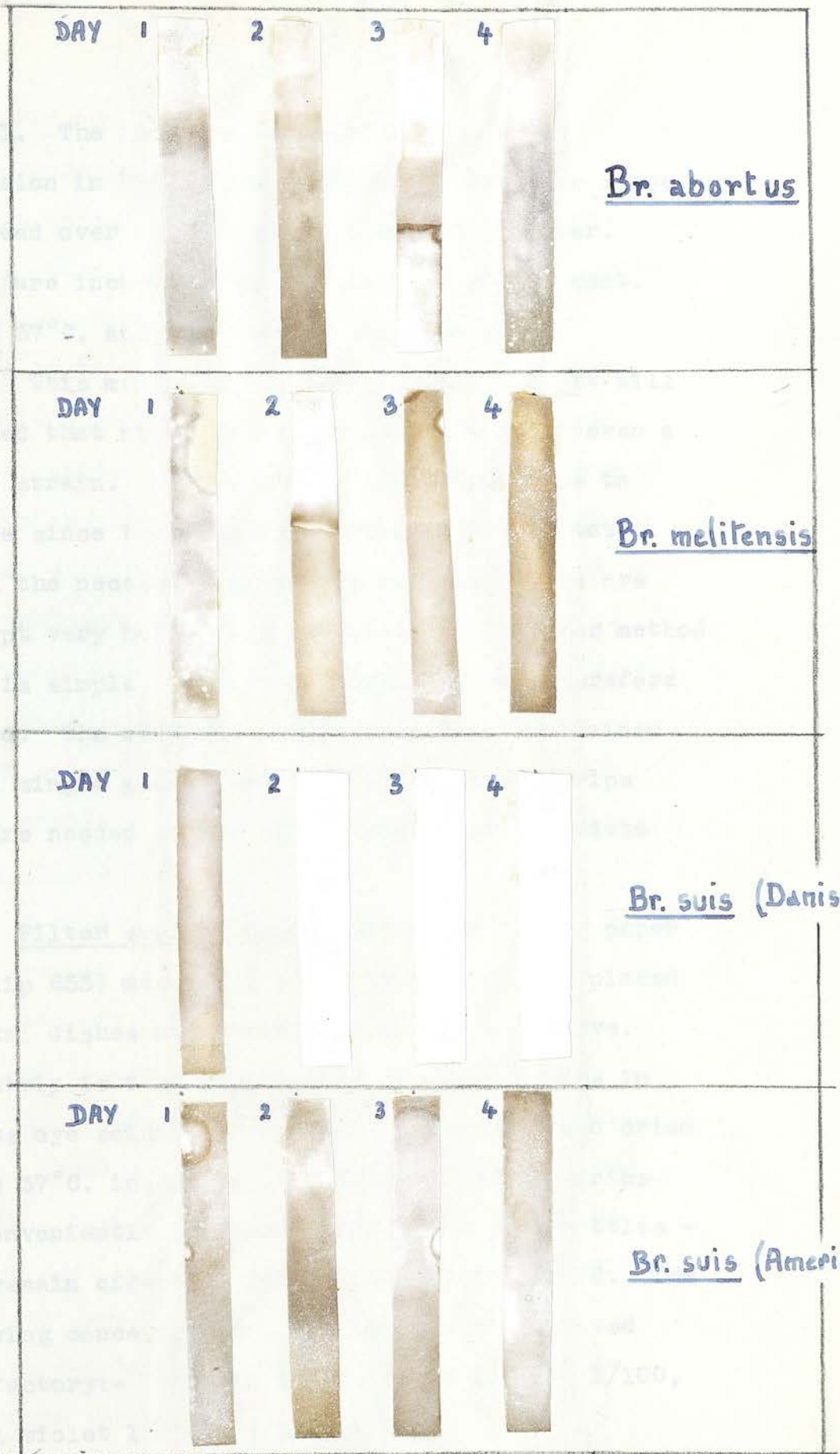


FIG. 4

control. The inoculum, a loopful of a thick suspension in broth from a 48-hour liver agar slope, is spread over an area about 1 cm. in diameter. Plates are incubated for 2-3 days in 10 per cent. CO₂ at 37°C. and the results are recorded.

This method gives good results, but it will be noted that nine plates are needed to test even a single strain. Further, they are troublesome to prepare since the dyes are liable to precipitate if all the necessary glassware and the medium are not kept very hot during preparation. Another method which is simple, rapid and economical was therefore devised. The required information can be obtained from a single plate, and the filter paper strips that are needed can be stored ready for immediate use.

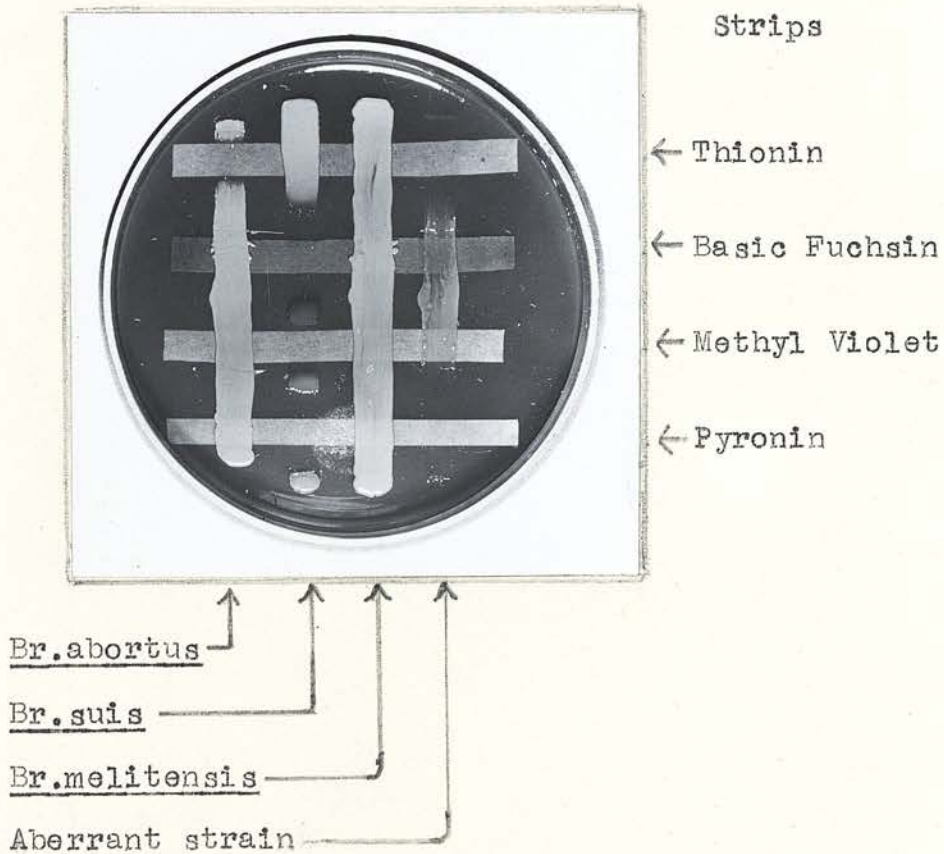
Filter strip method. Strips of filter paper (Postlip 633) measuring 6 cm. by 0.5 cm. are placed in Petri dishes and sterilized in the autoclave. Each strip is then dipped with sterile forceps in aqueous dye solution, replaced in the dish and dried in the 37°C. incubator overnight. The dye-strips can conveniently be stored in screw-capped bottles - they remain effective for an indefinite period. The following concentrations of the dyes have proved satisfactory:- thionin 1/600, basic fuchsin 1/100, methyl violet 1/200, pyronin 1/800.

For use four strips of the different dyes are laid in parallel on a liver agar plate, and 12 ml. of the same medium, melted and cooled to 50°C., are poured on top. When the agar has set, the plate is dried and is then ready for use. Dense suspensions of the strains to be tested are prepared by emulsifying part of a 48-hour growth on a liver agar slope into the condensation water (to which about one ml. of broth is added if necessary). The suspensions are then carefully inoculated across the plate at right-angles to the strips with a special loop. Up to seven strains can be tested on one 10 cm. plate. After drying, the plate is incubated at 37°C. in CO₂. Results can be read in 2-3 days. With each set of tests, control strains of Br.abortus, Br.melitensis and Br.suis of known behaviour should be included.

A typical result is shown in Plate 2. Organisms not susceptible to the dyes grow freely up to and across the strip. Sensitivity is indicated by inhibition of growth extending for distances up to 10 mm. from the edge of the strip. Typical strains of Br.abortus are inhibited only by thionin. Most strains of Br.melitensis grow on all the dyes. Br.suis grows only in the presence of thionin and is inhibited by the other dyes. Atypical reactions are given by certain strains, often from particular geographical areas: for example, Br.abortus strains from Southern Rhodesia

PLATE 2.

Brucella Dye Tests by the Filter Strip Method



BRUCELLA STRAIN

have unusual resistance to thionin. These aberrant strains have been described by Wilson and Miles (1946) and do not require further discussion here. It is to help in their identification that the four dyes are used; for ordinary work thionin and basic fuchsin would suffice.

The strip method has proved reliable and rapid in practice, but it was thought desirable to test a number of strains in parallel by the two methods, especially when it became necessary at a later stage to find out more about a variety of Br.abortus of unusual dye-sensitivity. For this purpose 24 strains were tested by the strip method and by the plate method of Huddleson, with the use of one instead of two different dye-concentrations. The actual dye-concentrations in the plates and the results of the tests are shown in Table 9. It is clear that the two methods give the same information, and that strains inhibited by one or other of the two routine concentrations of the dyes used in the plate method are inhibited by the corresponding strip over a zone 3 to 12 mm. wide. The strip method is now in constant use and saves much time and material.

(d) Agglutination with monospecific antiserum.

The antigenic relationship of Br.abortus and Br.melitensis is well-known. The organisms possess the same two antigens but the major antigen of Br.

TABLE 8

DYE PLATE METHOD

PAPER STRIP METHOD

BRUCELLA STRAIN	control No Dye	THIONIN				BASIC FUCHSIN				METHYL VIOLET				PYRONIN				DYES			
		30,000	60,000	120,000	240,000	25,000	50,000	100,000	200,000	50,000	100,000	200,000	400,000	100,000	200,000	400,000	800,000	T	BF	MV	P
Abortus B127	++	-	+	++	+++	+	++	+++	+++	+	+++	+++	+++	+++	+++	+++	+++	---	+	+	+
Meditensis B63	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Suis B64	+++	+++	+++	+++	+++	-	-	-	c	-	-	-	+	-	c	+++	+++	---	---	---	---
Abortus S19 B60	+++	(+)	+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	---	+	+	---
Abortus B22	+++	+	++	++	+++	+++	+++	+++	+++	+	+++	+++	+++	+++	+++	+++	+++	-	++	++	++
Abortus B40	+++	-	+	++	+++	+	+++	+++	+++	+	+++	+++	+++	+++	+++	+++	+++	-	++	++	++
Abortus D.S. B48	+++	-	+	++	+++	-	-	-	-	-	-	-	-	-	-	+++	+++	---	---	---	---
Abortus D.S. B96	+++	-	++	++	+++	-	-	-	-	-	-	-	-	-	-	++	+++	---	---	---	---
Abortus D.S. B101	+++	-	++	+++	+++	-	-	-	-	-	-	-	-	-	-	+++	+++	---	---	---	---
Abortus B103	+++	+	++	++	+++	+++	+++	+++	+++	+	+++	+++	+++	+++	+++	+++	+++	---	+++	+++	+++
Abortus B108	+++	-	++	++	+++	+++	+++	+++	+++	-	++	+++	+++	+++	+++	+++	+++	---	+	+	+
Abortus D.S. B126	+++	-	-	+	+++	-	-	-	+	-	-	-	-	-	+++	+++	+++	---	---	---	---
Abortus B135	+++	-	-	++	+++	+++	+++	+++	+++	+	++	+++	+++	+++	+++	+++	+++	---	++	++	++
Abortus D.S. B137	+++	-	-	++	+++	-	-	-	-	-	-	-	c	-	+	++	+++	---	---	---	---
Abortus D.S. B140	+++	(+)	+	++	+++	(+)	+	++	+++	-	-	+	++	+++	+++	+++	+++	---	+	-	+++
Abortus D.S. B161	+++	-	++	++	+++	-	-	-	+	-	-	(+)	+	+++	+++	+++	+++	---	---	---	+
Abortus B164	+++	(+)	++	++	+++	+++	+++	+++	+++	+	++	+++	+++	+++	+++	+++	+++	-	++	++	++
Abortus D.S. B167	+++	-	+	+++	+++	-	-	(+)	+	-	-	-	-	++	+++	+++	+++	---	---	---	-
Abortus B172	+++	(+)	++	+++	+++	+++	+++	+++	+++	+	+++	+++	+++	+++	+++	+++	+++	+	++	++	++
Abortus D.S. B197	+++	+	++	+++	+++	-	-	(+)	++	-	-	-	(+)	++	+++	+++	+++	---	---	---	-
Abortus B201	+++	(+)	++	++	+++	+++	+++	+++	+++	+	+++	+++	+++	+++	+++	+++	+++	---	++	++	++
Abortus B289	+++	-	-	+	+++	+++	+++	+++	+++	(+)	++	+++	+++	+++	+++	+++	+++	---	+	+	+
Abortus D.S. B303	+++	-	-	+	+++	(c)	(c)	+	+	-	-	(c)	+	(c)	(c)	+++	+++	---	---	---	---
Abortus D.S. B405	+++	-	-	±	+++	+	+	++	+++	-	-	(c)	+	+	++	+++	+++	---	+	-	+

- , + , ++ , +++ = Degrees of Growth
(c), c = Small numbers of discrete colonies

Zones of Inhibition of growth
- => 5 mm.
-- => 7 mm.
--- => 11 mm.

abortus is the minor antigen of Br.melitensis and vice versa. Br.suis cannot be distinguished serologically from Br.abortus. Antisera to Br.abortus and Br.melitensis usually agglutinate the heterologous organism to about half the homologous titre, but the two organisms can usually be clearly identified with the use of absorbed sera. Since both organisms possess the same antigens monospecific sera can only be produced by careful quantitative cross-absorption of antiserum with the heterologous strain.

Typical strains of Br.abortus and Br.melitensis are selected and antisera prepared by inoculation of rabbits with a heat-killed suspension. The minimum number of injections to produce a homologous titre of about 1/2,560 is given, since multiple injections are liable to stimulate antibodies to the minor antigen in excessive amount. Each serum is then absorbed with a suspension of the heterologous organism, standardized by opacity to match a 3,000 million Bact.coli standard tube. Equal amounts of diluted serum and suspension are mixed and kept, with occasional agitation, in the incubator at 37°C. for 3 hours then cleared by centrifugation. The appropriate serum dilution, which is about 1/32 - 1/64 of its homologous titre, should be determined by trial at several levels, as shown in the protocol in Table 10. The sera in this test were absorbed in dilutions of 1/20, 1/40, 1/80 and 1/160, and the

absorbed sera tested against two typical strains of Br.abortus and three of Br.melitensis. The absorbed serum which gives the highest titre with the homologous organism and no agglutination with the heterologous organism indicates the appropriate dilution at which the bulk absorption should be made. In the example given that level is 1/40 in each instance, i.e. the serum is to be absorbed at a dilution one-sixty-fourth of its "straight" titre of 1/2,560.

Monospecific sera are a valuable aid to species identification. Organisms to be tested must be antigenically smooth. Early signs of roughness are best detected by the thermo-agglutination test which consists in making a thick suspension (8,000 million organisms per ml.) in saline and placing it in a boiling water bath; rough strains will auto-agglutinate by mixing the suspension with equal parts of 1/500 acriflavine and incubating at 37°C.

(e) Other tests have been tried from time to time but none of these has given results which justified its adoption in routine work. For example, the urease activity of Br.abortus is feebler than that of the other two species, but the difference is quantitative rather than qualitative.

(f) H₂S production. All the abortus strains produced H₂S on each of the first 5 or 6 days of growth; with some strains the amounts were small but in all cases it was easily detectable. In the whole series there was only one melitensis or melitensis-like strain

2. Results of the examination of 403 Brucella Strains

(i) Classification of Strains

In Table 11 the source of the strains is shown, and they are classified according to the results of the laboratory tests. The total number of strains is sufficient to give a good idea of the consistency of the tests, and the numbers from Great Britain (368) and, to a less extent, from Italy permit an estimate to be made of the prevalence of the different types in these countries.

(a) CO₂ sensitivity. Of the 24 typical melitensis strains and the 17 melitensis-like strains, none required added CO₂ for growth; two strains grew less well in the presence of CO₂ than in air. Most of the 347 typical or almost typical abortus strains grew much better in the presence of 5-10 per cent. CO₂, but there was usually a little aerobic growth in 2 or 3 days (possibly initiated by small amounts of CO₂ from the washers of the medium containers). However, about 10 per cent. of the abortus strains, only subcultured two or three times since isolation, grew as well aerobically as in CO₂.

(b) H₂S production. All the abortus strains produced H₂S on each of the first 3 or 4 days of growth; with some strains the amounts were small but in all cases it was easily detectable. In the whole series there was only one melitensis or melitensis-like strain

TABLE 11

Classification of 403 recently isolated Brucella strains

Place of Isolation	Abortus Typical	Abortus Dye sensitive	Abortus Dye-sensitive except to pyronin	Abortus Thionin-sensitive	Abortus Inagglutinable	Abortus biochemically, Melitensis antigenically	Melitensis typical	Melitensis biochemically Abortus antigenically	Melitensis Inagglutinable	Total
BRITAIN	Blood	9	2	0	0	0	3 †	1 *	0	15
	Milk	279	36	4	0	0	14	0	0	351
	Other bovine sources	2	0	0	0	0	0	0	0	2
KENYA	Blood	3	0	0	1	2	3	0	0	9
MAURITIUS	Cow	2	0	0	0	0	0	0	0	2
ITALY	Blood	1	0	0	2	0	0	13	3	19
ISRAEL	Milk	3	0	0	1	0	0	0	0	5
TOTAL		299	38	4	4	2	24	14	3	403

† = 2 infected overseas, 1 laboratory infection.

* = Infected in Southern Italy.

that produced any H_2S , and this strain produced a trace on the first day only. These results confirm that a strain producing H_2S on the second day of incubation is unlikely to be Br.melitensis.

(c) Dye tests. In the dye tests all the melitensis and biochemically melitensis-like strains behaved characteristically, growing in the presence of the four test dyes. Of the Br.abortus strains, the great majority behaved typically, being inhibited only by thionin. However, there were definite varieties that gave aberrant reactions, the commonest being a variety of special sensitivity to all the test dyes. In the strip test there was complete inhibition of growth in their presence. Parallel tests were therefore made of a number of these strains in plates incorporating the dyes in concentrations up to one-quarter of the lowest strength employed in routine testing. The results are included in Table 9, the strains referred to being designated D.S. (dye-sensitive). It is evident that the degree of dye-sensitivity of these strains is so marked as to distinguish them sharply from typical strains. There was also epidemiological evidence of their constancy in that they were sometimes isolated from a group of cows in one herd. They constituted 11.4 per cent. of the total of 332 Br.abortus strains isolated in Britain from milk or human sources. A

less common type (1.2 per cent.) was sensitive to thionin, basic fuchsin and methyl violet but not to pyronin. It is of interest to recall that Wilson (1933) found 6 of 101 strains of Br.abortus sensitive to all the dyes. In the present series, four strains, all from overseas, were insensitive to thionin; strains of this type have been recorded previously and, as mentioned earlier, were noted by Wilson to be the usual type of Br.abortus in Southern Rhodesia.

(d) Tests with Monospecific Sera. The great majority of the Br.abortus and Br.melitensis strains from this country were serologically typical. Of the 351 strains isolated from milk, however, 14 (4 per cent.) were aberrant types; in the biochemical tests (CO₂ requirement, H₂S production, dye tests) they behaved as Br.abortus but they agglutinated to titre with monospecific melitensis and not with monospecific abortus antiserum. The disposal of cows excreting organisms of this type (invariably confirmed by the bacteriologists of the Ministry of Agriculture) was a difficult problem for the veterinary authorities; their decision was to take no risks and to apply the powers of slaughter permitted by the Brucellosis Melitensis Order (see below).

An interesting serological type showed the reverse characters to that just described. It behaved as melitensis biochemically but agglutinated

with an abortus monospecific serum. The table shows that of 19 strains obtained from human sources from Italy (mostly the southern part) 13 were of this type, and only one was a fully typical strain of Br.abortus. One strain of this type was isolated from a patient with undulant fever in the Middlesex Hospital, London, and inquiry disclosed that he had been infected while on holiday in Southern Italy. Wilson mentions that these strains have commonly been recorded from South Eastern France.

(e) Comment. These results warrant the following conclusions. Contagious abortion in Britain is due in the vast majority of cases to Brucella abortus, the infecting organism sometimes being of a type that is specially sensitive to the usual test dyes. Br.melitensis has been isolated from a small number of animals; the mode of sampling in the present series does not justify expressing this number as a percentage of the whole. Some bovine infections are due to aberrant strains behaving biochemically like Br.abortus but antigenically like Br.melitensis. In man there is no record of undulant fever in this country due to any organism other than Br.abortus, with the exception of patients infected abroad and of laboratory infections. In the present series a technician in the laboratory of a hospital for tropical diseases acquired an infection with Br.melitensis shortly after isolating that organism from an Oriental seaman.

The small number of strains from human cases in Kenya included both Br.abortus and Br.melitensis. The series included two Br.abortus strains isolated from foetal membranes from aborting cows in Mauritius, believed to be the first Brucella strains identified in that country. The prevailing strain causing undulant fever in Southern Italy is an aberrant type behaving biochemically like Br.melitensis but antigenically like Br.abortus.

(ii) Occurrence of Br.melitensis in Britain

The first report of Br.melitensis from this country was made by Menton from Staffordshire in 1940. He isolated the organism from the milk of an accredited herd by guineapig inoculation. It seemed to the authorities that a disease of grave veterinary and medical importance, new to this country, might have to be faced. Severe measures were justified, especially in time of war, and a new order, the Brucellosis Melitensis Order, 1940, was made empowering slaughter of infected animals. The implicated herd had been dispersed, but the cows were traced and five found to be excreting Br. melitensis were destroyed. It was hoped that the infection had been eradicated.

However, since that time the writer has identified Br.melitensis on 13 other occasions. The isolations in each instance have been from guineapigs

inoculated with milk samples, and no human infections are on record. Each "incident" has been reported to the veterinary officers of the Ministry of Agriculture and Fisheries who try by individual sampling to detect the infected cows in the herd. I am indebted to Dr. Stablerforth of the Ministry's laboratories for the information relating to the herds. Table 12 shows the time and place of each incident, and the number of cows found to be excreting Br.melitensis in each herd. It will be noted that there have been 13 incidents since 1940 - 4 in Staffordshire, 6 in East Anglia, 1 in Hertfordshire and 2 in Sussex. In the nine herds investigated, 18 cows were shown to be excreting Br.melitensis. Some are still under investigation. The localities are indicated in the spot-map (Fig. 6), but it is again emphasized that it would be incorrect to infer that this represents the geographical distribution of Br.melitensis in cattle in this country, since these are the particular areas in which the local bacteriologist has had a special interest in the epidemiology of brucellosis in man and animals. It is probable that the organism is in fact distributed much more widely in this country than has so far been demonstrated. By all laboratory tests the strains are completely typical, and it is not known why there appear to have been no cases of undulant fever in man due to milk-borne infection with this organism.

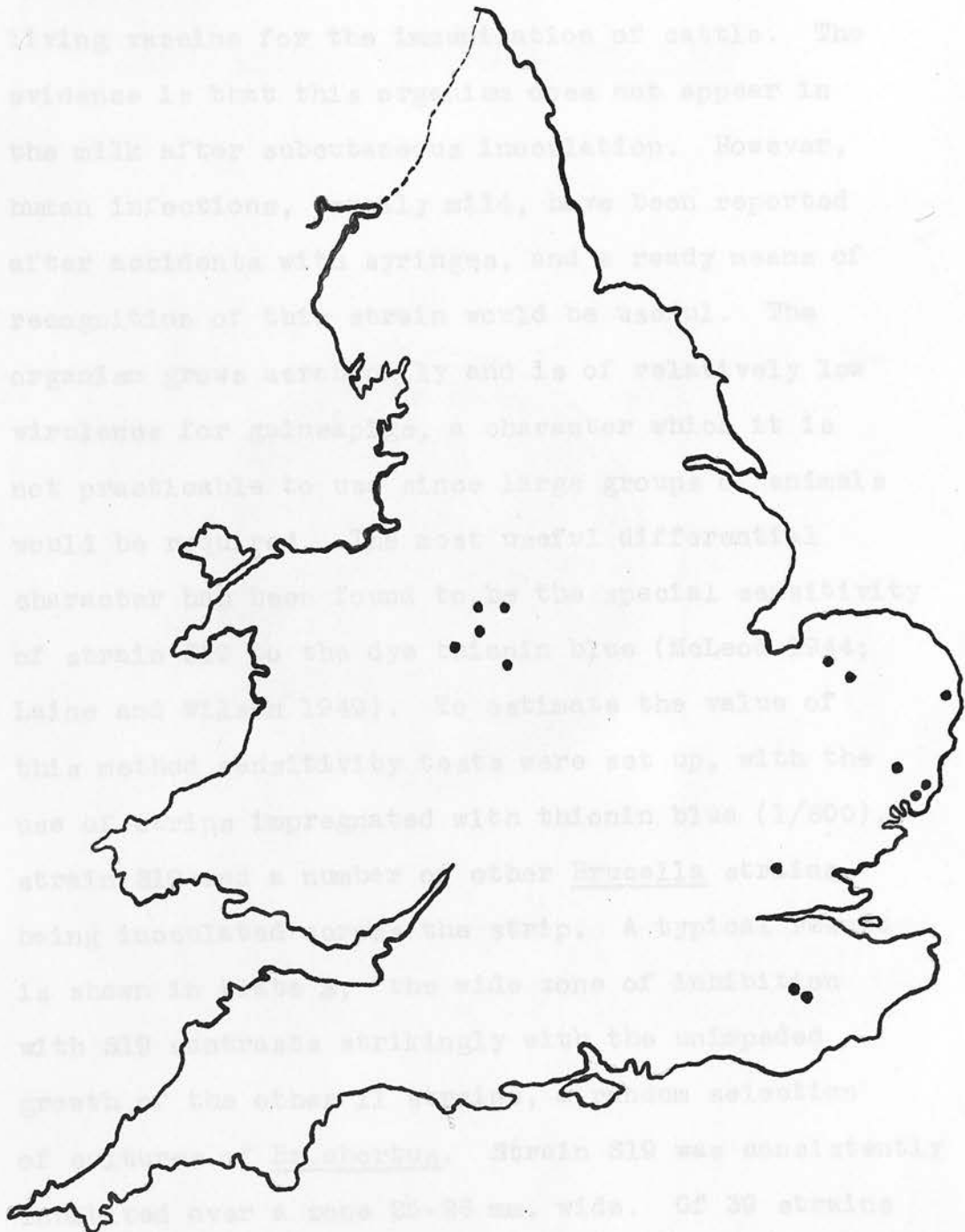
TABLE 12

Foci of *Br.melitensis* in cattle in England

Date	Place	Cows found to be infected
1940	Staffordshire.	5
1947, June	Trentham, Stoke-on-Trent, Staffs.	3 out of 28
" July	Felixstowe, East Suffolk.	1 " " 47
" "	Cannock, Staffs.	1 " " 54
" "	Newport, Salop.	1 " " 100
1950, Feb.	Deben, Suffolk.	3 " " 44
" "	Forehoe and Hensted, Norfolk.	1 " " 6
" June	Watton, Norfolk.	?
" Sept.	Cheadle, Stoke-on-Trent, Staffs.	1
" Nov.	Felixstowe, East Suffolk	Cultural tests on 40 animals negative
" "	Swaffam, Norfolk	?
1951, Jan.	Stevenage, Herts.	TT Bulk supply, source untraced
" Oct.	Wadhurst, Sussex.	1
" Nov.	Wadhurst, Sussex.	Under investigation

Fig. 5

Recorded foci of *Br. melitensis* in cattle
in England and Wales

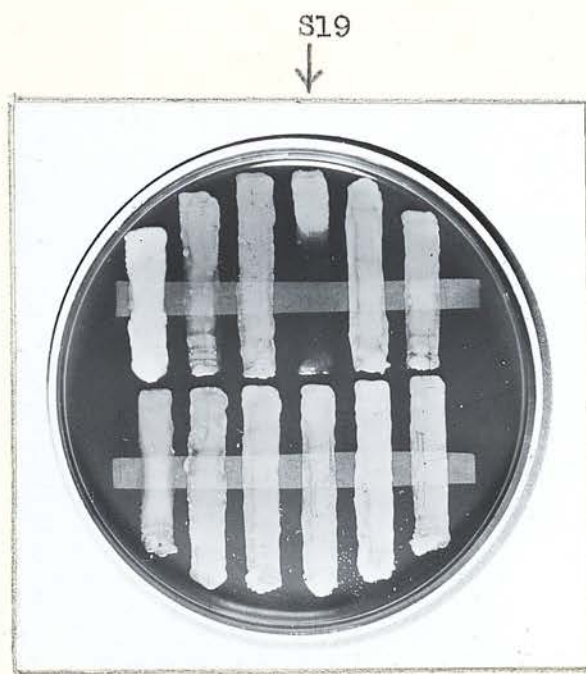


(iii) Identification of the Vaccinal Strain - S19

Br.abortus S19 is a strain of low virulence used by the Ministry of Agriculture as a living vaccine for the immunization of cattle. The evidence is that this organism does not appear in the milk after subcutaneous inoculation. However, human infections, usually mild, have been reported after accidents with syringes, and a ready means of recognition of this strain would be useful. The organism grows aerobically and is of relatively low virulence for guineapigs, a character which it is not practicable to use since large groups of animals would be required. The most useful differential character has been found to be the special sensitivity of strain S19 to the dye thionin blue (McLeod 1944; Laine and Wilson 1949). To estimate the value of this method sensitivity tests were set up, with the use of strips impregnated with thionin blue (1/600), strain S19 and a number of other Brucella strains being inoculated across the strip. A typical result is shown in Plate 3; the wide zone of inhibition with S19 contrasts strikingly with the unimpeded growth of the other 11 strains, a random selection of cultures of Br.abortus. Strain S19 was consistently inhibited over a zone 25-26 mm. wide. Of 39 strains of Br.abortus, typical except that some grew without CO₂, 37 showed no inhibition by thionin blue; the other two showed zones 8-9 mm. wide. Three Br.abortus

PLATE 3

Identification of Vaccinal Strain S19



The horizontal filter paper strips are impregnated with thionin blue (1/600). The plate has been inoculated with twelve Brucella strains, including S19. Strain S19 is the only one whose growth is inhibited.

strains of the type sensitive to all the usual test dyes were also sensitive to thionin blue, showing zones 11, 32 and 35 mm. wide. Of 7 strains of Br. melitensis and 3 aberrant strains, none were inhibited. Of 5 strains of Br. suis 4 were inhibited, showing zones 11, 13, 15 and 20 mm. wide.

It thus appears that, of the Br. abortus strains tested the only ones inhibited by thionin blue to an extent approaching S19 are those sensitive to all the dyes - and S19 is not sensitive to basic fuchsin or methyl violet. The justifiable conclusion seems to be that, if a suspected strain of Br. abortus is not inhibited by thionin blue, it is not S19; if it grows without CO₂ and is strongly inhibited by thionin blue and not by basic fuchsin or methyl violet, it may be S19.

Summary and Comment on Part II

This survey, comprising the examination of over 400 strains of Brucella from Great Britain and other countries, has shown that the criteria used in the differentiation of species are reliable for practical purposes. In general there is correlation between the different biochemical tests; and identification based on these tests is in most cases confirmed by serological tests with the use of monospecific antisera. However, there are occasional divergencies which make it necessary to base a conclusion

on the results of all the tests rather than on one method. Tests should be made on recently isolated strains, and special care should be taken to confirm the results in instances in which the bacteriological identification is at variance with the epidemiological evidence.

The method of performing the differential tests has been described. A simplified method of performing dye-sensitivity tests has been devised and has been shown to give the same information as is obtained by the orthodox method. The mode of preparation of monospecific agglutinating antisera by quantitative absorption is described, and it is confirmed that such sera are a reliable aid to species identification.

Information of epidemiological interest has emerged from the survey. It was well-known that the main cause of contagious abortion in cows and the only cause of undulant fever in man in this country is Brucella abortus. The strains are usually typical in their characters, but a type possessing special dye-sensitivity is not uncommon in cattle and may produce disease in man. Of greater interest is the occurrence of strains with all the characters of Brucella melitensis which have been recognized in cows in various parts of the country during the last 12 years. In spite of the fact

that milk containing this organism must have been consumed by many people, no human infections have been recorded. However, in view of the possibility that a severe disease new to these islands might spread in man and animals, the policy of slaughter adopted by the Ministry of Agriculture seems fully justified.

The survey has shown that the organism chiefly responsible for brucella infection in Southern Italy is an aberrant type with the biochemical characters of Br.melitensis and the antigenic structure of Br. abortus. The epidemiological value of precise identification is suggested by the isolation of such a strain in this country from the blood of a person infected in Italy.

An incidental observation was that the filter-strip dye test, with the use of thionin blue, was a useful aid to the recognition of Brucella abortus S19, the vaccinal strain used in the living state for the immunization of cattle against contagious abortion.

III THE BACTERIOLOGIST AND WORLD CONTROL
OF ENTERIC AND BRUCELLA INFECTIONS

It is perhaps appropriate to conclude by speculating upon the rôle of the bacteriologist in the future control of the two diseases under discussion. Enteric fever and brucellosis present widely differing problems. Salm.typhi and Salm. paratyphi A and B are strictly human pathogens, whereas Brucella abortus, Brucella melitensis and Brucella suis are the cause of diseases in farm animals that are communicable to man. In a less direct but no less serious fashion the health of a human community may suffer from the economic loss occasioned by brucella infection in farm stock.

It is true that the annual mortality due to typhoid and paratyphoid fever per million living in England and Wales declined from an average of 321 in the period 1871-80 to 5 in the period 1931-35, and that it has not exceeded 1 per million since 1944. The disease however has not been fully extinguished, and the large outbreak of typhoid fever in Aberystwyth in 1946 is a reminder that epidemic disease may suddenly appear in spite of the vigilance and the sanitary measures which have been responsible for the striking decline in incidence during the last 80 years. This point is also emphasized by the occurrence of extensive outbreaks of paratyphoid B

fever in several cities during and after the war, spread in most instances by synthetic cream in bakery products.

In this country where the incidence is relatively low the final abolition of the "unextinguished residuum" of enteric infection has been brought closer by recent advances in laboratory methods. The newer culture media and the improvements in serological technique should ensure that fewer cases are missed, and the Vi agglutination test should greatly assist the detection of carriers and ensure that convalescents who become chronic carriers are not discharged undetected into the community. The use of chloramphenicol should cut short the clinical disease and lessen the risk of development of the carrier state. The improved methods for detection of enteric bacilli in water and sewage, and the precise identification of infecting strains by Vi-phage typing, have rendered easier the elucidation of outbreaks or sporadic cases; the responsible carrier is now much more frequently traced. The central register of typed cases and carriers is becoming increasingly complete. This should have good results for it is rarely the known carrier who is responsible for spread of infection.

The satisfactory state prevailing in this country and the United States is unfortunately not reflected in all other countries. A recent

survey by Stowman (1948) for the World Health Organization described the recrudescence of typhoid fever in Europe after World War II. Before the war there was a large block of countries in which the incidence of typhoid fever had fallen to less than 5 per 100,000 and the mortality to less than 1 per 100,000. As a result of the destruction and disorganization caused by the war, only the United Kingdom, the Scandinavian countries, Switzerland and Iceland maintained their low incidence. High-incidence areas extended to the borders of the safe countries, and typhoid fever was commoner than paratyphoid. In Germany the incidence rose to about seventeen times the pre-war level. In some countries, such as Poland and the Netherlands, energetic preventive work went far to restore the pre-war level but an area of high incidence still stretched from the Mediterranean to the Baltic and the North Sea; over 250,000 cases occurred in Europe (excluding the U.S.S.R.) in each of the years 1946-47, and 25,000 lives a year, mostly of persons of working age, were lost. Happily the most recent report (W.H.O. 1950) shows that this picture was unduly pessimistic for there has been a downward trend in all European countries except France and Spain. In Africa the incidence has also fallen since 1947, but in Asia, except in Japan, there has been an increase.

A notable observation is that, in Bulgaria, where mass inoculation of inhabitants of villages with doubtful water supplies has been pursued since 1936, the steady fall in incidence was not interrupted by the war and the country enjoys a relatively low incidence. The mode of spread in the high-incidence areas must be due to conditions that can only be combatted by those basic sanitary engineering measures which have proved so successful elsewhere. Further, the Bulgarian experience suggests that, in the civil population of these areas as in the service communities elsewhere, mass inoculation on a large scale is a policy well worth pursuing with vigour. The development of a better vaccine (and, if it should ever become feasible, of an oral vaccine) is one of the main tasks of the bacteriologist.

Brucellosis presents a different problem. The estimates of 400-500 cases of undulant fever each year in England and Wales, and of 4,000 cases in the United States are believed to be too low, and the long period of incapacity makes it a disease not to be regarded lightly. However, the disease rarely occurs in epidemic form and prophylactic immunization is not indicated, especially in view of the satisfactory results given by the newer antibiotics in treatment. Aureomycin has been shown in experimental infections in guineapigs (Cruickshank 1949) and in clinical trials in man to be the most

promising substance produced so far. Infection with Br.abortus and Br.melitensis is conveyed to man from cows and goats by milk or by direct contact. The incidence of infection in adult cattle in this country was about 15-20 per cent. before the war, and a recent survey yielded Br.abortus from 12.4 per cent. of 336 samples of pooled milk from herds. There was no significant difference in the incidence in tuberculin-tested, accredited or undesignated milks; the organism is destroyed by pasteurization.

Veterinarians have shown (Report 1951) that brucellosis in animals in many parts of the world contributes largely to a low standard of living, resulting as it does in abortions, decreased milk flow and infertility of livestock. Vast sums of money have been saved in those countries such as the United States and Norway where contagious abortion has been eradicated or greatly reduced, and the prevention of human infection depends also upon these measures.

It is probable that the main task of the bacteriologist in human infections will be the development of improved methods of cultural and serological diagnosis, and the discovery of a fully potent antibiotic or chemotherapeutic agent. In the control of animal disease his task will be to co-operate with the veterinarian in the development of tests designed to detect infected animals and of

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- more effective types of vaccine. It has been shown beyond doubt that the elimination of animals reacting to sero-agglutination tests, the prophylactic vaccination of uninfected animals and the hygienic measures of good animal husbandry, can result in the eradication of brucellosis from herds, areas or countries.
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