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Serodiagnosis of Salmonellosis.

(Development and present status.)

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Introduction.

The name "*Salmonella*" is now commonly acknowledged for the genus of bacteria which comprises the typhoid-paratyphoid-enteritis group of bacilli. It was proposed by Lignières in honor of E. D. Salmon who in 1885 isolated and described the hog cholera bacillus, a member of this group. That name, by the way, was a misnomer. Isolated from pigs diseased with swine plague, it was considered by Salmon and Th. Smith as the causative agent of this disease. Later investigations, however, established the virus character of the malady and the hog cholera bacillus (synonyms: B. suipestifer, S. cholerae suis) as a regular concomitant, a "nosakoluth". It is pathogenic in itself as f.i. is the influenza bacillus that, in a similar sequence of events, has kept its name after being dethroned from its place in the etiology of influenza, now also recognized as a virus disease. All members of the Salmonella group are potential pathogens, able to infect man and animals. They are most widespread in the animal kingdom where they are found in fowl, swine, rodents, also in ruminants, horses, reptiles, etc. There they are the cause of sporadic disease or of large outbreaks; sometimes they are encountered as seemingly

harmless saprophytes, excreted with the feces or stored in mesenteric lymph nodes. Eggs, meat, milk, cheese, water, sewage have been found infected. Thus the opportunity for human beings to pick up Salmonella organisms from many sources, is amply given.

In the infected human a great variety of symptoms is observed. The malady "*Salmonellosis*" is by no means a clinical entity. It is a protean disease group which transcends by far the realm of food infection and typhoid or paratyphoid fever. Mild, often overlooked symptoms as well as severe forms of septicemia or localized inflammatory processes in all parts of the body have been described. The picture may simulate any of several clinical syndromes, such as gastroenteritis, from a mild diarrhea to dysentery- and cholera-like symptoms, cholecystitis, appendicitis, peritonitis, meningitis, salpingitis, pneumonia, pleurisy, endocarditis, osteomyelitis, abscesses, etc. Healthy carriers are known to harbor the organisms for some time. They may transmit them to other individuals, often with disease as the result. Thus the clinical and epidemiological importance of Salmonellosis is obvious. The exact diagnosis and identification of the incitant are a necessity for hospital and public health laboratories. The arsenal of diagnostic weapons, furthermore, includes the demonstration of antibody production in the serum of the infected person. Therefore, the serodiagnosis of Salmonellosis comprises two main methods: a direct one of isolating and identifying the causative agent by known antisera, and an indirect one of agglutination tests with the patient's serum against known Salmonella antigens.

I. Serological Diagnosis of Salmonella Organisms [1].

a. Historic development and definition of terms.

It is a far cry from the seemingly simple orthodox methods used some 40-50 years ago to the highly specialized intricate system of serological differentiation, which in our times has set up a number of almost 200 different types within the Salmonella group. The

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[1] Comprehensive presentations of the problem may be found in the following works: 1) *White, P. B.* (1926) Med. Res. Council Spec., Rep. Ser. 103, 2) *White, P. B.* (1929) Med. Res. Council System of Bact. 4, 86, 3) *Tesdal, M.* (1938) Die Salmonellagruppe, Presa Buna, Tassy (Roumania), 4) *Kauffmann, F.* (1941) Die Bakteriologie der Salmonellagruppe. Einer Munksgaard, Copenhagen, 5) *Edwards, P. R., Bruner, D. W.* (1942) Serological Identification of Salmonella Cultures. U. of Kentucky, Station Circular 54, 6) *Bornstein, S.* (1943) The State of the Salmonella Problem. J. Immunol. 46, 439, 7) *Wilson, G. S., & Miles, A. A.* (1946) Topley & Wilson Principles of Bacteriology and Immunology, 3rd edition. Baltimore, Williams & Wilkins Company.

development of the newer concepts is of general interest and so fascinating in itself, that a short historic review is warranted.

The typhoid bacillus was the first organism of the whole group to be isolated (cultured in 1884 by GAFFKY). Next in human pathology were the paratyphoid bacilli A and B, discovered around the turn of the century. These organisms, also incitants of enteric fever, showed slight cultural differences and serological cross-reactions which at that time were not easily understood. Meanwhile, other germs, almost identical culturally and very much alike in the then used serological tests, had been observed. Organisms found in food infection due to animal products were described, the first one in 1888 by A. GAERTNER in Frankenhausen (Germany). The germ, serologically different, received the name "Frankenhausen" from the place of its origin as did other bacilli isolated under similar conditions (Breslau, Aertryck, Morseele, Dublin, Essen, Moscow, etc.). Another group of related organisms was designated according to the authors' names (Gaertner, Schottmueller, Basenau, Drigalski, Dunbar, Danysz, Hirshfeld, etc.); a third group derived names from animals from which they were isolated (cholerae suis, suipestifer, typhi murium, typhi suis, pestis caviae, ratin, pullorum, gallinarum, psittacosis), the fourth finally from clinical symptoms they provoked in man or animals (paratyphoid A, B, C, as close to typhoid fever, enteritidis, morbificans bovis). Most of these designations were of merely casuistic interest. They did not give any clues for an exact species or type identification except for typhoid bacilli, paratyphoid and enteritidis bacilli which showed peculiarities in serological and cultural characteristics. The great majority of the strains was put into the large paratyphoid group, in spite of some more or less pronounced differences in serology. The trend to unification prevailed at that time, minor cultural or serological differences were looked upon as variations within the range of naturally occurring fluctuation. Therefore, B. typhosus, B. paratyphi A, B, paratyphi B and B. enteritidis represented the main recognized species in this group, during the first decade of the century. Serological identification was based on agglutination with immune sera, rarely on complement-fixation tests. Heat-killed and live organisms were employed for the production of antisera in rabbits. They covered the groups satisfactorily and gave evidence of some cross-reactions between the various members of the genus. Thus agglutination tables as those published by KUTSCHER and MEINICKE in 1906 provided a rather uniform picture.

The first break in this seemingly well-balanced structure was caused by observations of SOBERNHEIM and SELIGMANN (8) (1910),

who described deviations of cultural and serological features in known strains, so unusual and yet still inside the borders of the whole group, that deviating strains were interpreted as transition forms or even as "Artumwandlungen in der Enteritisgruppe". For the first time the authors described changes in colony form, combined with other minor cultural deviations and a substantially changed serology. Original antigens seemed to vanish, new ones made their appearance. Such a variant strain, plated out on a suitable medium, developed two kinds of colonies which the authors called "rund" (round), and "zackig" (crenated). The latter showed the newly acquired aberrant characteristics. BAERTHLEIN in 1912 and 1913 confirmed these colonial variants for a broad group of bacterial organisms. But it was only in 1921 that ARKWRIGHT (9) expanded these findings in the Salmonella group and raised them to the level of a general biological phenomenon. He explained the appearance of rough (zackig) colonies as a phase variation frequently occurring under still ill-defined conditions. Since "rough" variants were seen mostly in old laboratory cultures, or in strains which were being carried in the human intestines for some time, they might be regarded as products of degeneration. Some other cultural and morphological deviations, changes in agglutinogens and instability in physiological salt solution completed the picture of these products. Later experience (SCHÜTZE, WHITE) proved that sometimes the changes in agglutinability were the only sign of the altered condition, but these changes were so marked, and frequently of such a "cosmopolitan" character (SCHÜTZE), that the cultures were considered as "serologically rough", even without the appearance of cultural roughness. Some of the baffling results in SOBERNHEIM and SELIGMANN's studies might be explained by this smooth-rough variation.

Meanwhile, another kind of variation had been observed: the change from the normally motile Salmonella organism to a non-motile type, deprived of its flagellae. Although not frequently encountered, this change was destined to shed new light on the whole problem. The first observation of its kind was made on a culture of hog-cholera bacilli by TH. SMITH and REAGH in 1903 and studied in many details by BEYER and REAGH in 1904. Their results demonstrated the presence of two different kinds of antigens in normal Salmonella cultures, different in heat stability, in kind and form of agglutinability and related to different parts of the bacterial organism. The one was the *somatic* antigen, present in motile and non-motile bacteria, the other one the *flagellar* antigen, demonstrable in flagellated motile cultures, but missing in non-motile forms. Antibody analysis and absorption tests corroborated this

conception by the evidence of two different specific agglutinins in immune sera. Although similar experience with typhoid bacilli had been reported at the same time in Germany (Joos, 1903), and marked differences of sera, produced with live (flagellated) and dead (flagella-less) bacteria had been recorded by Sobernheim and Seligmann, the essential facts of these first observations fell almost into oblivion until they were rediscovered and thoroughly established by Weil and Felix (10) in 1917. These authors studied proteus bacilli in the course of their typhus-fever investigations and found that motile organisms, spread over the plates, possessed two different antigens, the one attached to the body of the bacillus, the other one being a part of the flagella. Non-motile organisms did not spread and contained only body antigens. The culture, that spread like a "Hauch" (haze) over the surface of the medium, had the flagellar antigenic substance now called "H", the non-motile, growing without spread (ohne Hauch) was characterized by the "O" antigen. These two symbols have been commonly accepted as abbreviations for flagellar and somatic antigens in bacteria, also in the Salmonella group, where Weil and Felix proved their presence (without the spreading phenomenon) in later studies (11). The O is heat and alcohol stable, the H, heat and alcohol labile. The O, as later studies revealed, is a polysaccharide complex, the H is a protein. The O-substance is slowly agglutinated in a granular form in a clear menstruum, the H clumps immediately, more heavily in flakes, while the surrounding fluid is not fully cleared. By heating bacterial emulsions or treating them with alcohol, pure O-antigens are obtained; by formolizing young broth cultures, H-antigens result together with the O-antigens. These two preparations are used for antibody production in rabbits. The resulting sera are either pure O-sera or mixed H + O-sera, with H-antibodies prevailing. With such sera on hand it could be proven that quite a number of organisms were identical, or related, as far as their O-antigen was concerned, but differed in H-agglutination. On the other hand there were types with identical H- but different O-antigens. These facts explained a good many of the cross-reactions which had so frequently interfered with attemps in differentiation and classification. Since various kinds of H-phases were found, some of them interrelated by common partial antigens, the clean-cut separation of two types from each other was still a matter of chance.

In 1922 a new variation was described by Andrewes (12) and followed up by Savage, Bruce White and others. Andrewes demonstrated that the flagella antigens of one culture were not always of one kind. Very frequently, two different H-phases could

be found side by side, the one characteristic of the type, the other one of a less specific character, since it was demonstrable also in other types. Previous concepts of the term "pure culture" had to be revised because of the amazing fact that these two phases, the specific and the non-specific one, were not both parts of *one* organism or *one* colony but were divided among them. A "pure culture" plated out to single colonies may develop colonies which contain the one phase only, and others endowed with the other phase exclusively. On subculture these two kinds remain constant for a longer or shorter period, until they split off the other phase again. Thus there occurs a permanent change of H-phases from one to the other in pure cultures. It was found later that not only the "group specific" (non-specific) phase might be present beside the "type specific" phase, but that also some phases believed to be of the specific type may occur as a second phase (KAUFFMANN and MITSUI, 1930). Therefore, the designation "specific" and "non-specific" phases was abandoned in favor of "first" and "second" phase. Strains which possess both kinds of phases are called bi-phasic, those with only one permanent phase, monophasic types. These are not the only possible variations of the flagella antigens. By special treatment "third phases" may be obtained, but in all probability these are biological artefacts without genuine or diagnostic importance. Permanent disappearance of one of the phases in biphasic cultures has been observed, obviously as a loss-variant, and the occurrence of inagglutinable phases in the cycle of phase variation has been described (SELIGMANN and SAPHRA).

These observations show that the limit of phase variation possibilities may not yet have been reached, but their general importance should not be overestimated.

Suffice it to stress that of the three heretofore mentioned variations: S-R (O-antigens), O-H (O- and H-antigens), H-H (H-antigens), the latter two provide the keys for a sound classification of the Salmonella group and for the explanation of almost all observed cross-reactions. The threefold variability of antigens O, phases 1, and 2 of H, yields a great number of possible combinations and therewith differentiations. Since cultures determined in this way seem to be permanent and not changing to other patterns, they have been called types. Their occurrence under various regional, epidemiological and clinical conditions, has been studied in many parts of the world and has given a practical aspect to this highly scientific problem after a technical agreement on the nomenclature had been achieved. BRUCE WHITE and FRITZ KAUFFMANN submitted their suggestions to the Salmonella Subcommittee of the International Society of Microbiologists which *in 1934* (13)

accepted the so-called "Kauffmann-White Schema", now in a modified form and used as a table for the identification of about 200 different Salmonella types and still open to further additions. Roman numerals have been chosen as symbols of the O-antigens, small letters and arabic numerals represent the H-phase.

A last phase variation, although of a more limited scope, is to be mentioned. It refers to the *Vi-phenomenon* which may be of definite diagnostic importance, and involves a type of antigen of a specific nature. It has been a well-known fact that freshly isolated typhoid bacilli, culturally typical in every respect, often are not agglutinated by a specific anti-typhoid O-serum. Although most of the cultures acquire agglutinability after a couple of days or weeks, this is a serious set-back for diagnostic purposes. In 1934, Felix and Pitt (14) discovered that those strains contained a special antigen which probably enveloped the original typhoid O-antigen. The antigen is part of the soma, not of the flagella, it is alcohol stable but heat labile and produces antibodies of its own. With the reappearance of the usual typhoid O-antigen it vanishes more and more. Felix and Pitt called this antigen the Vi-antigen because they believed the new antigen to be a virulence factor. Again a change of phases was observed. Transmutation from Vi-colonies to Vi-free ones occurs and intermediate stages are frequent. Kauffmann, who studied these biological phenomena, named the typhoid bacillus V (abbreviated from Vi), when a Vi-antigen but not the somatic typhoid antigen was demonstrable. W became the name of a strain without any Vi-content, but with well developed somatic antigen. Between them are the VW forms containing both antigens. There are a few more Salmonella types known to possess the Vi-antigen, which thus becomes a diagnostic aid also for S. paratyphi C and S. ballerup, recently omitted from the K-W Table.

All the foregoing is apt to stress that foremost consideration should be given to serological methods in identifying Salmonella cultures. Indeed it was so impressing that it induced Kauffmann to the following definition of Salmonellae: "Salmonella bacteria are gram negative bacteria which by their antigenic structure can be fitted into the Kauffmann-White Schema." This definition, which neglected the cultural characteristics of the cultures involved and led for a time to the inclusion of several "Salmonella coli" into the system, did not meet with general approval and has later been modified by Kauffmann. The necessity remains to combine cultural and serological features for the final diagnosis of a Salmonella organism.

b. Cultural Characteristics.

The members of the genus Salmonella are gram negative, non-sporing, motile [2] bacilli. They form acid and gas [3] from dextrose, mannite, maltose and sorbitol, and usually produce H_2S. They fail to ferment lactose, sucrose, and salicine. They do not liquefy gelatine [4], nor do they form indol from peptone. Cultures which follow this pattern and display established Salmonella O-, H-, and Vi-antigens are classified as Salmonellae. Biochemically characteristic cultures—even when isolated from sick individuals—with *no* serological relation to any known type, need painstaking investigation before admission to or rejection from this group.

In rare variants there are some biochemical properties that do not fit into the ordinary cultural pattern of Salmonella. These are: lactose fermentation, indol production, anaerogenicity and non-motility.

Lactose fermentation has been observed in a variant of an old laboratory culture of S. anatum by KAUFFMANN (15), and in 2 freshly isolated coliform cultures with the complete antigenic pattern of S. newington by SELIGMANN and SAPHRA (16).

Indol formation has been observed in variants of SS. eastbourne (KRISTENSEN and KAUFFMANN, 17), panama, enteritidis, (SELIGMANN and SAPHRA, 18), and of S. oregon by HINSHAW.

Anaerogenicity was observed in cultures of SS. paratyphi A, B, typhi murium, montevideo, enteritidis, sendai, anatum by KAUFFMANN, SELIGMANN, and SAPHRA, and a number of other workers.

Loss of motility is usually of a temporary nature. It can frequently be re-estabilished by culturing at room temperature in suitable fluid media, e.g. brain-heart infusion broth.

When such deviations from the rule concern type characteristics (anaerogenic SS. typhi, gallinarum, gelatin liquefying SS. abortus bovis, schleissheim, dar-es-salaam, texas, etc., non-motile SS. gallinarum), they do not pose a diagnostic problem. Nor are there particular difficulties in cases, where lactose and salicine fermentation, loss of gas formation or non-motility occur in variants of known parent cultures.

The problem of classification, however, becomes more delicate when aberrant cultural features are observed in nature without evidence of genetic relationship to a typical Salmonella of identical *serology* (16).

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[2] except for SS. gallinarum-pullorum.

[3] except for SS. typhi, gallinarum, whose type characteristic is anaerogenicity.

[4] except for SS. abortus bovis, schleissheim, dar-es-salaam, texas, hidalgo, memphis, canastel, georgia, and a few recent types.

On the whole these rare deviations from the rule are almost negligible for general diagnostic practice. Tests with dextrose, lactose, sucrose, sorbitol, salicine, suitable media for indol and H₂S production usually precede and are corroborated by the serological Salmonella diagnosis. The cultural investigation may even be condensed to some combined media as e.g. KRUMWIDE's triple sugar agar slants (dextrose, lactose, sucrose), KLIGLER's medium (lactose, dextrose, iron salt), and BRAUN and SILBERSTEIN's media A and B, which contain lactose, sucrose, mannite as differentials (with paper reagents for indol and H₂S).

On the other hand, some more fermentative tests with carbohydrates, alcohols, and organic acids may be used to characterize certain types or to differentiate some types which are antigenically identical or closely related. A striking example is given in Table I. It demonstrates the cultural delimitation of types which cannot be differentiated by serological means.

TABLE I.

Cultural Differentiation of Serologically Closely Related Types of Group C.

	Arabi-nose	Acid from Inosi-tol	Trehal-ose	Dulci-tol	d-Tar-trate	H₂S Formation	Growth on Agar
S. paratyphi C	(+)	—	+	+	+	+	abundant
S. cholerae suis. A.	—	—	—	(—)	(—)	(—)	abundant
S. cholerae suis. E.	—	—	—	(—)	(—)	+	abundant
S. typhi suis.	+	—	+	+	+	—	sparse
S. thompson	+	+	+	+	+	+	abundant

Legend. ( ): exceptions occur. A.: American Variety. E.: European Variety (Kunzendorf).

c. The diagnostic table of Kauffmann and White.

Salmonella bacteria are endowed with somatic (O) and flagellar (H) antigens; few with Vi-antigen. The combination of O und H in a certain strain characterizes the type. To facilitate the determination of a given type, a diagnostic table, the so-called *Kauffmann-White Schema* has been set up, using a now generally accepted nomenclature and arranging the great many types according to their prevailing O-antigens. This seemingly complicated table is in fact an abbreviation. It simplifies the antigenic mosaic for the sake of diagnostic clarity and omits minor differences of no practical bearing.

Kauffmann-White Table (1946).

d. The Antigens.

This Kauffmann-White Table (1946) was submitted in July 1947 at the Fourth International Congress for Microbiology and

was published in 1949 as a reprint. It is a simplified version of the previous ones. The types are newly arranged in part, and some of the formerly listed types have been omitted for specific reasons. A list of new types, published since 1946, has been added by us as an appendix.

The Schema establishes 9 groups, A, B, C, D, E, F, G, H, I, according to the predominant *O-antigens,* and a collection of further groups of all those types, which do not fit into the A-I-groups.

TABLE II.

The Kauffmann-White-Table (April, 1950).

Type	O-Antigen	H-Antigen	
		Phase 1	Phase 2
Group A			
S. paratyphi A	I, II, XII	a	—
Group B			
S. kisangani	I, IV, V, XII	a	1,2
S. arechavaleta	IV, V, XII	a	1,7
S. bispebjerg	I, IV, XII	a	e,n,x
S. abortus equi	IV, XII	—	e,n,x
S. tinda	I, IV, XXVII, XII	a	e,n,z_{15}
S. paratyphi B	I, IV, V, XII	b	1,2,
S. abony	I, IV, V, XII	b	e,n,x
S. abortus bovis	I, IV, XXVII, XII	b	e,n,x
S. schleissheim	IV, XXVII, XII	b,z_{12}	—
S. abortus ovis	IV, XII	c	1,6
S. altendorf	IV, XII	c	1,7
S. stanley	IV, V, XII	d	1,2
S. schwarzengrund	I, IV, XXVII, XII	d	1,7
S. salinatis	IV, XII	d,e,h	d,e,n,z_{15}
S. saint paul	I, IV, V, XII	e,h	1,2
S. reading	IV, XII	e,h	1,5
S. kaposvar	IV, V, XII	e,h	1,5
S. kaapstad	IV, XII	e,h	1,7
S. chester	IV, V, XII	e,h	e,n,x
S. san diego	IV, V, XII	e,h	e,n,z_{15}
S. derby	I, IV, XII	f,g	—
S. essen	IV, XII	g,m	—
S. california	IV, XII	g,m,t	—
S. budapest	I, IV, XII	g,t	—
S. typhi murium	I, IV, V, XII	i	1,2
S. texas	IV, V, XII	k	e,n,z_{15}
S. bredeney	I, IV, XXVII, XII	l,v	1,7
S. brandenburg	IV, XII	l,v	e,n,z_{15}
S. heidelberg	IV, V, XII	r	1,2
S. coeln	IV, V, XII	y	1,2
S. stanleyville	IV, XII	z_4,z_{23}	—
*S. haifa	I, IV, V, XII	z_{10}	1,2
S. brancaster	I, IV, XII	z_{29}	—

* not yet published (April 1, 1950).

TABLE II (continued).

Type	O-Antigen	H-Antigen Phase 1	H-Antigen Phase 2
Group C_1			
S. san juan	VI, VII	a	1,5
S. oslo	VI, VII	a	e,n,x
S. edinburg	VI, VII	b	1,5
S. leopoldville	VI, VIII	b	1,6
S. georgia	VI, VII	b	e,n,z_{15}
S. paratyphi C	VI, VII, Vi	c	1,5
S. cholerae suis	VI, VII	c	1,5
S. typhi suis	VI, VII	c	1,5
S. birkenhead	VI, VII	c	1,6
S. mission	VI, VII	d	1,5
S. amersfoort	VI, VII	d	e,n,x
S. lomita	VI, VII	e,h	1,5
S. norwich	VI, VII	e,h	1,6
S. braenderup	VI, VII	e,h	e,n,z_{15}
S. montevideo	VI, VII	g,m,s	—
*S. menston	VI, VII	g,s,t	—
S. thompson	VI, VII	k	1,5
S. daytona	VI, VII	k	1,6
S. singapore	VI, VII	k	e,n,x
S. concord	VI, VII	l,v	1,2
S. irumu	VI, VII	l,v	1,5
S. potsdam	VI, VII	l,v	e,n,z_{15}
S. colorado	VI, VII	l,w	1,5
S. jerusalem	VI, VII	l,w	z_{10}
S. makiso	VI, VII	l,z_{28}	z_6
S. oranienburg	VI, VII	m,t	—
S. virchow	VI, VII	r	1,2
S. infantis	VI, VII	r	1,5
S. papuana	VI, VII	r	e,n,z_{15}
S. richmond	VI, VII	y	1,2
S. bareilly	VI, VII	y	1,5
S. hartford	VI, VII	y	e,n,x
S. mikawasima	VI, VII	y	e,n,z_{15}
S. aequatoria	VI, VII	$z_4.z_{23}$	e,n,z_{15}
S. mbandaka	VI, VII	z_{10}	e,n,z_{15}
S. tennessee	VI, VII	z_{29}	—
Group C_2			
S. narashino	VI, VIII	a	e,n,x
S. gatuni	VI, VIII	b	e,n,x
S. muenchen	VI, VIII	d	1,2
S. manhattan	VI, VIII	d	1,5
S. newport	VI, VIII	e,h	1,2
S. kottbus	VI, VIII	e,h	1,5
S. takoradi	VI, VIII	i	1,5
S. bonariensis	VI, VIII	i	e,n,x
S. litchfield	VI, VIII	l,v	1,2
S. manchester	VI, VIII	l,v	1,7
S. fayed	VI, VIII	l,w	1,2
S. bovis morbificans	VI, VIII	r	1,5

* not yet published (April 1, 1950).

TABLE II (continued).

Type	O-Antigen	H-Antigen Phase 1	H-Antigen Phase 2
Group C₂ (continued)			
S. hidalgo	VI, VIII	r	e,n,z_{15}
*S. praha	VI, VIII	y	e,n,z_{15}
S. duesseldorf	VI, VIII	z_4,z_{24}	—
S. tallahassee	VI, VIII	z_4,z_{32}	—
S. glostrup	VI, VIII	z_{10}	e,n,z_{15}
S. sanga	(VIII)	b	1,7
S. virginia	(VIII)	d	—
*S. emek	(VIII), XX	g,m,s	—
S. kentucky	(VIII), XX	i	z_6
S. corvallis	(VIII), XX	z_4,z_{23}	—
S. amherstiana	(VIII)	l,(v)	1,6
Group D			
S. sendai	I, IX, XII	a	1,5
S. miami	I, IX, XII	a	1,5
S. loma-linda	IX, XII	a	e,nx
S. durban	IX, XII	a	e,n,z_{15}
S. onarimon	I, IX, XII	b	1,2
S. typhi	IX, XII, Vi	d	—
S. ndolo	IX, XII	d	1,5
S. eastbourne	I, IX, XII	e,h	1,5
S. berta	IX, XII	f,g,t	—
S. enteritidis	I, IX, XII	g,m	—
S. blegdam	IX, XII	g,m,q	—
S. pensacola	IX, XII	g,m,t	—
S. dublin	I, IX, XII	g,p	—
S. rostock	I, IX, XII	g,p,u	—
S. moscow	IX, XII	g,q	—
*S. neasden	IX, XII	g,s,t	—
S. claibornei	I, IX, XII	k	1,5
S. panama	I, IX, XII	l,v	1,5
S. goettingen	IX, XII	l,v	e,n,z_{15}
S. dar-es-salaam	I, IX, XII	l,w	e,n
S. napoli	I, IX, XII	l,z_{13}	e,n,x
S. javiana	I, IX, XII	l,z_{28}	1,5
*S. shoreditch	IX, XII	r	e,n,z_{15}
S. canastel	IX, XII	z_{29}	1,5
S. gallinarum	I, IX, XII	—	—
S. pullorum	IX, XII	—	—
Group E₁			
S. butantan	III, X, XXVI	b	1,5
S. shangani	III, X, XXVI	d	1,5
S. vejle	III, X, XXVI	e,h	1,2
S. muenster	III, X, XXVI	e,h	1,5
S. anatum	III, X, XXVI	e,h	1,6
S. nyborg	III, X, XXVI	e,h	1,7

* not yet published (April 1, 1950).

TABLE II (continued).

Type	O-Antigen	H-Antigen Phase 1	H-Antigen Phase 2
		Group E$_1$ (continued)	
S. meleagridis	III, X, XXVI	e,h	l,w
S. zanzibar	III, X, XXVI	k	1,5
S. london	III, X, XXVI	l,v	1,6
S. give	III, X, XXVI	l,v	1,7
S. uganda	III, X, XXVI	l,z$_{13}$	1,5
S. simi	III, X, XVII	r	e,n,z$_{15}$
S. weltevreden	III, X, XXVI	r	z$_6$
S. amager	III, X, XXVI	y	1,2
S. orion	III, X, XXVI	y	1,5
S. lexington	III, X, XXVI	z$_{10}$	1,5
S. macallen	III, X, XXVI	z$_{36}$	—
		Group E$_2$	
S. newington	III, XV	e,h	1,6
S. selandia	III, XV	e,h	1,7
S. cambridge	III, XV	e,h	l,w
S. canoga	(III), (XV)	g,s,t	—
S. new brunswick	III, XV	l,v	1,7
S. illinois	(III), (XV)	z$_{10}$	1,5
		Group E$_3$	
S. chittagong	I, III, XIX	b	z$_{35}$
S. niloese	I, III, XIX	d	z$_6$
S. senftenberg	I, III, XIX	g,s,t	—
S. taksony	I, III, XIX	i	z$_6$
		Group F	
S. marseille	XI	a	1,5
S. luciana	XI	a	e,n,z$_{15}$
S. pharr	XI	b	e,n,z$_{15}$
S. chandans	XI	d	e,n,x
S. aberdeen	XI	i	1,2
S. veneziana	XI	i	e,n,x
S. pretoria	XI	k	1,2
S. senegal	XI	r	1,5
S. rubislaw	XI	r	e,n,x
S. solt	XI	y	1,5
		Group G	
S. atlanta	XIII, XXIII	b	—
S. mississippi	I, XIII, XXIII	b	1,5
S. grumpensis	XIII, XXIII	d	1,7
S. wichita	I, XIII, XXIII	d	—
S. havana	I, XIII, XXIII	f,g	—

TABLE II (continued).

Type	O-Antigen	H-Antigen Phase 1	Phase 2
Group G (continued)			
S. borbeck	XIII, XXII	l,v	1,6
S. worthington	I, XIII, XXIII	l,w	z
S. poona	XIII, XXII	z	1,6
S. cubana	I, XIII, XXIII	z_{29}	—
Group H			
S. heves	VI, XIV, XXIV	d	1,5
S. florida	(I), VI, XIV, XXV	d	1,7
S. onderstepoort	(I), VI, XIV, XXV	e,(h)	1,5
*S. caracas	I, VI, XIV, XXV	g,m,s	—
S. horsham	(I), VI, XIV, XXV	l,v	e,n,x
S. carrau	VI, XIV, XXIV	y	1,7
S. madelia	(I), VI, XIV, XXV	y	1,7
S. sundsvall	(I), VI, XIV, XXV	z	e,n,x
Group I			
S. hvittingfoss	XVI	b	e,n,x
S. vancouver	XVI	c	1,5
S. gaminara	XVI	d	1,7
*S. nottingham	XVI	d	e,n,z_{15}
S. szentes	XVI	k	1,2
S. orientalis	XVI	k	e,n,z_{15}
*S. shanghai	XVI	l,v	1,6
*S. salford	XVI	l,v	e,n,x
Further Groups			
S. kirkee	XVII	b	1,2
S. memphis	XVIII	k	1,5
S. cerro	XVIII	z_4,z_{23}	—
S. minnesota	XXI, XXVI	b	e,n,x
S. kibusi	XXVIII	r	e,n,x
S. pomona	XXVIII	y	1,7
S. tel-aviv	XXVIII	y	e,n,z_{15}
S. urbana	XXX	b	e,n,x
S. donna	XXX	l,v	1,5
S. adelaide	XXXV	f,g	—
S. monschaui	XXXV	m,t	—
S. inverness	XXXVIII	k	1,6
S. champaign	XXXIX	k	1,5
S. rio-grande	XL	b	1,5
*S. allandale	I, XL	k	1,6
S. waycross	XLI	z_4,z_{23}	—
S. weslaco	XLII	z_{36}	—

* not yet published (April 1, 1950).

The main O-antigen of the *A-group* is II, regularly combined with XII and variably with I.

The only member of this group is S. paratyphi A.

The main antigen of the *B-group* is IV, also in combination with XII. The partial antigens I, V, XXVII characterize some members of the group. Of its 22 members, SS. paratyphi B, typhi murium and derby are the most widely distributed types.

The main O-antigen of the *C-group* is VI, in combination with VII in the C_1 sub-group, or with VIII in the C_2 sub-group. 36 types are covered by the two sub-groups. SS. paratyphi C, cholerae suis, oranienburg, montevideo, and newport are the most important types. It may seem inconsistent to include in this group three strains devoid of the VI (SS. amherstiana and virginia and kentucky). The probability, however, that these types with the sole VIII antigen, have lost the originally present VI and thus are only loss variants, may give an excuse for the apparent arbitrariness. On the other hand, the VI occurs also in the H-group in combination with several other antigens (I, XIV, XXIV, XXV). The types concerned have not been included in the C-group, since the VI plays there only a minor role as a partial antigen.

The *D-group* is characterized by the IX-antigen, combined as in the A- and B-groups with XII. The I-antigen is present in some types. SS. typhi, enteritidis, panama, and the animal pathogens SS. gallinarum-pullorum, belong to the group that contains 22 members.

The *E-group* is characterized by the antigen III. Combinations with X, XV, XIX, XXVI, XXXIV provide the pattern for some subdividing of the 21 membres of this group.

The other groups display a great variety of types with various O-antigens. Some of these—I, VI, etc.—occur also in other combinations in the groups A-E.

Whereas the O-antigens determine the group in the *Kauffmann-White* Schema, the *H-antigens* determine the types *within* the groups. Like the O-antigens, the H-antigens may consist of only *one* component, f.i. "a", "b", "d", or they may be as complex as "e, n, x", or "g, s, t" or "1,2".

The table illustrates that most of the Salmonella types are biphasic in their H-antigenicity, and that relatively few are monophasic, as f.i. S. typhosa (IX; d), S. oranienburg (VI, VII; mt). While most known phases may occur in biphasic as well as in monophasic types, there are some phases which so far have been found in monophasic types only: the various g compounds, mt and several z phases. A great number of monophasic as well as biphasic types, regardless of their O-antigenic group, have identical or clo-

sely related H-antigens, as f.i. S. enteritidis (IX; g, m) and S. montevideo (VI, VII; g, m, s), or S. derby (IV; f, g) and S. havana (I, XIII, XXIII; f, g). Other types have one identical and one different phase as f.i. S. san diego (IV, V; e, h—e, n, z_{15}) and S. st. paul (IV, V; e, h-1,2). Or types have no common H-antigen at all, although they belong to the same O-group as f.i. S. typhi and S. panama (d and l,v-1,5).

The *Vi-antigen* is rare. It differs from O-antigens by its heat-lability. Its alcohol stability differentiates it from H-antigens. Its presence has been recognized in SS. typhi, paratyphi C, SS. ballerup and hormaechii. The latter two types have been eliminated recently from the Salmonella group. The Vi-antigen occurs in varying amounts. It may be very strong, it may be altogether absent. Its presence effaces the group specific antigen in proportion, a strong Vi-antigen fully suppressing the group specific agglutinability. A culture of S. typhi may, therefore, present itself in 3 antigenic forms:

IX	VI	d	
—	+++	+++	"V" form
++	++	+++	"VW" »
+++	—	+++	"W" »

Freshly isolated cultures of S. typhi show often a strong Vi- and no IX-antigen. In the course of time, Vi becomes weaker, while the O-antigen appears proportionally, until, with the complete loss of the Vi-antigen, the IX-antigen dominates exclusively. Of practical importance is the fact that boiling for 20 minutes destroys the Vi completely, whereupon the previously non-detectable O-antigen becomes manifest [5].

e. The Antisera.

The described Salmonella antigens are determined with the help of agglutinating sera. These antisera are either group-specific (anti-O) or type specific (anti-H) or anti-Vi-sera. Single factor sera are needed to identify the various partial antigens of some O- or H-antigens; phase specific antisera give the means for identification of biphasic types. Rabbits are generally used for the preparation of agglutinating sera; although good sera have been prepared from horses. Even human convalescent sera may be used for some pur-

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[5] The presence of the Vi-antigen in S. newport was reported but not confirmed. *Felix* and *Pitt* believe it to be present in S. paratyphi B. This is contested by *Kauffmann*, who considers this special antigen as a heat labile fraction of the somatic V-antigen. While the Vi in the above-mentioned types is identical, this so-called Vi is different. Vi-antigen identical with that of S. typhi, has been found in a colon strain by *Kauffmann*.

poses. A medium sized rabbit yields 50 c.c. and more of antiserum at one bleeding, an amount sufficient to perform more than ten thousand agglutination tests.

For the preparation of anti-O-sera smooth single colonies are selected. The grown cultures must be freed of their H-antigens [6]. This is done by boiling the bacterial suspensions. To prepare an agglutinogen for immunization, we take a 24-hour agar slant culture, wash it off with 10 c.c. of saline, boil the suspension in a water bath for 2½ hours, replace the evaporated water and store the antigen in the ice box. Alcohol treatment is not suitable. Although alcohol completely eliminates H-agglutinability, the H-agglutinogens are not completely destroyed and may elicit some interfering H-agglutinins on immunization.

Intravenous injections are given in intervals of 6 or 7 days. We start with 0.1 or 0.2 c.c. and increase the dose gradually, up to 1.5 or 2.0 c.c. if necessary. 3-4 injections usually suffice to produce a serum of adequate titer. Trial bleeding after 10 days after the last injection, and, if satisfactory, final removal of blood by heart puncture are performed. The properly separated serum is mixed with equal parts of sterile glycerin and further preserved with merthiolate 1 : 10,000. Thus conditioned, it keeps sterile, clear and potent for years (we have in our laboratory sera which have not lost their titer for more than 8 years).

Every O-serum should be tested for tube and slide agglutination titer, before it is used, and should be rechecked from time to time. The minimal titer of the glycerinated sera in the test tube should be no less than 1 : 320 for the homologous antigen; higher titers (1 : 2,500 and more) are easily obtained. Further quantitative tests should be done with antigenically related types of other groups, in order to appraise the degree of any cross-reaction. F.i. a serum prepared with S. panama (I, IX, XII) may display cross-agglutination with B group and S. paratyphi A types which have the I or the XII antigen in common with S. panama. A serum prepared with S. newport (VI, VIII) may cross-agglutinate with all VI, VII types and with S. kentucky (VIII, XX) as well as with SS. virginia, amherstiana (VIII) and the whole carrau-onderstepoort group (VI, XIV . . .). Some more cross-reactions have been observed for which an explanation by the labelled antigens cannot be advanced. A table of all kinds of occurring cross-reactions may be found in KAUFFMANN's book (4).

Quantitative appraisal of such cross-reactions will facilitate the

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[6] Only the naturally flagellaless type S. pullorum and occasionally occurring flagellaless variants of motile types may be used without further treatment.

use of the O-serum in question and indicate the proper serum dilution for diagnostic use [7].

The same procedure is to be followed for the determination of the titer for slide (or spot) agglutination. It is essential to secure the proper dilution of the serum which still gives a distinct slide agglutination of the homologous type and its affiliated O-group members, but no, or only a negligible, agglutination of related O-antigens or groups.

A serum with a homologous tube agglutination titer of 1 : 320 will give an appropriate slide agglutination in a dilution 1 : 5—1 : 10, a titer of 1 : 640—1,280 will correspond to a slide agglutination titer of 1 : 10—20. However, the condition of antigens and antibodies may scale these figures considerably up and down. Only thorough-going and repeated testing with a variety of homologous and heterologous antigens will determine the optimal slide agglutination titer of a serum. The slide test has many advantages; it saves time and material, thus allowing the testing of more antigens with more sera. The slide agglutination takes only a few seconds, whereas a tube agglutination needs at least 2 hours for a reliable reaction. For this reason the determination of the O-antigen is done primarily by the slide agglutination test. Doubtful results should be checked by tube agglutination.

For the spot agglutination a drop of serum is placed on a slide; so much material of a fresh agar slant culture, as can be taken up with the tip of a platinum wire, is carefully emulsified in the drop. Slides are tilted for a few seconds, then observed either with the naked eye or with the help of a low-power hand lens or a binocular with up to 12-fold magnification. A concave mirror (microscope-mirror reflector or shaving mirror), put underneath the slide, reflects well the enlarged image of the drop, thus facilitating the reading.

The preparation of H-antisera and the problem of H-agglutination is more involved than that of the O-group. It has not been possible as yet to produce an O-free pure H-antigen. Attempts have been made (ORCUTT, JENKINS) but, so far, the antigenicity of these products has not been proven. All ordinary H-antisera, therefore, contain varying amounts of group specific O-antibodies beside the desired H-agglutinins. Two possible methods present themselves, for the elimination of O-antibodies; both, however, are far from

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[7] Some unwanted reactions may be excluded by the proper choice of types for immunization. Members of the A, B and D groups, free of the partial antigen I, as SS. paratyphi A, var. durazzo (II), S. reading (IV) and S. pullorum (IX) will minimize cross-reactions with I containing types; selection of SS. virginia or amherstiana (VIII) for the C_2 group excludes reactions with the C_1 group.

perfect. Rough cultures, which had lost the original O but retained
the H, were tried. The sera, however, prepared with such strains,
frequently developed anti-rough agglutinins which were not at all
specific, but gave overlapping reactions with any strain in a more
or less rough condition. The other method consists in the use of
O + H containing antigens and in absorbing the O-antibodies of
the serum by a pure, H-free O-antigen of the group. This technic
works, but it usually lowers the H-titer of the serum and introduces
a new factor that does not simplify the serum preparation. A fur-
ther difficulty concerns biphasic Salmonella strains. There the
single phases must be isolated and kept in such a condition that
they do not split off the other phase. The isolation is sometimes
hampered by poor development or even absence of one or both
phases. This deficiency may be overcome by growing the culture
in suitable broth at room temperature. If such a broth culture does
not yield a well motile antigen within 24 hours, the broth is centri-
fuged for one hour at high speed, thereby eliminating all the less
motile organisms. A drop of the supernate then is brought into a
new tube of broth. This procedure—repeated if necessary—will
result eventually in a well motile culture by selection of the most
motile organisms. Semisolid agar (½%) may be substituted for
brain heart infusion or other broth. The semisolid agar medium is
particularly helpful in regaining a missing phase. Mixed with
diluted antiserum against the other, present phase, it suppresses
the motility of organisms with just this phase but does not impede
the development and swarming of the few organisms of the seem-
ingly missing phase. These multiply and gather, if plates are
employed, at the periphery of the cultural growth, or, if agar sticks
are used at the walls of the tube. This ingenious method, devised
by GARD, may also be adapted to cultures in U-shaped tubes. One
arm of the tube filled with the mixture of semisolid agar and anti-
serum is inoculated by stabbing. The organisms accessible to the
H-antiserum, will be arrested around the site of inoculation, where-
as the non-inhibited members of the other phase (if present at all)
will swarm out and may be caught at the other arm of the U-tube.
Thus the isolation and maintenance of single phases as antigens
is necessary for any H-antiserum production. It is helpful to have
available several H-identical antisera of different O-types, in order
to avoid unwanted O-antibodies which might interfere in the prac-
tical diagnosis. Therefore, the selection of the proper cultures for
immunization is of importance. A few examples will suffice: S.
oranienburg, a type frequently encountered, has the antigenic
formula VI, VII; m,t. Until recently all m,t-antisera were prepared
with this type. It was not always easy to differentiate O- and H-

agglutinations and to avoid embarrassing cross-reactions with all
members of the C_1 group. The discovery of a new type—S. mon-
schaui—with the same H but a totally different O (XXXV; m,t)
provided the means for another m,t-antiserum, not interfering with
any other O-antigen. S. paratyphi B (B-group) is endowed with a
strong b-phase. To identify it, without undesirable O-reaction, a
b-antiserum is prepared with S. hvittingfoss (O; XVII). S. derby
(B-group) has the f,g-phase; S. adelaide (XXXV; f,g) is suited for
unhampered type determination. For the i of S. typhi murium an
antiserum derived from S. aberdeen (XI) may be used; for the d
of S. typhi or S. muenchen, antisera prepared with S. virginia
(VIII) or S. wichita (I, XIII, XXIII) are helpful. The reverse proce-
dure takes place, to identify the rare types just mentioned: b-serum
from S. paratyphi B is ideally suited for the recognition of S.
hvittingfoss, fg.-serum from S. derby for S. adelaide, etc.

The general method, used by us for the preparation of anti-H-
sera from *diphasic* types is the selection of a single colony, sub-
cultured in 10 c.c. of infusion broth and incubated at $37°$. After 4-5
hours, 0.05 c.c. formalin (1 drop) is added to the rubber stoppered
test tube. The material is kept in the icebox for 2 days, then tested
for sterility. Centrifugation, washing of the sediment, resuspension
in 10 c.c. saline follow. After renewed serological testing the antigen
is ready for use. In *monophasic* strains living cultures or formolized
antigens older than 4-5 hrs. can be used.

Dosage, timing, bleeding, serum preparation, titration with the
homologous and related antigens are the same as described above
for O-antisera. The H-sera titers determined in the test tubes are
generally high, 1 : 8,000, 16,000, 32,000 and more, whereas their
O-titers are usually below 1 : 1,000. The proper dilutions for slide
agglutination (H) range between 1 : 100 and 1 : 1,000. Positive
reaction occurs almost immediately, while any O-agglutination, if
occurring at all, is delayed.

Monophasic H-antisera may be prepared by the use of all those
types which are listed as monophasic in the table. In addition there
are some variants of biphasic strains which display only one phase.
S. paratyphi B, var. java, has only the b-antigen, S. newport, var.
puerto rico, only the 1,2, S. cholerae suis, var. kunzendorf usually
only the 1,5. With these strains, too, monophasic antisera may be
produced.

The slide agglutination method is used for the H-determination
in our laboratory, as in those of Kauffmann, Hormaeche, et al.
Edwards and his associates prefer the test tube agglutination in
order to exclude any interfering O-reaction. Both methods have
their advantages. Quantitative determinations have to rely on the

tube test. For further identification of closely related H-antigens, single factor antisera are necessary (see below). *Vi-antisera* are especially needed for the serological identification of inagglutinable strains of S. typhi. Although boiling of such cultures may restore the agglutinability to O-antiserum, the method is laborious and requires a good deal of material and time and does not always result in well-agglutinable, stable suspensions. It is, therefore, advantageous to possess a potent and reliable Vi-antiserum, the use of which dispenses with the boiling procedure.

The preparation of such a serum is rather delicate. Single colonies have to be selected which present pure V-forms. These may be suspended in saline and injected directly, or broth and agar cultures may be prepared from these single colonies. They may be used for immunization in the living stage or after formolization or alcoholization. Vi-antibodies develop readily, although to a lower titer. In addition the antisera contain H-antibodies and O-antibodies. It must be kept in mind that the O-antigen, seemingly missing in Vi-cultures, is suppressed in its agglutinability only, but has not lost its capacity of stimulating antibodies. A Vi-antiserum, prepared with S. typhi, therefore, must be freed of the anti-O- and H-agglutinins by specific absorption. The best antigen for the production of a potent Vi-antiserum without disturbing cross-reactions is S. ballerup. Its O- and H-antigens are of a seldom encountered kind. Their antibodies, if present, do not give any diagnostic difficulties. Another Vi-antiserum, prepared with KAUFFMANN's special B. coli (No. 1), is also valuable for the agglutination of typhoid Vi-cultures, but less so for S. paratyphi C. It has the H-antibody 1.5, which reacts with the second phase of that type. Titers of suitable Vi-antisera vary from 1 : 5—1 : 20 on slide agglutination and from 1 : 160—1,280 in the test tube.

Further methods of differential diagnosis: With O-, H- and Vi-antigens on hand, one is able to classify the known Salmonella organisms into groups and types. There remain, however, some features both in O- and H-antigens, which demand further differentiation for the final establishment of certain types.—For instance: members of the C_1 (VI, VII) and C_2 (VI, VIII) group usually react with both, anti-VI,VII- and anti-VI,VIII-sera, due to the common VI-antigen. Sera that do not contain an anti-VI, as those against S. virginia or S. kentucky, but possess the VIII-antibody, will agglutinate the C_2 (VI, VIII) group and not the C_1 group (VI, VII). Another example: the m,t-antigen (S. oranienburg) and the g,m-antigen (S. enteritidis) cross-react due to the mutual m-fraction. An f,g-serum will agglutinate the g,m-antigen but not the m,t.

The availability of similarly related antisera is limited. For the

most part one has to resort to *single factor sera*, in order to eluci-
date the often complicated antigenic mosaic of O and H. The minor
part of the Salmonellae contains only one kind of O-antigen; the
great majority is composed of several kinds. Although they seem to
represent an inseparable chemical entity, their partial antigens give
rise to differentiated antibodies in the immunized animal. Thus a
VI,VIII-antigen elicits a combination of VI- and VIII-antibodies.
It is possible to remove the one without touching the other. A
VI,VII-antigen added in an adequate amount to a VI,VIII-anti-
serum will, therefore, absorb the VI-antibody and leave the VIII-
antibody in a now "single factor" serum. Such a serum differen-
tiates the two subgroups of C as readily as a virginia-antiserum.
The principle, as demonstrated in this example, consists of re-
moving, by proper choice of antigens, the mutual antibodies and
isolating in this way the characteristic and differentiating one.
Single factor sera against V or VI or X or XV or XXVII and many
others have been prepared, but only a few of them are of major
diagnostic importance.

Exactly the same absorption and production of single factor
antibodies is employed in H-antisera. There the diagnostic value is
greater. Differentiation of the closely related antibodies against 1,2,
1,5, 1,6, 1,7 is made possible by anti-2, 5, 6, 7 single factor sera.
Single factor h, x, z_{15} antisera differentiate the phases e,h, e,n,x,
e,n,z_{15}. In the same way the various g-compounds may be analyz-
ed. The technic of the absorption method varies according to the
strength of the antiserum and the avidity of the employed antigens.
Detailed data may be found in KAUFFMANN's (4), and EDWARDS and
BRUNER's (5) publications.

f. The diagnostic procedure.

The use of a screening, *multivalent serum*, combining all or se-
lected groups of antibodies, has been recommended and is in use at
many places. It should exclude enterobacteria of other species, as
f.i. Colon and Paracolon bacilli, Shigellae, B. proteus, and confine
further investigations to those organisms that are agglutinated by
this serum.

Multivalent sera may be prepared either by immunizing rabbits
with a variety of O- and H-antigens simultaneously (KAUFFMANN,
FELSENFELD, EDWARDS, BRUNER, et al.) or by pooling a number of
selected univalent antisera. We are using a pooled O-antiserum
made up by sera for the groups B-E. Its preparation is illustrated in
the following Table:

TABLE III.

Preparation of a Pooled O-Serum.

(Merthiolated 1 : 10,000.)

Serum against Type	Antigenic Formula	Homologous Titer in Slide Agglutination	Undiluted Serum	Saline
			ml.	ml.
S. paratyphi B	I, IV, V, XII	1 : 20	5.0	
S. oranienburg	VI, VII	1 : 40	2.5	
S. virginia	VIII	1 : 50	2.0	85.5
S. enteritidis	(I), IX, XII	1 : 25	4.0	
S. anatum	III, X	1 : 100	1.0	

This serum, tested against almost 5,000 Salmonella cultures, covered approximately 99 %. It fails with some rare types and with cultures in a pure Vi-state. If positive, the preliminary test is to be followed by agglutination with individual O-sera (group A-E first) and by the determination of the flagella antigens.

Sometimes, however, reactions with these naturally or artifically combined sera seem ambiguous. A positive reaction may be found with non-Salmonella strains. It is known that a number of O- and a few H-antigens, and even the Vi-antigen, occur in members of the colon-paracolon, Shigella, proteus groups. The O-antigens, I, VI, XIII are not very rare in other enteric organisms, the H-antigens e,h, and 1.5, g . . . , have been occasionally demonstrated in some coliform organisms. The XXXIII-antigen is particularly frequent in certain paracolon types [8]. Positive reactions in rare instances have also been registered without confirmation by individual sera. Convenient as a screening serum of the described sort may be, its use requires experience and discrimination.

The average laboratory which is not doing Salmonella work regularly, is better served with a set of O-antisera of the groups A, B, C_1 C_2, D and E, and an additional Vi-antiserum. The anti-A-serum should be prepared with S. paratyphi A, var. durazzo (II). A positive reaction with this serum secures the diagnosis of a suspected S. paratyphi A, since no other Salmonella possesses the II. The other anti-O-sera may be produced with any member of the respective group, however, a pure VIII-serum (SS. virginia, amherstiana) for C_2 is recommended.

With such a set of antisera, one is able to group a very high per-

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[8] S. arizona has this O-antigen. The position of this type in the Salmonella group was somewhat problematic, due to the observation of very belated lactose fermentation and gelatine-liquefaction of some strains. It has now been omitted from the K-W-Table, and inserted into the arizona group of paracolon bacteria by *Edwards, West* and *Bruner* (Bulletin [1947] 499, Kentucky Agricult. Exper. Sta., Univ. Kentucky, Lexington).

centage of the occurring Salmonella strains by slide agglutination. Thus, provided the ordinary cultural features are in agreement, not only the diagnosis Salmonella is achieved but also the classification into the most common groups. S. typhi may be recognized without further tests, if lack of gas production in dextrose coincides with the presence of IX- and/or Vi-antigen [9]. In all other cases *positive reacting cultures* should be sent to a laboratory equipped with the technical means for identification of the individual type. This sort of co-operation has proved rather successful in our experience.

Some laboratories, more frequently confronted with Salmonella problems, may go a step further und use an additional limited number of H-antisera in order to identify the most widely spread endemic types. The prevalence of certain Salmonella types varies geographically [10]. The most commonly encountered types in human beings anywhere are the following: S. paratyphi B, S. typhi murium, S. derby, S. cholerae suis, S. oranienburg, S. montevideo, S. newport, S. typhi, S. enteritidis, S. panama, S. anatum. Special geographic conditions seem to influence the distribution of S. paratyphi A, S. sendai and S. paratyphi C. For the differential diagnosis of the more frequent types the following H-sera are required: a, b, d, i, f,g, g,m, m,t, l,v, e,h, 1,2 and 1,5. BORNSTEIN recommended a very similar set-up for use in the U.S.A., proposing 6 anti-O-, 11 anti-H-sera and a Vi-antiserum. Recently KAUFFMANN and EDWARDS have propounded a simplified serological diagnosis for the most frequent Salmonella types. They use 5 O-sera: I, II, IV, V, VI, VII, VIII (SS. thompson + newport), IX and III, X, XV (SS. anatum + newington). The H-sera are: a, b, c, d, i, 1, 2, 3, 5 (SS. thompson + newport). A ballerup Vi-serum is added and for bacteriologists particularly concerned with animal Salmonellosis H-sera e,n,x and g,p for SS. abortus equi and dublin are provided (20).

Judicious use of the H-agglutinins in combination with the A-E group O-sera will give a high percentage of correct diagnoses of the most important types. On the other hand, some confusion with antigenically closely related types cannot be prevented because of insufficient differentiation of the "non-specific" second phases 1,2, 1,5, 1,6, 1,7, or because of cross-reactions between g,m, m,t, f,g, etc. Moreover, a considerable number of established agglutinins, for instance, c, e,n,x, etc., are missing in this set-up. An exact antigen analysis of all types of the Kauffmann-White Schema or the establishment of new types, can only be attempted by laboratories spe-

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[9] There is a faint risk of mistake if one of the very rare gasless variants of members of the D group is involved or the animal pathogen S. gallinarum.

[10] A good survey on geography of Salmonella has been published by *Felsenfeld* and *Young* (19).

cializing in this field. They must possess a complete set of all agglutinating sera including single factor agglutinins, a complete type culture collection, and experienced workers. This was facilitated by the International Salmonella Center in Copenhagen (FRITZ KAUFFMANN) and National Salmonella Centers in many countries, which received their first sets of sera and cultures from Copenhagen. The U. S. Army has equipped a series of its laboratories with complete "typing kits" for Salmonella diagnosis. Results have been very satisfactory. Some more typing centers have been established at various places, and commercial manufacture of adequate sera has been attempted.

Agglutination tests dominate the methods of Salmonella diagnosis. Some other tests have been employed, which, in a general way, confirm the results derived from agglutinations. These are: complement-fixation, precipitation, specific agglutinin-inhibition (SELIGMANN, 21), and typing with phages. The latter method was tried in the Salmonella group by BURNET (22), CRAIGIE and YEN (23) developed it for the subdivision of typhoid bacilli by means of different type specific Vi-phages. FELIX and CALLOW (24) attempted its use in a similar way for S. paratyphi B. The epidemiological bearing of phage typing has been acknowledged; but for strictly diagnostic purposes all the above-mentioned methods are of secondary importance only.

II. Serological response of the infected.

The Gruber-Widal serum reaction which for several decades has been in use to determine the specific antibody production against typhoid and paratyphoid bacilli in infected, diseased, and vaccinated individuals, has set the pattern for identical tests in the whole group of Salmonella. During the course of a Salmonellosis the formation of antibodies may take place, whenever generalized symptoms develop or when a mere gastroenteric syndrome is moderately severe and of some duration. As in typhoid fever, a positive serum reaction may not be expected before the end of the first week; from then on the titer rises for the next days and weeks. A slow decrease follows with the end of the disease, leading eventually to weak remnants of agglutinins or negativity. Mild cases of a transitory character fail to stimulate antibody production; healthy carriers, with no history of previous disease, are usually devoid of specific agglutinins. Infants, although critically ill, rarely have a positive serum reaction; their poor ability to produce antibodies of any kind is a known fact.

Some restrictions are to be applied for the correct appraisal of tests with the patient's serum. Non-specific agglutinations, even with a rising titer, may be observed under the impact of a non-related infection. Residuals of formerly present specific antibodies (especially H-agglutinins) will become reactivated (*anamnestic* reactions). As a rule such a reaction recedes rather quickly. False positive serum reactions may be given by patients who previously have undergone prophylactic immunization against typhoid and paratyphoid bacilli (vaccines TAB or TABC). Those individuals develop the specific O- and H-antibodies and retain them often for many months.

The classical GRUBER-WIDAL test used to be performed with living bacteria or formolized broth antigens. No differentiation of antigens could be attempted at a time when such antigen differences were not known. As a consequence, misleading reactions may have been observed. The antigens of S. typhi or paratyphi B are agglutinated by somatic antibodies against all other members of the D or B group. If they contain flagellar antigens, as they usually do, cross-reactions may occur with all types that possess the H-antigen d (SS. muenchen, virginia and others) or the second phases 1,2, 1,5, 1,6, 1,7, which are shared in part with S. paratyphi B and are rather common in many groups. For this reason FELIX (25) in 1930 proposed the use of selected formolized (H) and alcoholized (O) antigens side by side, in order to facilitate the differentiation and the diagnostic combination of antibodies. Elaborating on this suggestion and adapting it to general practical use, we have prepared a variety of such antigens for routine tests in diseases of unknown origin. The choice of the antigens depends mainly on the organisms prevailing in the respective area. Since SS. typhi (IX: d), paratyphi B (IV; b-1,2), typhi murium (IV; i-1,2) are widely spread all over the world, as are members of the C group (SS. cholerae suis, oranienburg, montevideo, newport), a routine serum test should include the following antigens: IV, V, IX, VI, VII for the determination of O-antibodies, and b, d, i, 1,2 or 1,5 for H. According to regional requirements antigens II, VIII, III, X, or other O- and H-antigens may be added. Stable, well-agglutinable O-antigens are best prepared by alcoholization. A 24-hour agar slant is washed off with 2 c.c. of saline, 6 c.c. of 98% alcohol is added, the mixture is kept at room temperature for 2 days in a rubber-stoppered bottle, and then centrifuged. The decanted alcohol mixture is replaced by 3-4 c.c. of formolized saline solution, and stored in the ice box. H-antigens of maximal agglutinability are prepared by adding 0.5% formalin to a well motile 24-hour culture in broth, in a rubber-stoppered flask. 48 hours at room

temperature ordinarily suffice to sterilize the antigen, which then is centrifuged and resuspended in buffered 0.5% formalin. It is possible, by selection of special H-antigens, to eliminate interfering O-reactions. SS. urbana, virginia, kentucky, newport var. puerto rico, will furnish suitable antigens for b, d, i, and 1,2 respectively.

The serum in question is diluted with saline 1 : 40. To tubes with 0.5 c.c. of this dilution the afore-mentioned antigens are added and kept at 37° for 2 hours. Tentative reading, followed by final appraisal after 24 hours at room or ice box temperature. If positive reactions are recorded, higher serum dilutions are tested with the reacting antigen (up to 1 : 400 and more). While high titers may be found on occasion, a definite positive agglutination in dilutions of 1 : 40-1 : 80 against O or H may be rated as indicative of specific antibodies. In doubtful cases (negative, or 1 : 40 only) a repetition of the serum test after 4-7 days is advisable. Rise or decline of the titer or no change are of diagnostic value.

A previously reported experience with a young child (SELIG-MANN, SAPHRA, WASSERMANN, 26) demonstrates the usefulness of the test. This child displayed a picture of recurrent diarrhea and attacks of fever of "unknown origin". Stool examination was negative at the beginning, but the serum reaction showed high titers against alcoholized S. derby (IV), formolized S. kentucky (i) and formolized S. newport var. puerto rico (1,2). This combination of agglutinins was suggestive of an infection with S. typhi murium. One week later this organism was isolated from the stool, proving the etiological correlation of the sero-logical and belated bacteriological findings.

The serum test may prove to be a valuable diagnostic help, also in cases, where a Salmonella organism has been isolated from the sick person. Under these conditions the living homologous strain (cultured on moist agar slant or sediment of a broth culture) may be used as antigen. It easily demonstrates the sum of O, H (and Vi) antibodies in the patient's serum and thus confirms the etiological correlation of the Salmonella and the disease condition. Positive agglutinations in dilutions up to 1 : 6,400 have been observed in our laboratory. If a negative reaction is obtained in clinically suggestive cases, repetition of the test after a few days is recommended.

If agglutinins are persistently missing in patients, whose symptoms are unusual in Salmonellosis, the etiological bearing of the isolated organism becomes questionable. Since casual carriers are not rare and the possibility of alimentary excretion of recently ingested Salmonellae exists, the bacteriological finding may be

coincidental in a non-related disease. The lack of antibody production is a strong argument against etiological connections.

Special problems arise, when disease befalls individuals with a history of previous *TAB-vaccination.* The evaluation of positive reactions requires great care. Since in vaccinated persons the O-antibodies are usually weak, with a titer rarely exceeding 1 : 100, while the H-antigens are better developed (up to 1 : 1,000 and more) and of greater persistence, a differential diagnosis is often possible. A discrepancy between H- and O-agglutinins should lead to inquiries about previous vaccination.

Another problem concerns the search for *Vi-agglutinins* in typhoid fever. The patient displays, almost regularly, specific O- and H-antibodies, therefore, a test for Vi-agglutination is not needed. Vi-antibodies may or may not be demonstrable; in either case they hardly influence the diagnosis. But it is different with carriers of typhoid bacilli. Here the O-antibody is frequently absent, only the H-antibody is detectable. The anti-d-agglutinin covers other Salmonella types too; thus a definite type diagnosis without the corresponding O-antibody is not possible. In such cases a positive Vi-agglutination is of major diagnostic importance. The test is widely used in carrier surveys, again either in test tube or slide agglutinations. A simple and practicable form of spot-agglutination has been devised by DESRANLEAU (27). Alcohol-killed agar suspension, supended in a buffered glycerine-saline solution, provides the antigen for the slide test, suspensions of living or formalin-killed bacteria are used for the macroscopic agglutination. Positive reactions on the slide with serum dilutions 1 : 2—1 : 5 are already suggestive, while in the test tube 1 : 5—1 : 10 is considered as conclusive. These are minimum values; positive Vi-reactions up to 1 : 160 have been recorded in typhoid carriers.

The antigens are to be derived from Vi-strains of SS. typhi, ballerup or E. coli 1 (KAUFFMANN).

For the final serodiagnosis of Salmonellosis the direct method of isolating and identifying the causative agent is superior to the indirect method of measuring the antibody response of the infected. Nevertheless the patient's serum test may become a valuable help in a case with questionable etiology.

Summary.

The authors describe the historical development of the Salmonella problem since the days of the discovery of the typhoid-, paratyphoid- and enteritis bacteria, including the epidemiological, clinical and serological features. Briefly they discuss the cultural characteristics of this group of bacteria,

more in detail the serology. They deal with the differentiation of bacterial antigens, somatic and flagellar as well, their qualities and the possible combinations which form the basis of a large diagnostic table, the so-called Kauffmann-White Table, an internationally approved method of differential diagnosis. All types recognized until April 1, 1950 (almost 200), are included in the list printed in this paper. The identification of the various types, so different in clinical and epidemiological respect, is performed with the help of specific antisera. The preparation of somatic and flagellar antisera and sera against single antigenic factors is detailed and their selection for final serological identification is discussed. In this way the methods and procedures for practical diagnosis are demonstrated, as far as the average or the specialized laboratory is in a position to employ them.

There are serological reactions developing during the course of the disease in the infected organism. They may be checked through agglutination of various Salmonella antigens by the patient's serum, a method similar to the Widal reaction in typhoid fever. The diagnostic importance of these reactions, however, is far inferior to the direct isolation and serological identification of the causative bacterial agent.

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Résumé.

Les auteurs décrivent le développement du problème de la salmonellose, y compris les faits historiques, épidémiologiques et cliniques depuis la découverte des bacilles typhiques, paratyphiques et de l'entérite. Ils discutent brièvement les caractères de culture de ce groupe de bactéries et plus en détail leur sérologie. La différentiation des antigènes bactériens, des somatiques aussi bien que des flagellaires, est discutée ; leurs qualités et les combinaisons possibles sont démontrées, basées sur une grande table diagnostique, celle de Kauffmann-White. La table publiée ici contient tous les types connus jusqu'au 1er avril 1950 (près de 200). L'identification des antigènes différents se fait à l'aide d'antiséra spécifiques, dirigés contre les antigènes somatiques, flagellaires et contre des parties spéciales des antigènes originaux. Leurs réactions combinées déterminent l'identité des divers types de Salmonella qui diffèrent en qualité clinique et épidémiologique. On décrit en détail la préparation de ces antiséra et leur sélection pour l'identification définitive. Le procédé diagnostique est développé respectivement pour le laboratoire ordinaire et pour le laboratoire spécialisé. — C'est le malade qui développe des anticorps au cours de la maladie. D'après l'exemple de la réaction de Widal dans la fièvre typhoïde, on peut détecter ces anticorps par réaction agglutinatoire. Mais cette réaction n'a pas la même importance diagnostique que l'isolement direct des bacilles et leur identification sérologique.

Zusammenfassung.

Die Autoren geben eine Darstellung der geschichtlichen, epidemiologischen und klinischen Entwicklung des Salmonellaproblems, wie es sich seit der Entdeckung der Typhus-, Paratyphus- und Enteritis-Bazillen ergeben hat. Sie besprechen kurz die Eigenschaften und das Verhalten dieser Bakteriengruppe in der Kultur und gehen dann ausführlicher auf die Serologie ein. Sie beschreiben die Differenzierung der bakteriellen Antigene in Körper- und Geißelantigene, ihre Eigenschaften und die Kombinationsmöglichkeiten, die zum Aufbau einer großen diagnostischen Tabelle, des international anerkannten Kauffmann-White-Schemas, geführt haben. Die hier abgedruckte Tabelle enthält alle bis zum 1. April 1950 verifizierten Typen (beinahe 200). Die Identifizierung der verschiedenen Antigene durch spezifische Antisera (Körper- und Geißelantisera, solche gegen Einzelteile der Antigene), ermöglicht die Erkennung der verschiedenen Salmonella-Typen, deren klinische und epidemiologische Bedeutung durchaus verschieden ist. Die Herstellung solcher Antisera und ihre Auswahl für die endgültige Identifizierung wird eingehend besprochen. So wird die diagnostische Methode beschrieben, welche im Einzelfall zu befolgen ist, je nach den im betr. Laboratorium verfügbaren technischen Möglichkeiten. — Die Reaktion des erkrankten Organismus gegenüber dem eingedrungenen Krankheitserreger kann auch an Hand von Serumreaktionen des Patienten geprüft werden, ähnlich wie bei der Gruber-Widal-Reaktion des Typhuskranken. Die diagnostische Bedeutung dieser Reaktionen, deren Technik besprochen wird, steht jedoch weit zurück hinter derjenigen der direkten bakteriologisch-serologischen durch Bakteriennachweis.