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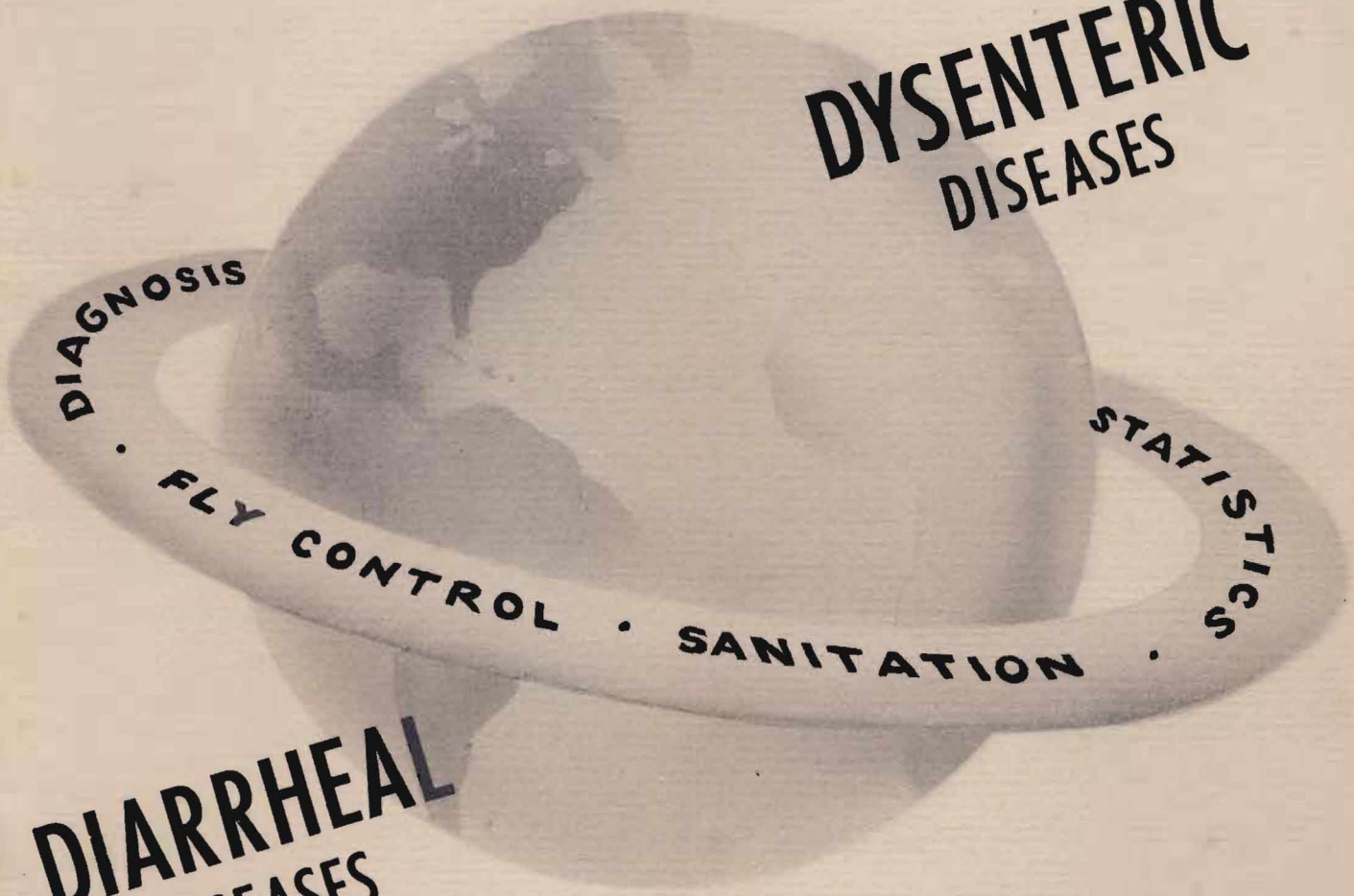

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Contents

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SELECTION OF SALMONELLA AND SHIGELLA CULTURES FOR SEROLOGICAL CLASSIFICATION

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Among the microorganisms received for confirmation as *Salmonella* or *Shigella* types, a large number belong to neither group but are paracolon, coliform, or other enteric bacteria. For example, Ewing and Bruner (8) reported that 39 percent of the cultures submitted to them were *Proteus*, paracolons, etc., rather than *Salmonella* or *Shigella* types.

The aim of this paper is to outline procedures which should aid in the elimination of extraneous bacteria in the laboratory in which they are isolated and by which probable *Salmonella* and *Shigella* cultures may be differentiated.

The procedures outlined here are based upon the writers' experience gained during the bacteriological examination of fecal specimens, rectal swabs, urine sediments, and cultures derived from these sources. The subject will be presented first in a general way and then the differentiation of salmonellae and shigellae will be discussed separately.

There are several important points to be considered in the original treatment of the specimen. In bacillary dysentery and gastroenteritis or enteric fever caused by salmonellae, it is important to make cultures in the acute stage of the disease, since the number of causative microorganisms in the stool diminishes rapidly and late cultures are likely to be negative. Early in the disease, patients' stools are fecal in character and are formed. This stage is followed by a series of watery evacuations which contain shreds of mucous, and often, especially in bacillary dysentery, macroscopic blood. If the mucous shreds are washed in sterile physiological saline solution and then cultured directly by streaking over the surface of the plating medium, the inciting microorganism usually can be isolated. Further, it is very important that the specimen be cultured as quickly as possible after its collection. One of the best procedures is to deliver the stool in the pan directly to the laboratory, so that the bacteriologist can select suitable material for microscopic examination and for cultural work. Otherwise, some means for rapid delivery and prompt treatment of the specimen should be devised. If conditions are such that specimens cannot be cultured immediately, suitable portions, containing mucous shreds if present, are placed in a preserving medium until the specimen can be cultured. The buffered glycerol-saline preservative medium devised by Sachs (14) can be recommended. This medium should be tinted pink by the addition of phenol red, and it should not be used if it becomes acid. Other such media are reviewed by Bangxang and Eliot (2).

It is often a distinct advantage to collect and culture rectal swabs. An experienced team of two or three workers can collect and inoculate several hundred rectal swabs in a short space of time. The technic is valuable in the investigation of outbreaks, for examining food handlers, and for collecting specimens from dispensary or office patients.

In chronic cases where stool cultures may be negative, swabs collected directly from the lesions during proctoscopic examination often reveal the inciting microorganisms.

Plating media. The selective *Shigella-Salmonella* Agar (Difco) and Desoxycholate Citrate Agar (BBL) are good plating media and they have the advantage that a large inoculum may be used. Kauffmann (10) recommended the use of brilliant green agar for the isolation of salmonellae, and bismuth sulfite agar (Wilson-Blair) surpasses all others for the detection of typhoid bacilli (4). Fecal material or mucous on a swab may be inoculated over the entire surface of plates of these media since coliform bacteria are greatly inhibited. When fishing colonies from such media, care must be taken because coli-

form bacteria on the plate may be viable. This point is stressed because triple sugar iron agar (TSI) slants are received frequently which have two or three microorganisms present because of carelessness in fishing colonies. In routine work it is advisable to use a less inhibitory differential plating medium in addition to selective media. MacConkey agar or eosin methylene blue agar (enteric) is good for this purpose. Some knowledge of the general intestinal flora may be ascertained by this procedure and it allows for isolation of an occasional pathogen which may not grow well on the more selective media.

Enrichment media. Tetrathionate broth is preferred for isolation of salmonellae. Galton and Quan (9) found a 164 percent increase in *Salmonella* isolation attributable to the efficacy of a combination of tetrationate enrichment (Kauffmann modification) and brilliant green plating agar. Unfortunately, tetrathionate broth is not a favorable medium for shigellae or for *Salmonella typhi*. Thus, selenite broth is probably the enrichment medium of choice for routine work, since salmonellae (including *S. typhi*) and shigellae usually can be isolated from it. Primary plating media are inoculated with mucous or fecal material on cotton swabs. Then the swabs, containing 0.1 to 1.0 gram of inoculum, are placed in tubes of enrichment media. The specimen must be emulsified in the enrichment broth. Large inocula can be used with enrichment media because coliform bacteria are inhibited while enteric pathogens may increase in number. Enrichment media are incubated for 16 to 18 hours and if primary plates are negative, a large loopful of enrichment broth culture is streaked on an additional (secondary) plate.

The primary plates usually are positive in acute cases of both salmonellosis and shigellosis so that enrichment media are of greatest value with specimens collected after the acute period of disease is passed, in chronic cases, and in carrier studies. Enrichment media may be used to advantage to culture blood, urine, or gall bladder drainage specimens in those cases in which a *Salmonella* type may be the etiological agent.

Differential media. If plates are found which contain colonies of lactose negative microorganisms several such colonies are picked and inoculated into TSI agar slants. After 18 to 20 hours incubation at 37° C., the TSI agar slants are examined and all those showing acid and gas throughout the medium are discarded. Occasionally slants may be observed that are acid throughout, without evidence of gas formation. These are usually anaerogenic coliform bacteria. Fecal streptococci produce a similar reaction. Cultures on TSI agar slants that exhibit a typical acid butt, with or without gas, and an alkaline slant are inoculated onto Christensen's (3) urea medium for the detection of *Proteus* cultures. The latter medium is incubated from 2 to 4 hours at 37° C., then a preliminary reading is made. Specimens that are negative at this reading then are tested in *Salmonella* and *Shigella* antiserum by the slide agglutination method. The urea agar cultures are reincubated after the preliminary reading. Positive reactions are characterized by complete alkalinization of the medium within 20 hours' incubation and may be recorded and discarded if desired. Negative urea agar slants should be retained for 48 hours since some paracolon bacteria yield a delayed reaction which is doubtful or weakly positive. Such a reaction aids in the differentiation of these cultures. Christensen's medium remains unchanged or becomes slightly acid when inoculated with *Shigella* or *Salmonella* cultures. For details of the use of Christensen's medium see Ewing (6). If preferred, the rapid urease test devised by Stuart, Van Stratum, and Rustigian (17) may be substituted for Christensen's medium.

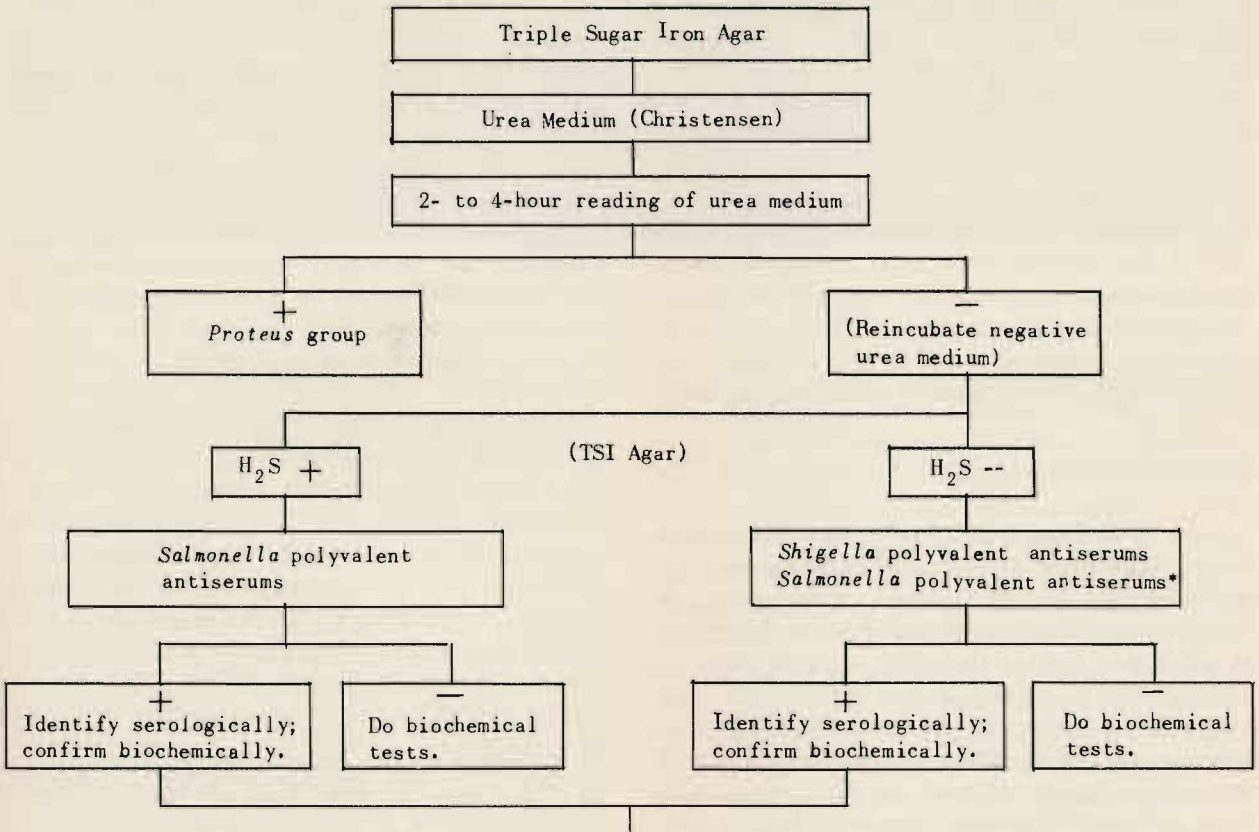
Biochemical reactions. A study of their biochemical reactions is the only method available at present for the final elimination of most paracolon bacteria. Any culture thought to be a member of either the *Salmonella* or the *Shigella* genus should be confirmed as such by the application of a few biochemical tests before it is forwarded to a central laboratory for serologic examination. The essential reactions are listed in table 1.

For hydrogen sulfide and indol tests a medium composed of 2 percent Bacto peptone and 0.5 percent sodium chloride is used. After inoculation, a strip of oxalic acid paper (Gnezda's test for indol) and a strip of lead acetate paper are inserted into a tube and held in place by the cotton stopper.*

* Oxalic acid papers may be made by soaking strips of filter paper (about 3 by ¼ inches) in a warm saturated solution of oxalic acid. The lead acetate papers are made by impregnating similar strips in a warm, saturated solution of neutral lead acetate. After drying, the paper strips may be stored in petri dishes or screw-capped bottles; they do not require further sterilization.

Table 1

OUTLINE OF PROCEDURE FOR IDENTIFICATION OF SALMONELLA AND SHIGELLA CULTURES



	Salmonella	*Shigella*
Glucose	AG	A
Lactose	-	V (positives delayed)
Sucrose	-	V (positives delayed)
Mannitol	not needed	V
Salicin	-	-
Adonitol	-	-
Citrate (Simmons')	V	-
M R	+	+
V P	-	-
H_2S (paper)	+ (usually)	V
Indol	-	V
Motility	V	-

*Occasional *Salmonella* cultures may fail to produce hydrogen sulfide in TSI agar. Also certain salmonellae and shigellae cross agglutinate (see text). *Salmonella typhi* and *Salmonella gallinarum* are anaerogenic. Rarely anaerogenic cultures of other types appear.

The papers must not come into contact with the medium. It is advisable to test for indol with Kovac's or Pringsheim's reagent after the papers are observed 48 to 72 hours. This procedure affords confirmation of negative findings by Gnezda's method.

Motility of microorganisms belonging to the family Enterobacteriaceae is determined by the use of a semisolid medium. This is a far more accurate method than direct microscopic examination. Edwards and Bruner (5) described a modification of the motility medium of Jordan, Caldwell, and Reiter that is an excellent one both for motility determination and for separation of phases. As an alternative, a medium of peptone or tryptone water to which 0.25 percent agar is added, may be used.

Salmonella

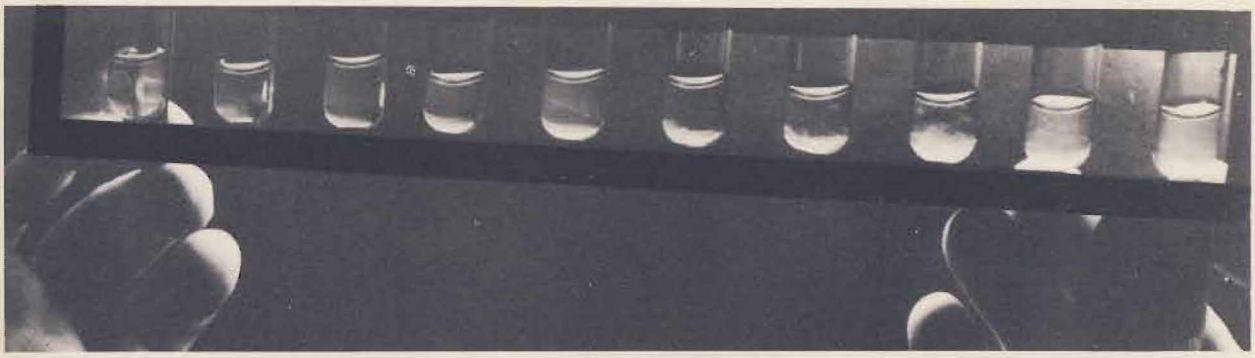
Most of the organisms isolated from feces which bear a superficial resemblance to *Salmonella*, but which actually are not members of the genus, can be eliminated from consideration in the laboratory in which they are isolated. The currently accepted definition of the genus is as follows: "A large genus of serologically related, Gram-negative and non-sporing bacilli; 0.4 – 0.6 microns by 1 – 3 microns in usual dimensions, but occasionally forming short filaments; showing, with certain exceptions, a motile peritrichous phase in which they normally occur; in fact adhering to the pattern of *S. typhi* in staining properties and morphology. Barely fermenting lactose or sucrose, liquefying gelatin or producing indole, they regularly attack glucose with, but occasionally without, gas production. All the known species are pathogenic for man, animals, or both." (15).

The above definition admits certain cultures which ferment lactose or sucrose or which produce indol to the genus, and it is true that *Salmonella* cultures possessing these characteristics have been found. However, they are extremely unusual forms and for practical purposes it may be said that ANY CULTURE WHICH FERMENTS LACTOSE, SUCROSE, SALICIN, OR ADONITOL, OR WHICH PRODUCES INDOL IN AMOUNTS DETECTABLE IN THE USUAL TESTS IMMEDIATELY CAN BE EXCLUDED FROM THE GENUS. The only exceptions to this statement are cultures which, in spite of possessing aberrant biochemical characters, possess both O and H antigens characteristic of *Salmonella* types and thus can be oriented in the Kauffmann-White scheme. In addition, *Salmonella* cultures are methyl red positive and Voges-Proskauer negative. Further, in the case of *Salmonella*, the urea agar of Christensen exhibits absolutely no evidence of urease activity after 24 or 48 hours' incubation. Certain paracolon organisms which show no urease activity after the short incubation period used to detect *Proteus* cultures will produce a distinct alkalinity in the medium after 24 to 48 hours' incubation. Many such cultures belong to the Bethesda or Ballerup groups which otherwise are extremely difficult to distinguish from *Salmonella* except by prolonged incubation of fermentation tests. Such cultures often have a pronounced putrefactive odor which is absent in *Salmonella*.

Fermentation of lactose, sucrose, or salicin may be delayed, and tests should be incubated for 30 days before being discarded. The tubes may be stoppered with corks soaked in hot paraffin to decrease the time necessary for the production of detectable acid in fermentation tubes. By corking the tubes, cultures which ferment lactose or sucrose slowly often can be eliminated in one-half the time necessary if uncorked tubes are incubated.

The majority of *Salmonella* strains other than *S. typhi* produce gas and abundant hydrogen sulfide in TSI slants. In addition to being anaerogenic, certain cultures of *S. typhi* produce insufficient hydrogen sulfide to blacken TSI agar. Also, certain other types of *Salmonella* infrequently encountered in the United States fail to produce hydrogen sulfide. Among these are *S. choleraoe-suis* (diphasic), *S. paratyphi A.*, *S. berta* and certain strains of *S. senftenberg*. Further, anaerogenic strains of any *Salmonella* type may appear. When gas production and hydrogen sulfide formation are apparent in TSI medium it is logical to suspect the presence of a *Salmonella* type other than *S. typhi*. Such cultures may be tested at once with polyvalent *Salmonella* serum.

Polyvalent *Salmonella* serum may be prepared in several ways. Probably the simplest serum that is at all serviceable is a pure O serum which contains agglutinins for *Salmonella* O groups A through E_3. Experience has shown that 98 to 99 percent of the *Salmonella* types isolated from man belong to these groups. If desired, a serum which contains agglutinins for selected H antigens in addition to the above mentioned O antigens may be made. Finally, it is possible to produce a polyvalent serum for all the O and H antigens of the genus. Such a serum



Positive and negative H agglutination tests.

is difficult to prepare and has a greater tendency to react with paracolon cultures than does a serum containing O agglutinins for only groups A through E_3.

Agglutination tests with polyvalent serum can be performed by suspending a small amount of growth from a TSI agar slant in a droplet of physiological saline solution on a slide, adding a small drop of properly diluted serum, and tilting the slide back and forth a few times. If *Salmonella* is present agglutination quickly should become apparent. If agglutination occurs, and if the necessary serums are available, the next step is the grouping or typing. If these procedures are not carried out in the laboratory, the culture should be confirmed by biochemical study and sent to a laboratory in which *Salmonella* typing is done. Biochemical study is necessary since a certain number of *Shigella* and paracolon strains are agglutinated by polyvalent serum. However, if typical agglutination is obtained and preliminary biochemical results are characteristic, it is justifiable to report, "*Salmonella*, type undetermined."

If the organism fails to agglutinate in polyvalent serum, it should be subjected to the biochemical tests outlined above and listed in table 1. If the organism cannot be eliminated from the genus *Salmonella* by these tests it should be sent to a laboratory specializing in the study of enteric bacteria. In the absence of agglutination in polyvalent serum it is justifiable to report that probably no *Salmonella* type was found.

Shigella

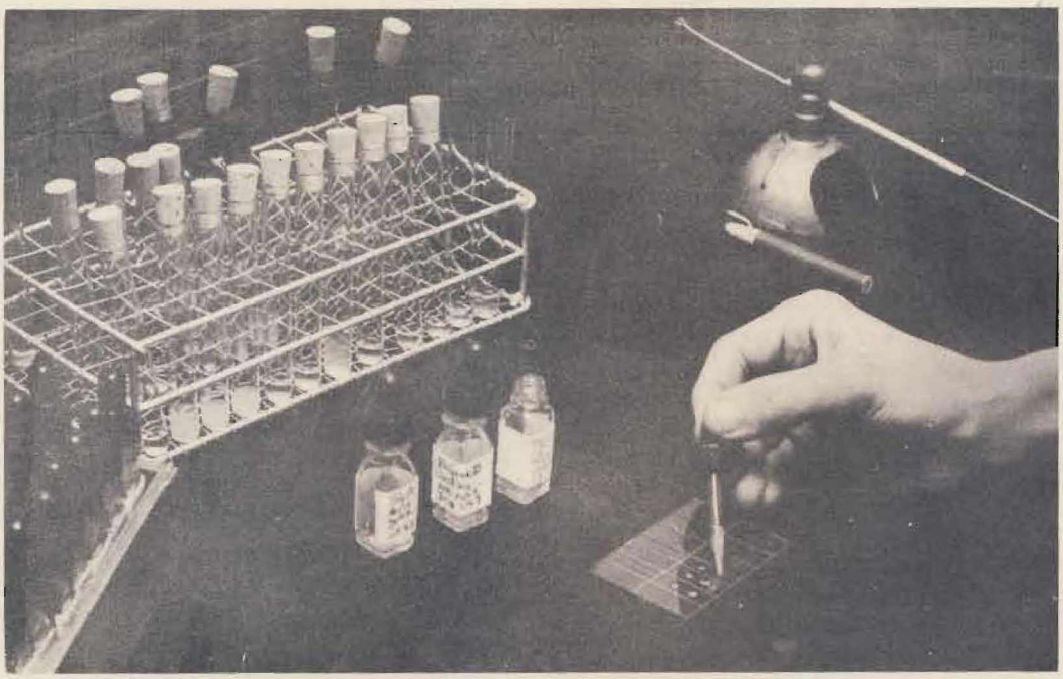
The following definition of the genus *Shigella* (7) provides a working basis for a discussion of the differentiation of shigellae: "gram-negative bacteria that are aerobic, nonsporulating, nonmotile, and, with a few exceptions, nonproductive of gas from fermentable substances. They do not utilize salicin, adonitol, or citrate, or hydrolyze urea, liquefy gelatin, or form acetylmethylcarbinol. Lactose is utilized by only two recognized species (*Shigella sonnei* and *Shigella dispar*), and by these only upon prolonged incubation."

After cultures which appear suspicious on TSI agar slants are tested for urease activity, differentiation of shigellae may be begun. Those cultures which are anaerogenic, hydrogen sulfide negative (TSI) and urease negative are selected for examination as possible shigellae. A small amount of gas along the line of stab or at the bottom of a tube of TSI agar should not lead one to eliminate the culture from examination if there is no evidence of hydrogen sulfide production. Certain biochemical variants of *Shigella flexneri VI* (Newcastle, Manchester) produce gas from fermentable substances and sometimes enough gas is evolved to become apparent in TSI agar. *Shigella* cultures are not known to produce blackening of TSI agar or Kligler's iron agar in the ordinary 20- to 24-hour incubation period. However, many *Shigella* types do produce various degrees of discoloration of lead acetate papers suspended over 2 percent peptone water, and such reactions do not eliminate microorganisms from the genus *Shigella;* the lead acetate paper test is simply a more sensitive method.

Suspected shigellae then should be tested in polyvalent or grouping antiserums by the spot plate technic. Thick suspensions of the bacteria are prepared by suspending them in formalinized normal physiological saline solution. Triple sugar iron agar slants may be utilized for this purpose, or infusion agar slants may be inoculated and the growth examined following 6 hours' incubation or more.

Polyvalent antiserums are prepared against members of group A, B, and C in the *Shigella* schema (7). To prepare an antiserum for members of group A, for example, smooth cultures of each of the seven members of the group are cultivated and the growth pooled into a mixed vaccine. Then a single rabbit, or group of rabbits, is injected with the mixed vaccine. It is advisable to test-bleed the animals near the end of the immunization period to determine whether or not the agglutinin content of the antiserum is satisfactory for each constituent of the mixed vaccine. If antibody for any one of the microorganisms is unsatisfactory, the rabbit is injected again with a vaccine made from that particular microorganism. Such grouping antisera are used in slide tests and should be employed in a dilution that permits prompt and complete agglutination of microorganisms belonging to the group. Delayed, incomplete cross reactions are seen between certain members of the three groups but cognizance is taken only of those agglutination reactions that are prompt and complete. When such reactions occur, the suspension then is tested in specific antiserums prepared against each member of the group. In addition to the polyvalent antiserums prepared against members of groups A, B, and C, three other antiserums are employed for preliminary grouping of shigellae. If a suspension does not react in polyvalent A, B, or C antiserum, or is weakly agglutinated by one of them, the suspension then is tested with *Shigella alkalescens*, *Shigella sonnei* (mixed form I and II), and *Shigella dispar* (mixed I and II) antiserums.

Use of the above-mentioned six antiserums permits preliminary grouping of all except a few of the rarer *Shigella* types. Cultures which appear to be shigellae but which do not react in the antiserums should be suspended in plain saline and heated (100° C., ½ hour) and then retested. Certain *Shigella* may contain heat labile antigens, some of which belong to the class of antigens designated K by Kauffmann (11,12). One member of this group of antigens is designated L, and it is this type that is found in some microorganisms included in the *Shigella* group. The heat labile L antigen is found in *S. alkalescens* cultures (13). When present, L antigen markedly or completely inhibits O agglutination. For other references to these inhibitory antigens see Archer (1), Stuart *et al.*, (16), and Schuetze (19).



Method of preparing slide agglutination tests with *Shigella* polyvalent serums.

If a laboratory cannot prepare grouping antiserums, serological examination of suspected cultures may be omitted and primary differentiation made by the use of a few essential biochemical reactions

(table 1). Those cultures which conform to the foregoing description of the *Shigella* group may then be selected for serological examination either in the laboratory where they are isolated or in other laboratories equipped for such work. Further, it is to be emphasized that cultures that appear to be shigellae according to their reactions in polyvalent or grouping antiserums must be subjected also to the same biochemical tests in order to confirm them as members of the genus *Shigella*.

Shigella cultures are nonmotile, and if a simple test in semisolid medium is made, many extraneous microorganisms can be eliminated from consideration as possible shigellae by this test alone. All members of the genus produce acid from glucose. Microorganisms of the *Alcaligenes* group are sometimes found in feces, and cultures of *Alcaligenes fecalis* are sometimes submitted for typing as shigellae. *A. fecalis* does not ferment glucose, but it sometimes is confused with shigellae because it produces increased alkalinity on the slant of TSI agar. This gives the impression that acid is present in the butt. If such slants are compared with an uninoculated tube of TSI it is observed that there is no change of reaction in the butt. Likewise, *Pseudomonas aeruginosa* produces increased alkalinity on the slant, and in this case there is a purplish cast to the medium caused by diffusion of pigment. *P. aeruginosa* cultures produce a characteristic aromatic odor.

Mannitol is included in the list of biochemical test substances because it aids in the subdivision of the genus *Shigella* into groups. Members of group A do not utilize this substrate, while with a few exceptions, members of group B, C, D, and E ferment it. Mannitol negative cultures of *S. flexneri* IV and *S. flexneri* VI sometimes occur.

The fact that *S. sonnei* and *S. dispar* serotypes ferment lactose and occasionally attack sucrose after 48 hours or more serves to distinguish these microorganisms from other shigellae. The average time required for *S. sonnei* and *S. dispar* cultures to ferment lactose is 6 or 7 days. If lactose and sucrose tubes are plugged (see above, under *Salmonella*) fermentation is generally accelerated. It should be mentioned that if fermentation tubes are to be plugged, an indicator which is not an oxidation-reduction indicator must be used. Early fermentation of lactose or sucrose, i.e., within 24 hours, means that the culture is not a *Shigella* type. Ancillary evidence to this is afforded when gas is also present. Most paracolon cultures produce relatively large amounts of gas but anaerogenic forms are not uncommon (16,21). Regardless of their action toward lactose and sucrose or their gas producing propensities, most paracolon cultures can be differentiated from shigellae on the basis of other biochemical tests, motility, and serology.

Members of the genus *Shigella* do not utilize salicin or adonitol, and cultures which produce acid from these test substances may be eliminated from consideration as possible shigellae. None of the described *Shigella* types grow on Simmons' citrate agar. For this reason Simmons' citrate medium is useful in separating paracolons that are able to utilize citrate and ammonium salts. However, most *Salmonella* cultures utilize citrate, *S. typhi* being among the exceptions.

Use of the tests listed in table 1 should allow laboratory personnel to confirm microorganisms that react in antiserums as shigellae, to confirm cultures which do not agglutinate in antiserums for the more common shigellae as possible *Shigella* types, and to eliminate most paracolon cultures from consideration as members of the genus *Shigella*. Microorganisms that conform to the definition as regards their biochemical reactions but which cannot be typed serologically can be sent to laboratories equipped for complete serological studies.

Summary

Serological and biochemical tests are outlined which will guide workers in their identification of *Shigella* and *Salmonella* types. Use of these notes will aid in the elimination of many paracolon and coliform bacteria in the laboratory where they are isolated, and should decrease the percentage of error in cultures sent for typing as salmonellae and shigellae.

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SIMPLIFIED SEROLOGIC IDENTIFICATION OF SALMONELLA CULTURES

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The growing multiplicity of *Salmonella* types and the numerous diagnostic serums required for the recognition of all of them make impractical complete serologic typing of salmonellae in the average laboratory. However, this situation should not deter the small laboratory from making accurate diagnoses of *Salmonella* infections or from exact recognition of those *Salmonella* types which are of greatest importance in the epidemiology of salmonelloses of man. To persons not familiar with the typing of *Salmonella* cultures, the Kauffmann-White schema of the antigenic types thus far recognized seems very complicated and the fast growing numbers of types add to the confusion of the average technician. Actually, the pioneer work of White and the meticulous systematic studies of Kauffmann provide the only sure basis for recognition of types encountered frequently in diagnostic laboratories. By taking advantage of the knowledge of the genus *Salmonella* which has been gained through the studies of many workers over a period of years which has culminated in the Kauffmann-White system of classification, it is possible to evolve a simplified method of diagnosis which can be carried on even in the small diagnostic laboratory.

Simplified *Salmonella* diagnosis is by no means new. As early as 1930 Kauffmann (1) proposed a set of 12 OH serums which permitted recognition of the principal types in the genus. Bornstein (2) made a similar proposal in 1942. Kauffmann and Edwards (3) in 1947 described a simple method for the recognition of the most important *Salmonella* types. Other workers have made similar suggestions.

The method described here, with a few exceptions, is similar to that of Kauffmann and Edwards. It is based on the necessity for the exact identification of those *Salmonella* types particularly adapted to man, which are disseminated from man to man, which produce severe enteric fevers, and which tend to become endemic in the population since numerous permanent carriers result from the infections. Among these types are *S. typhi*, *S. paratyphi A*, *S. paratyphi B*, *S. paratyphi C*, and *S. sendai*. It is true that *S. paratyphi A* and *S. paratyphi C* occur only infrequently in the United States and that *S. sendai* has been found only in the Orient. Nevertheless, these types are important human pathogens in various parts of the world, and it is desirable that they be recognized so that they may be dealt with promptly and efficiently when they appear. The importation of *S. paratyphi C* from the Pacific area is already a matter of record. In addition to the types particularly adapted to man, *S. cholerae-suis* should be identified exactly since it so often produces septicemia and localizations in man; and *S. typhi-murium* should be recognized since it occurs more frequently in man than does any other of the numerous types which produce acute gastroenteritis.

Fortunately the great majority of *Salmonella* cultures isolated from man, including the types mentioned above, are members of the first five somatic groups of the Kauffmann-White classification. Edwards and Bruner (4) found that 98.3 percent of 3,019 cultures from man and animals were members of these five groups, and Seligmann, Saphra, and Wassermann (5) obtained an identical figure in 2,916 cultures from man. Bruner and Joyce (6) classified 99.5 percent of 1,007 cultures isolated from man in Italy in these groups. Thus, it is necessary for the average laboratory to have O serums only for groups A through E of the genus.

In the light of these facts the following diagnostic scheme for the average routine laboratory is proposed. A polyvalent serum which at least contains agglutinins for antigens I, II, III, IV, V, VI, VII, VIII, IX, X, XII, XV, XIX and Vi should be available. Such a combination is a minimum requirement for a polyvalent serum. The serum may be prepared from smooth cultures of *S. para-*

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typhi A, S. paratyphi B, S. thompson, S. virginia, S. gallinarum, S. anatum, S. newington, and *S. senftenberg,* and a culture of the Ballerup type which is in the Vi form. Antigens of the eight first-mentioned cultures are prepared by the method described below for the production of O serums and are mixed in equal amounts. Usually six injections of the mixture in amounts of 0.5, 1.0, 2.0, 4.0, 5.0 and 5.0 ml are given to rabbits at intervals of 4 days. To the last four injections, dried organisms of the Vi form of the Ballerup strain should be added just before injection. (The method of preparation of the Vi organisms and amounts administered are described in a subsequent paragraph.) After four injections a trial bleeding may be taken. If it is found that agglutinins for a particular component are lagging the amount of that particular antigen in the mixture may be doubled. The rabbits are bled on the 6th day following the last injection and the serum preserved with 0.25 percent tricresol. The serum is then standardized against all the antigens injected as described below under standardization of O serums. It is used in slide tests in the highest dilution in which it readily will agglutinate all the injected antigens. It also should be tested against a living Vi culture of *S. typhi* to assure the presence of Vi agglutinins.

Six O serums for groups A to E, respectively, should be prepared as follows:

A	I, II, XII	*S. paratyphi A*
B	IV, V, XII	*S. paratyphi B*
C_1	VI, VII	*S. thompson*
C_2	VIII	*S. virginia*
D	IX, XII	*S. gallinarum*
E	III, X, XV	*S. anatum* and *S. newington*

These serums may be prepared from smooth cultures of the indicated types. Twenty-four-hour infusion broth cultures should be heated at 100° C. for 2 hours. After cooling, 0.3 percent formalin is added. These formolized antigens are injected into rabbits in amounts of 0.5, 1.0, 2.0, and 4.0 ml at intervals of 4 days. The rabbits are bled 6 or 7 days following the last injection. If desired a trial bleeding may be taken before the rabbits are exsanguinated. If a sufficient titer has not been obtained an additional injection of 4.0 ml may be given. Experience has shown that additional injections are without effect. Although not necessary, it is desirable to inject two rabbits with each antigen since certain animals fail to develop a satisfactory titer. The antigens should be injected as soon after preparation as convenient and should be refrigerated during the period of immunization. Antigens which have been held for long periods before injection are not as effective as freshly prepared suspensions.

The serums should be tested with homologous antigens by slide agglutination. A convenient method of preparing antigens for slide agglutination is to scrape the organisms from the surface of a slant and suspend them in 1.0 ml of absolute alcohol. After heating the alcoholic suspensions at 60° C. for 1 hour, the tubes are centrifuged, the alcohol decanted, and the organisms suspended in 0.5 ml of carbolized saline. Such antigens, when kept in the refrigerator, can be used for several weeks. The serums to be tested are diluted 1 to 5, 1 to 10, and 1 to 20. A small drop of each dilution is placed on a slide and mixed with a drop of antigen of equal size. The slide is then tilted back and forth and the reactions observed. A satisfactory serum when diluted 1 to 10 should produce pronounced agglutination of alcohol-treated organisms within 30 seconds to 1 minute.

After the rabbits are bled and the serum collected it should be preserved by addition of an equal amount of glycerol. Preservation with glycerol has two advantages: it produces no precipitate in the serum, and it prevents rapid drying of slide tests. Serums refrigerated at 4° C. retain their potency for several years.

After the serums have been preserved they should be standardized. This is accomplished by preparing different dilutions of each serum and determining the highest dilution in which it will give pronounced reactions with the homologous antigen within a given time limit, usually about 30 seconds after tilting of the slide is begun. It should be remembered that alcohol-treated organisms are more resistant to agglutination than are living cultures. Therefore, the serums should be standardized against living antigens if living organisms are to be used in routine tests.

As a rule, cross reactions between the various O groups are not pronounced but certain minimal cross reactions should be expected. If one is careful to observe with which serum an antigen first reacts it is not difficult to determine to what O group an organism belongs. Representative reactions of antigens with the grouping serums are illustrated in table 1.

In addition to the O grouping serums, a Vi serum

should be prepared. Freshly isolated cultures of *S. typhi* do not agglutinate readily with O serum since the reaction is masked by Vi antigen (7). A potent Vi serum can be prepared from the organism described by Kauffmann and Moeller (8) as *S. ballerup*. Vi colonies of this organism are decidedly more opaque than O colonies. Vi colonies should be selected repeatedly until no O colonies appear upon plates. After a pure Vi form is obtained, the are administered. This procedure rarely fails to yield a potent Vi serum which will agglutinate freshly isolated typhoid bacilli in a dilution of 1 to 20 or 1 to 40 in slide tests. Such a serum has a distinct advantage over serums prepared with *S. typhi* since it contains no O or H agglutinins for typhoid bacilli and may be used in the unabsorbed state. It does contain O and H agglutinins for the Ballerup strain but such antigens occur very rarely

Table 1

REACTIONS OF REPRESENTATIVE ANTIGENS WITH O GROUPING SERUMS

Antigens	Serums					
	Group A I, II, XII	Group B IV, V, XII	Group C_1 VI, VII	Group C_2 VIII	Group D IX, XII	Group E III, X, XV
I, II, XII	++	-	-	-	±	-
I, IV, XII	+	++	-	-	±	-
I, IV, V, XII	+	++	-	-	±	-
IV, V, XII	-	++	-	-	±	-
VI, VII	-	-	++	-	-	-
VI, VIII	-	±	±	++	-	-
VIII	-	±	-	++	-	-
VIII, XX	-	±	-	++	-	-
IX, XII	-	±	-	-	++	-
I, IX, XII	+	±	-	-	++	-
III, X	-	-	-	-	-	++
III, XV	-	-	-	-	-	++
I, III, XIX	+	-	-	-	-	++

± to ++ indicates different degrees of agglutination.

growth from the plates is suspended in absolute alcohol, centrifuged, and the alcohol decanted and replaced with fresh alcohol. The culture is suspended in the alcohol, again centrifuged, and the fluid poured off. The organisms are dried in a vacuum desiccator, ground to a fine powder, and stored until needed. The bacteria are suspended in physiological saline just before injection. The amounts administered are the same as those used for the production of O serums. The first injection should be of a density comparable to a 24-hour broth culture; but in succeeding injections the suspension may be increased rapidly in density as well as in amount so that toward the end of the period of immunization quite dense suspensions in enteric organisms. The use of the serum is explained below.

In addition to the polyvalent, O, and Vi serums, it is necessary to prepare six H serums as follows:

a – *S. paratyphi A*
b – *S. paratyphi B*, phase 1 or *S. minnesota*, phase 1
c – *S. cholerae-suis*, phase 1
d – *S. typhi*
i – *S. typhi-murium*, phase 1
1, 2, 3, 5 – *S thompson*, phase 2, and *S. newport*, phase 2

All the above-named organisms occur in monophasic form so that one need not isolate the desired

phases. In order to prepare the serums the organisms are passed through two or three successive tubes of semisolid agar to insure maximum motility. They are then inoculated into infusion broth, incubated overnight, and an equal amount of physiological saline solution containing 0.6 percent formalin is added to the cultures. The formalinized suspensions are injected into rabbits in the same manner and amounts as are the antigens used to produce O serums. The rabbits are bled on the 6th day following the last injection and the serums are preserved with glycerine. It is unnecessary to take trial bleedings because satisfactory titers are almost invariably obtained. The serums should be titrated against the homologous antigens. A titer of 5,000 or more is adequate.

EXAMINATION OF CULTURES

It is usually convenient to examine cultures which give reactions typical of *Salmonella* on triple-sugar-iron-agar (TSI) slants and which produce no alkalinization of Christensen's urea agar. The growth from the TSI slants may be used to make a dense saline suspension. This suspension should be tested on a slide with polyvalent serum. In performing slide agglutination tests it is advisable to use amounts of antigen and serum approximately equal to a 3 mm loopful. If larger drops are used nonspecific reactions are more likely to appear. Also, in the use of the O grouping serums, the use of large drops emphasizes cross reactions between groups. Suspensions which are agglutinated by polyvalent serum should then be tested with the O grouping serums.

Any culture which is agglutinated in a typical manner by polyvalent serum and by one or more of the O grouping serums may be reported as a probable *Salmonella* provided preliminary biochemical examination indicates that it is a member of the genus. Results should be confirmed by extended biochemical tests since many paracolon bacteria contain antigens related to the O antigens of *Salmonella*.

If a culture reacts with polyvalent serum but not with the O grouping serums, it should be tested with Vi serum since most freshly isolated strains of *S. typhi* are not agglutinated by O serum for group D, to which they belong. If the culture reacts with Vi serum a portion of the suspension should be heated in a boiling water bath for 10 minutes, and after cooling, again should be tested with the O serums and with Vi serum. After boiling, *S. typhi* cultures should react with group D serum; and *S. paratyphi C* cultures, which also may contain Vi antigen, should react with group C_1 serum. From a practical standpoint, cultures which react with Vi serum, and which, after heating, react with group D serum may be reported as *S. typhi*, although in order to complete their identification the H antigens should be identified and biochemical tests performed. Rarely rough cultures of *S. typhi* are isolated which, after boiling, agglutinate neither in Vi or O serums. Such cultures also may be nonmotile. They are identifiable only by their content of Vi antigen and by their biochemical characteristics.

Cultures which continue to agglutinate in Vi serum after heating and which are not agglutinated by the O grouping serums may be presumed to contain O antigens related to those of the Ballerup strain. Such cultures now are classified as intermediate paracolon bacteria.

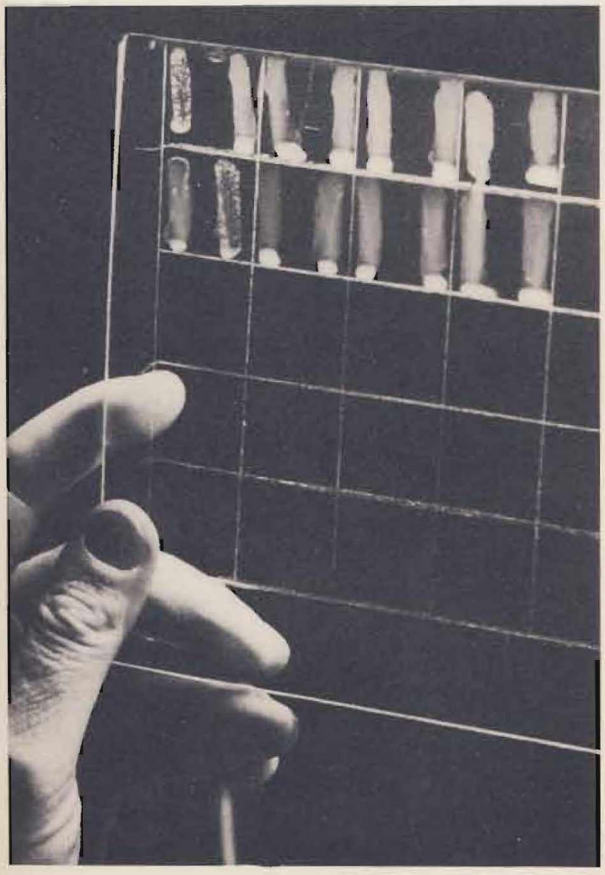
After the O group of the culture is determined its H antigens should be examined. This may be done by testing growth from an agar slant on a slide with H sera which are diluted 1 to 50 or 1 to 100. An alternate method is that recommended in 1942 by Edwards and Bruner (10), in which an infusion broth culture is diluted with an equal amount of saline containing 0.6 percent formalin and tested with appropriate sera at 50° C. Only one dilution (1 to 1,000) is used and results are read after incubation for 1 hour in a water bath.

The group A cultures should be tested with serum a to assure that they are *S. paratyphi A*, the only known type in that group. In addition, they should be subjected to differential biochemical tests such as nonfermentation of xylose, and inability to utilize tartrates and citrate. The majority of *S. paratyphi A* strains produce little or no H_2S.

Group B cultures should be tested with H serums for antigens b, i, and 1,2,3,5. In the event that agglutination occurs in b or i serum, the culture is almost certainly *S. paratyphi B* or *S. typhi-murium*, respectively. Diphasic cultures often occur only in one phase when recently isolated. If phase 2 only is present, it is necessary that phase 1 be obtained to identify the culture. Thus, if the culture is agglutinated only by 1,2,3,5 serum, it should be planted on a Gard plate (9) with 1,2,3,5 serum or placed in a tube containing 2 or 3 ml of semisolid medium to which a loopful of that serum has been added. Phase 1 will migrate through medium and can be isolated from the spreading growth.

Useful modifications of the Gard technique have been described by Edwards and Bruner (10), Hajna (11), and Juenker (12). After phase 1 is obtained it can be determined whether the culture is *S. paratyphi B*, *S. typhi-murium*, or some other type within group B.

Group C cultures should be tested with c and 1, 2, 3, 5 serums. If agglutination occurs in c serum, the culture may be identified as *S. paratyphi C* or *S. cholerae-suis* depending upon its biochemical reactions. *S. paratyphi C* usually ferments arabinose and always ferments trehalose promptly, whereas *S. cholerae-suis* attacks neither of these sugars. *S. paratyphi C* ferments dulcitol promptly while *S. cholerae-suis* gives delayed or negative reactions in dulcitol mediums. If phase 2 only is present, phase 1 should be isolated as previously described and tested with c serum. It should be remembered that the majority of cultures of *S. cholerae-suis* belong to the Kunzendorf variety in which phase 1 is more or less completely suppressed; so any group C culture which agglutinates in 1, 2, 3, 5 serum, fails to ferment arabinose and trehalose, and gives a negative or delayed result in dulcitol may be reported as *S. cholerae-suis*.



Reactions of group A and group B *Salmonella* cultures with grouping serums.

Group D cultures should be tested in a, d, and 1, 2, 3, 5 serums. Cultures which agglutinate only in d serum, which are anaerogenic, and which possess the usual biochemical attributes of *S. typhi* may be reported immediately. The organism will have been examined already for the presence of Vi antigen so that its identity is fairly well established. Some recently isolated cultures of *S. typhi* are very poorly motile and may fail to flocculate in d serum. Such cultures can be typed as *S. typhi* provided they possess IX, XII, and Vi antigens and the biochemical properties of *S. typhi*.

Identification of *S. sendai* depends upon the recognition of both phases and biochemical examination. It is necessary that the organism agglutinate both in a and 1, 2, 3, 5 serums. If only one phase is present the second must be isolated as previously described. Further, this type is H_2S and citrate negative and gives a negative result in Stern's glycerol-fuchsin broth. Arabinose is fermented immediately but xylose and sorbitol give delayed reactions. Cultures with similar antigens but different biochemical reactions belong to another type. *S. sendai* occurs frequently only in the Orient.

Cultures which belong to group E are not examined further but are reported simply as group E *Salmonella*.

By adoption of the system outlined above, or a similar one, the diagnosis of *Salmonella* infections in the routine laboratory should be greatly improved. The small laboratory should not attempt the exact typing of all *Salmonella* cultures but should identify only the more important types. The remaining cultures should be designated "*Salmonella*, type undetermined" and sent to a center for identification. The adoption of such a system permits the rapid diagnosis of 98 to 99 percent of *Salmonella* infections. At present many laboratories continue to rely on ordinary agglutination tests in which O and H reactions are not distinguished and upon biochemical reactions. Reports on enteric cultures therefore are often long delayed and inaccurate.

The system proposed above is flexible and may be modified to suit the needs of the individual laboratory. Further, as workers gain familiarity with the method it is likely that they will wish to extend the number of types which they identify. This may be done by increasing gradually the

number of H serums to the point that all the types which occur frequently may be recognized. Any proposal short of complete typing must be a compromise and each laboratory must decide to what length they wish to go in the identification of *Salmonella* cultures. The methods proposed here are based upon experience, with a view toward the employment of a minimal number of serums.

SUMMARY

A simplified method for the serological grouping of *Salmonella* is described. Methods for the exact identification of the principal human pathogens of the genus are included. Methods for the preparation of the necessary serums, for the preparation of antigens, and for the performance of the tests are given in some detail.

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SIMPLIFIED METHODS FOR THE SEROLOGICAL IDENTIFICATION OF SHIGELLA CULTURES

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A number of new *Shigella* types were added to the genus during recent years so that a relatively large number of microorganisms are now classified in the genus *Shigella* (6). The purpose of this paper is to outline serological methods by which those shigellae that are most common in the United States may be grouped and typed. The needs of many laboratories may be fulfilled by simply identifying a culture as a member of one of the groups, reporting it as such, and then forwarding the culture to a laboratory that is equipped to complete the typing. Other laboratories may desire to carry the identification beyond the grouping stage and to determine the type of the microorganisms. Methods designed to meet the requirements of both kinds of laboratories are given herein. The methods are based in part upon the work of Boyd (3,4), Wheeler (21,22), and upon the writer's studies.

A definition and the essential biochemical reactions of members of the genus *Shigella* are given by Ewing and Edwards (5) and need not be discussed here. However, it should be emphasized that cultures that are grouped or typed should be confirmed as members of the genus *Shigella* by the performance of biochemical tests.

Group A of the *Shigella* schema (fig. 1) is composed of those shigellae which characteristically do not form acid from mannitol. These are *Shigella dysenteriae* I (Shiga), *Shigella dysenteriae* II *(Shigella ambigua* or *schmitzii),* and the microorganisms described by Large and Sankaran in 1934 (14) *(Shigella dysenteriae,* types III to VII*).*

The microorganisms formerly called *Shigella paradysenteriae,* Flexner and now referred to as *Shigella flexneri,* types I to VI, make up group B. With a few exceptions, cultures belonging to this group ferment mannitol within 24 hours. Certain biotypes of *Shigella flexneri* VI do not utilize this substrate. Also, mannitol negative variants of *Shigella flexneri* IV may occur. Nelson (15) described a small outbreak caused by such a variant. The microorganisms described under the names of *Shigella rabaulensis* and of *Shigella rio* (20) appear to be varieties of *S. flexneri* IV that do not utilize mannitol.

The members of group C of the *Shigella* schema are similar to *S. flexneri* cultures as regards their cultural and biochemical characteristics but described types bear little serological relationship to members of group B. However, intragroup relationships among group C cultures are known (23,24).

Group D is composed of those shigellae which utilize lactose after continued incubation. These are *Shigella sonnei* and *Shigella dispar.*

The final group of the schema is made up of *Shigella alkalescens,* the commonly occuring forms of which were described by Andrewes (1) and by Stuart *et al.,* (19). Coliform and paracolon cultures that are serologically identical to *S. alkalescens* cultures were described by Stuart *et al.* (19) and Wheeler *et al.* (23).

S. alkalescens and *S. dispar* are retained in the genus *Shigella* for the purpose of the present discussion. However, it is probable that they will be removed from the genus and placed with the *Escherichia* because of biochemical and serological relationships to members of the coli group (7,9,12).

Cultures which appear to be shigellae are tested first in polyvalent or grouping serums to determine the group to which they belong. Six such antiserums are needed for grouping purposes. These are groups A, B, C, *S. sonnei* mixed, *S. dispar* mixed, and *S. alkalescens.*

The preparation and use of polyvalent *Shigella* antiserums is described by Ewing (8). Polyvalent A is prepared by the injection of two or more rabbits with a mixed vaccine made by pooling 20-hour broth cultures of each of the seven *S. dysenteriae* types. Polyvalent antiserums for group B and for group C are prepared in a similar manner, using mixed vaccines. All six of the *S. flexneri*

types are included in the pooled vaccine for group B because agglutinins for the specific antigen of each type should be present in the serum. For the preparation of a mixed *S. sonnei* antiserum broth cultures of form I (smooth) and form II ("rough") are pooled to make the vaccine. Mixed *S. dispar* serum is prepared in a similar way by mixing young broth cultures of types I and II. An antiserum for *S. alkalescens* is included with the grouping serums because of the high incidence of this type. Broth cultures to be used for the preparation of antiserums for *S. dispar* and *S. alkalescens* should

Figure 1.
THE GENUS SHIGELLA
Dysentery and Related Bacteria

Proposed Designation	Other Designations					
Group A. *Shigella dysenteriae,*	I	*Shigella dysenteriae* (Shiga-Kruse bacillus) *Bacterium shigae.*				
	II	*Shigella schmitzii, Schigella ambigua, Bacterium ambiguus,* etc.				
	III	Q 771 of Large-Sachs group, Type 8524 Gober, *et al. Shigella arabinotarda A.* Christensen and Gowan.				
	IV	Q 1167 of Large-Sachs group; *Shigella arabinotarda B.*				
	V	Q 1030				
	VI	Q 454				
	VII	Q 902				
Group B. *Shigella flexneri,*		**Kauffmann & Ferguson**	**Boyd-Wheeler**	**Andrewes & Inman**	**Weil**	**Other**
	I	1a	F.I	V	F.I	
	I	1b	F.I	VZ	F.I.III	
	II	2a	F.IIa	W	F.II	
	II	2b	F.IIb	WX	F.II,VII	
				X	F.VII	
				Y	F.VIII	
	III	3	F.III	Z	F.III	
	IV	4a	F.IV		F.IV	Boyd 103
	IV	4b	F.IV		F.III,IV	
	V	5	F.V		F.V.	Boyd P.119
	VI	6	F.VI		F.VI	Boyd 88, Newcastle and Manchester bacilli, *Shigella newcastle*
Group C. *Shigella boydii,*	I		B.I		F.IX	Boyd 170
	II		B.II		F.X	Boyd P.288
	III		B.III		F.XI	Boyd D.1
	IV		B.IV		F.XIV	Boyd P.274
	V		B.V		F.XIII	Boyd P.143
	VI		B.VI		F.XII	Boyd D.19
	VII					"Lavington," Type T, *Shigella etousae*
Group D. *Shigella sonnei* *Shigella dispar*		Sonne - Duval bacillus, Kruse - E - Ruhr, *Shigella ceylonensis A*				
	I	Serotype I, Carpenter & Stuart; *Shigella madampensis* (Castellani)				
	II	Serotype II, Carpenter & Stuart; *Shigella ceylonensis B* (Castellani)				
Group E. *Shigella alkalescens*		Andrewes, Stuart *et al.*, Type I, DeAssis.				

B. - Boyd; F. - Flexner

From Ewing, Bact., 57: 633-638 (modified) (1949).

be heated at 100° C. for 2½ hours to destroy L antigens which may cause cross reactions with coliform cultures that contain related antigens (10,12). All newly prepared serums should be tested with a living culture of a bacterium known to contain alpha antigen (17,11). If alpha agglutinins are present, the antiserum should be absorbed with the alpha antigen culture.

While it is possible to use the grouping serums in the unabsorbed state if cognizance is taken only of rapid and complete agglutination, it usually is desirable to free them of intergroup reactions by absorption with appropriate microorganisms. *S. alkalescens* cultures may react in A antiserum because of a relationship of this type to *S. dysenteriae* I. Also, *S. alkalescens* reacts in B and C antiserums. The agglutination in B serum is brought about by minor relationships to certain *S. flexneri* types and the reaction in polyvalent C is caused by the relationship of *S. alkalescens* to *S. boydii* I and IV. Further, *S. dispar* I and II cultures are agglutinated by B and C grouping serums. Certain *S. flexneri* types, particularly *S. flexneri* IV and the "X" and "Y" varieties, react slightly in C antiserum. *S. sonnei* II cultures are agglutinated by antiserum C because of the relationship of that type to *S. boydii* VI. See table 1 for method of absorption of polyvalent serums.

Mixed *S. sonnei*, mixed *S. dispar*, and *S. alkalescens* antiserums are employed in the unabsorbed condition.

The following technic of agglutinin absorption may be used for the preparation of absorbed polyvalent antiserums and for absorption of specific antiserums. Smooth cultures of the microorganisms to be employed for absorption are inoculated into infusion broth. After incubation for 15 to 18 hours at 37° C., the broth cultures are used to seed infusion agar plates. Standard 90 mm petri dishes containing 20-25 ml of infusion agar are employed. Each plate is seeded with 0.3 to 0.4 ml of broth culture and the inoculum is spread over the entire surface of the agar. Such plates are incubated in an upright position for 18 to 20 hours at 37° C. The growth is removed with formalinized (0.3 percent of 40 percent formaldehyde solution) physiological saline solution. If the suspension is to be boiled, plain physiological salt solution should be used. The microorganisms are sedimented by centrifugation and the supernatant fluid discarded. Diluted antiserum is added to the packed bacteria and the cells are resuspended. Antiserums to be absorbed are diluted 1:5 or 1:10 and added to an absorptive dose calculated to be in excess of that required. The mixtures are incubated at 37° C. for 6 hours and placed in an icebox (about 4° C.) overnight. They are then centrifuged and the antiserum removed. In most cases, the number of plates indicated in the tables effectively removes the heterologous agglutinins from 1.0 of antiserum. There are exceptions, of course, in which antiserums must be reabsorbed by additional bacteria to remove all of the heterologous agglutinins.

If a culture suspected of being a *Shigella* type is not agglutinated by any of the six grouping serums in slide tests, a suspension made with plain saline solution should be heated for ½ hour, cooled, and retested. Some shigellae, particularly *S. alkalescens* cultures, contain thermolabile antigens that inhibit O agglutination (2,12,16).

The use of the grouping antiserums described above and the biochemical criteria given by Ewing and Edwards (5) will enable laboratory personnel to determine the group to which an unknown *Shigella* type belongs and to confirm it as a member of the genus *Shigella*. The needs of many laboratories will be fulfilled by this procedure. Such cultures, together with information as to source, clinical diagnosis, age and sex, should be forwarded to a laboratory equipped for typing of

Reaction of group B *Shigella* cultures in slide agglutination tests with polyvalent serums.

shigellae, *e.g.*, a State health department laboratory.

Those laboratories which desire to make specific identification of cultures belonging to the various groups should have type specific antiserums for this purpose. Such antiserums may be prepared in the same way as that mentioned for grouping serums except that single types are employed as vaccines.

The incidence in the United States of *Shigella* types belonging to group A is not high. In most cases antiserums for *S. dysenteriae* I, II, and III are all that are needed for this group. Newly prepared antiserum for *S. dysenteriae* I should be tested with a boiled suspension of *S. alkalescens* to determine the amount of cross reaction. If the titer for *S. alkalescens* is in the magnitude of 1:320 or higher, the antiserum should be absorbed to remove the heterologous agglutinin. Antiserums for *S. dysenteriae* II and III are usually specific and may be employed in the unabsorbed state in slide tests.

The shigellae of group B are common in the United States and if typing is to be done, it is advisable to have antiserums for all of the six types. Of the six, *S. flexneri* II, III and VI appear to occur most frequently. Since there are extensive intragroup relationships among *S. flexneri* cultures it is necessary to use absorbed type specific serums to identify them. The one exception of this is in the case of *S. flexneri* VI. By using a freshly isolated culture of this type it is sometimes possible to obtain an antiserum which may be used in a dilution of 1:10 or 1:20 without absorption.

Each *S. flexneri* type contains a specific or major antigen and a number of common group or minor factors. It is possible to absorb antiserums in such a way as to remove all of the group agglutinins and thus render the serum specific. The manner in which *S. flexneri* serums may be treated to render them specific is shown in table 2.

At present, group C of the *Shigella* schema includes seven types (fig. 1). With the exception of *Shigella boydii* VII, these were isolated originally in India by Boyd (3,4) and his collaborators. *S. boydii* types since have been recognized in various parts of the world and most of the types have been found in the United States. *S. boydii* VII was isolated originally in North Africa in 1943 by Stock *et al.* (18). This serotype was found later in Italy, England, and France. As yet it is unreported in the United States.

Some *S. boydii* types may be identified by the use of unabsorbed serums in slide tests. When an antiserum for *S. boydii* I is prepared, it should be tested with a suspension of *S. alkalescens* and if the titer of the latter is 1:320 or more, the serum should be absorbed with *S. alkalescens*. Unabsorbed *Shigella boydii* II and III serums may be employed in slide tests if trials indicate little or no cross reactions with other shigellae. It usually is necessary to absorb *S. boydii* IV antiserum with a culture of *S. alkalescens* because these microorganisms cross agglutinate to considerable degree. The reciprocal of this absorption is necessary also, that is, *S. alkalescens* serum should be absorbed by *S. boydii* IV.

Of the members of group C, *S. boydii* I, II, and IV appear to be more common in the United States. It is probable that most laboratories will find that antiserums for these three types will meet their requirements. The manner in which *S. boydii* and *S. alkalescens* antiserums should be absorbed in order to render them specific is given in table 3. If *S. boydii* V and VI serums are employed, they should be absorbed as indicated in table 3.

S. sonnei is one of the most common *Shigella* types isolated in the United States, and in other

Table 1

ABSORPTION OF POLYVALENT ANTISERUMS

Serum	Absorbing Cultures	Number of Plates
Polyvalent A (*S. dysenteriae* I-VII) 1.0 ml	*S. alkalescens*	Growth from 10 plates
Polyvalent B (*S. flexneri* I-VI) 1.0 ml	*S. alkalescens* *S. dispar* I *S. dispar* II	Growth from 10 plates do do
Polyvalent C (*S. boydii* I-VII)	*S. alkalescens* *S. dispar* I *S. dispar* II *S. sonnei* II	Growth from 10 plates do do do

Table 2

PREPARATION OF SPECIFIC S. FLEXNERI ANTISERUMS

Antiserum 1.0 ml.	Absorbing culture(s)	Number of plates	Specific factor
I	III	10	
	IV	10	I
	V	5	
	VI	10	
II	I	10	II
	"X" variant	10	
	"Y" variant	10	
III	I	10	III
	II	10	
	IV	5	
	V	5	
IV	I	10	IV
	II	10	
	III	10	
V	I	10	V
	"X" variant	5	
	III	10	
VI	I	10	VI

parts of the world, as well. This type exists in two "phases," I and II (24). The phases are sometimes referred to as smooth and rough but this terminology is inaccurate and confusing, for in addition to forms I and II there exists a true rough variant. We prefer to designate these variants as form I, form II, and R(ough) rather than as phases. The term phase should be reserved for variation in the flagellar antigens of Enterobacteriaceae. Transitional forms between the three variants often are encountered and it is not uncommon to find cultures which are mixtures of forms I and II. Such cultures react in antiserum for form I, form II, and *S. boydii* VI. Pure form I and form II cultures cross-react very little or not at all, but pure form I and form II antiserums are difficult to obtain so that it usually is necessary to cross absorb the antiserums if one wishes to separate the two forms by means of slide tests. As indicated in table 3, *S. boydii* VI antiserum should be absorbed with a form II *S. sonnei* culture before it is utilized for diagnostic purposes, because the two types cross-react to considerable degree. Such an absorption leaves the specific agglutinins in *S. boydii* VI antiserum but the absorption of form II *S. sonnei* antiserum by *S. boydii* VI results in the removal of all agglutinins from the antiserum. Thus, it appears that form II *S. sonnei* contains the group antigens of *S. boydii* VI.

S. dispar I and *S. dispar* II antiserums should be cross-absorbed if specific typing is desired. While these microorganisms occur relatively frequently, it is likely that a mixed antiserum which agglutinates both I and II will meet the needs of many laboratories. Serotype II of *S. dispar* appears to be more common than type I.

If laboratories, such as those of the level of State health department laboratories, have specific antiserums for the first three members of group A (fig. 1), the six members of group B, *S. boydii* I, II and IV (of group C), *S. sonnei*, *S. dispar*, and *S. alkalescens*, most *Shigella* types found in the

Table 3

PREPARATION OF SPECIFIC S. BOYDII AND S. ALKALESCENS ANTISERUMS

Antiserum 1.0 ml.	Absorbing culture	Number of plates per ml
S. boydii I	*S. alkalescens*	5
IV	*S. alkalescens*	10
V	*S. dispar* I and II	5 (of each)
VI	*S. sonnei*, Form II	10
S. alkalescens	*S. boydii* IV	10

United States may be identified. Cultures belonging to other types and microorganisms that appear to conform to the description of the genus *Shigella* but fail to type, may be forwarded to a laboratory especially equipped to identify them.

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DIARRHEA - DYSENTERY — Use of Mortality Data as a Guide for Field Investigations

IDA L. SHERMAN, Statistician

Through his work in Hidalgo County, Tex., Dr. James Watt has shown that fly control will reduce the incidence of dysentery-diarrhea and its resultant mortality. Since extension of his findings might be of benefit to other areas in the United States which have an unduly high dysentery-diarrhea mortality, a study was undertaken to determine whether such areas existed.

To locate affected areas, epidemiological criteria were expressed in the form of statistical indices. Through these indices, places with a sufficiently large number of summer deaths could be cited for further investigation to determine whether effort to reduce the mortality level might be expended advantageously.

Since only mortality data are available on diarrheal diseases, the study was necessarily limited to deaths. Previous study of dysentery-diarrhea data for the years 1941-1946 had shown that the highest mortality from these diseases occurred among children 2 years of age or younger; that there was a pronounced seasonal variation; and that for the United States as a whole the incidence reached a peak in the summer months. Examination of the data for the years 1946-1947 showed marked changes in the previous pattern of seasonal incidence. The change in the curve of seasonal incidence for the United States as a whole was the resultant of marked change in the seasonal pattern of many northern States, some western States, and several southern States. In some States the prevalence continued to show a high summer incidence; in others, excess of deaths during summer months not only decreased but reverted to an excess of deaths during the winter months. The change in the seasonal incidence was repeated in 1947 and, from available 1948 data, there are indications that the prevalence will follow the 1946-1947 pattern rather than that of earlier years.

The mortality data to be studied in localizing areas of high incidence were limited therefore to dysentery-diarrhea deaths of children under 2 years old for the years 1946-1947, and consisted of mortality tabulations prepared especially for the Communicable Disease Center by the National Office of Vital Statistics.*

The first step was to eliminate those areas with no apparent summer dysentery-diarrhea problem. To accomplish this, the following two criteria were established for State-wide data:

1. Evidence of a summer excess of deaths coded to diarrhea and dysentery among children under 2 years of age, based on the ratio of deaths occurring during the summer months (June, July, August, and September) and those occurring in the winter months (December, January, February, and March). The extent to which the ratio of S/W (summer/winter) exceeds 1.0 provides a rough index of the degree of summer excess mortality.
2. An age-specific diarrhea and dysentery death rate among children under 2 years of age (based on live birth data and deaths during the summer months of 1946-1947) above the median rate for the 48 States and the District of Columbia.

Application of these two criteria resulted in the elimination of all but 16 States; but since the United States data by population-size groups showed that almost half the deaths occurred in "rural areas" (farm areas and communities less than 2,500 population), the data for the 16 States were examined by urban-rural classification, and it was found that in four of these States, the dysentery-diarrhea problem was essentially one of rural areas.

* International List of Causes of Death, Codes 27 and 119 (Fifth Revision, 1938) under 2 years of age, by State, county, population-size group, race and sex, place of death by residence of decedent.

TABLE I.- NUMBER OF DYSENTERY-DIARRHEA DEATHS, ALL AGES, BY STATES
1948

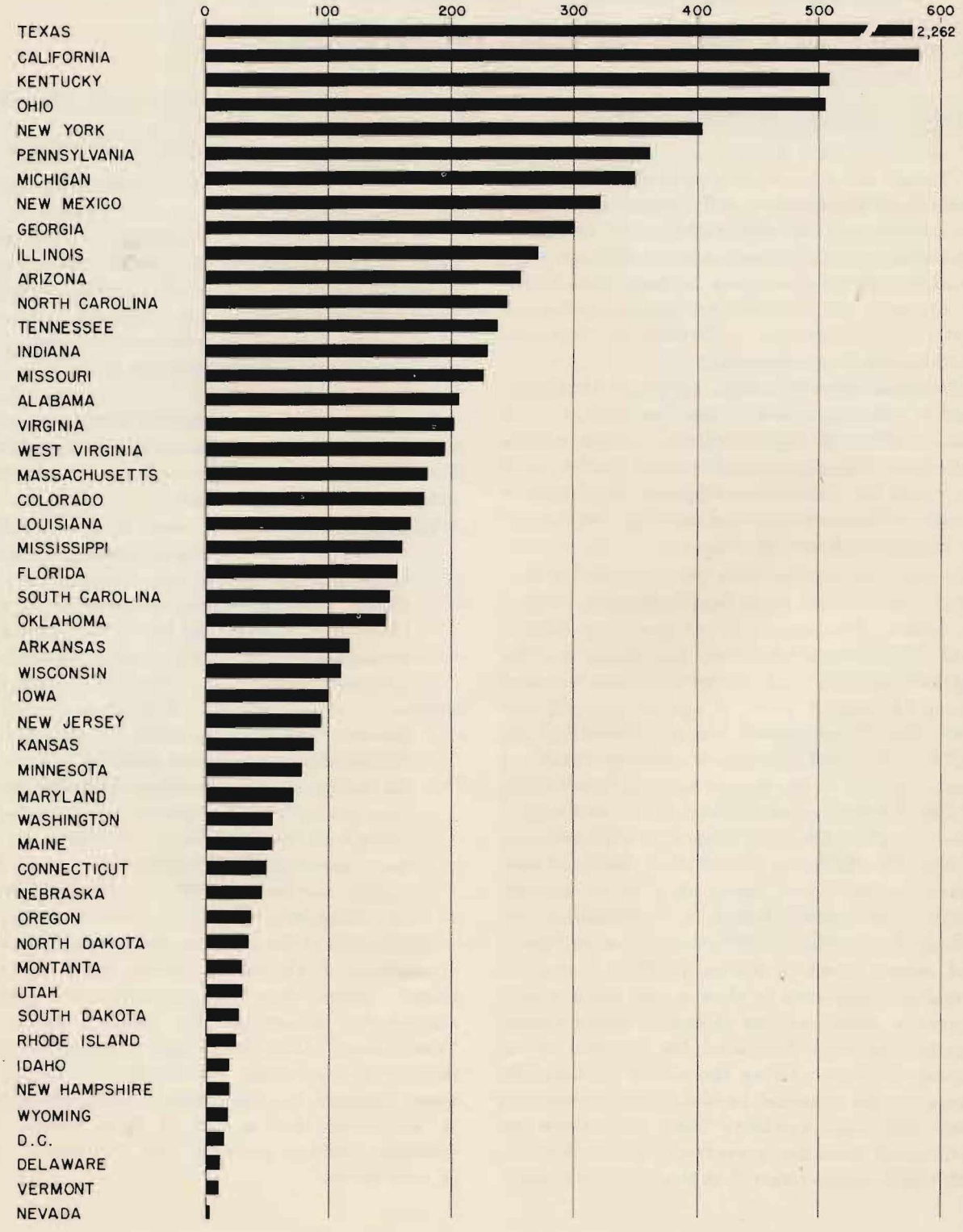
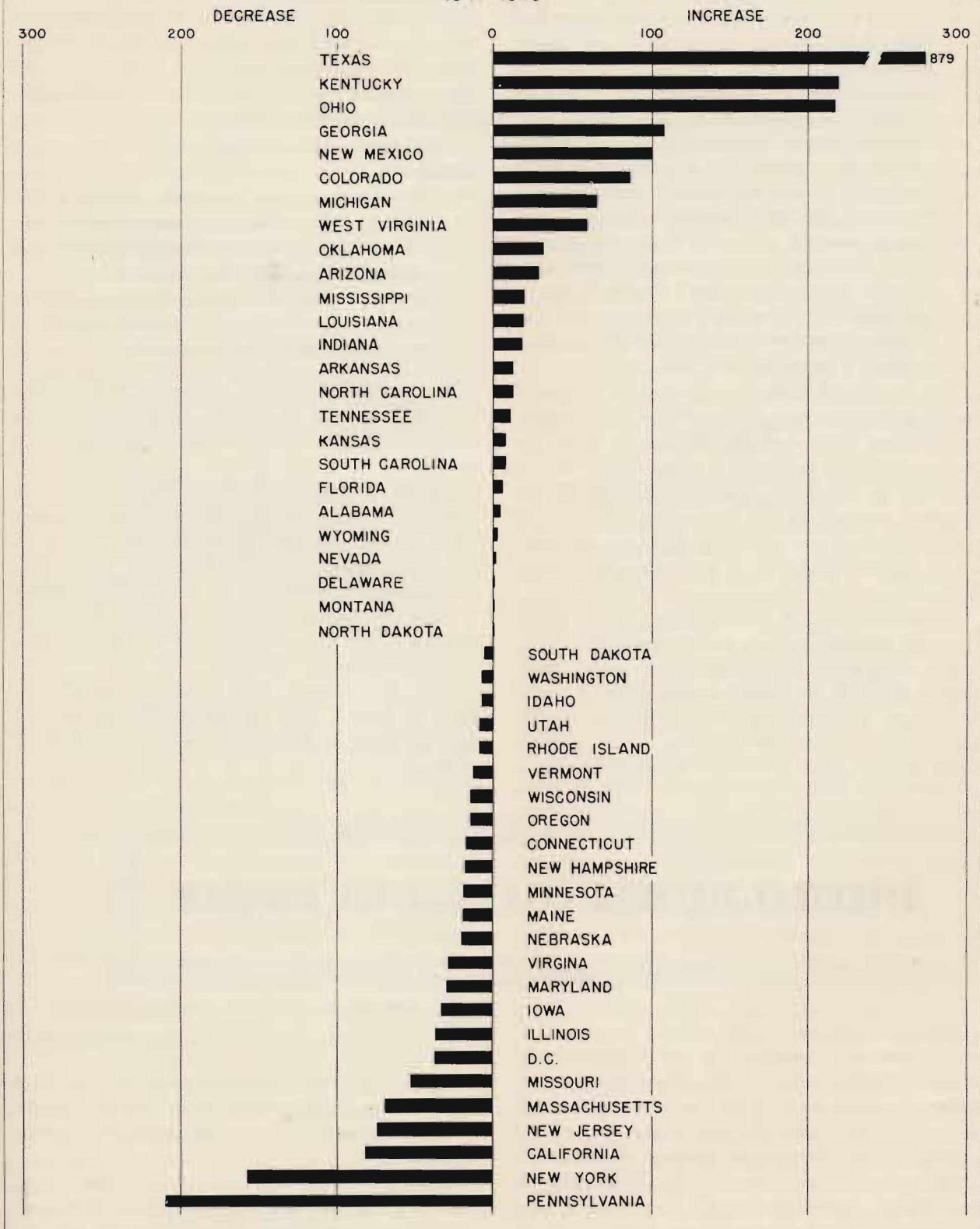


TABLE II.- CHANGE IN DYSENTERY-DIARRHEA MORTALITY BY STATES
1947-1948



In carrying the analysis to a county level, statistical criteria appropriate for small areas were established as follows:

1. **Number of Deaths:** Unless a county showed a total of four or more deaths during the "summer months" of 1946 and 1947 combined, it was eliminated for further study.
2. **Season:** A S/W ratio of 2 to 1 or greater.
3. **Mortality Rates:** Age-specific summer dysentery-diarrhea rate of 200 or more per 100,000 estimated population under 2 years of age, based on 1946-1947 "summer" deaths.
4. **Age at Death:** If a total of 25 percent or more of the 1946-1947 deaths among children under 2 years of age were under 1 month of age, it was considered presumptive evidence that the dysentery-diarrhea mortality figures reflected epidemics among the newborn.

The data for all States were examined by county and by population-size groups within each county. In 12 States there were 68 counties in which the problem appeared to be one of urban areas, and 28 counties in which the dysentery-diarrhea deaths occurred primarily in the rural areas. Of these 96 counties, there was evidence in 4 that the mortality figures might be a reflection of nursery epidemics.

In the remaining 36 States, there were 24 counties with indications of a dysentery-diarrhea problem in urban areas; but among these counties, there were 9 in which the deaths among infants 1 month or younger accounted for 29 – 59 percent of the total number of dysentery-diarrhea deaths under 2 years of age. The 36 States showed only three counties in which the problem was one of the rural areas.

The list of States and counties, with all pertinent information, was transmitted to Engineering Services for their use in field study and investigation. The following suggestions with respect to the consideration of environmental factors were made by the epidemiologist:

That field studies should not be undertaken unless

1. The community, or substantial areas within it, has privies or other accessible and widely disseminated sources of human excreta with which flies might become contaminated.
2. The population lives in an urban-type environment where effective fly control operations can be instituted and maintained.

Since mortality data as a measure of incidence have their obvious limitations, field investigations must supplement the conclusions of the statistical analysis.

Figures available for 1948 (for all ages) show an increase in mortality from the diarrheal diseases as compared with the figures for 1947. The total number of deaths for all ages in 1947 was 8,938, of which 6,527 or 73 percent were among children 2 years of age or younger. The total figure for 1948 is 9,909 or an increase of 971 deaths from these diseases over 1947.

Two accompanying tables show the total number of deaths from dysentery-diarrhea for all ages for 1948 by State; and the change from 1947 by State.

DYSENTERY-DIARRHEA (FLY) CONTROL PROGRAM

JOSEPH H. COFFEY, Sanitary Engineer

April 1, 1950, marked the official opening of a new operational program for the Communicable Disease Center, namely, the Dysentery-Diarrhea Control Program in which fly control measures will play an important part. The new program is the outgrowth of the well-known studies of Watt and Lindsay in Hidalgo County, Tex., [Pub. Health Rep. 63(41): 1319-1334 (1948)] from which it may be concluded with reasonable certainty that in areas of high dysentery-diarrhea morbidity and high fly density, a significant reduction in disease transmission may be effected by control of domestic flies.

The new program is organized in the same manner which has worked so successfully in the malaria and typhus control programs; that is, the projects

are operated through the State and local health departments.

On the basis of the findings by the Statistics Section, Epidemiologic Services, only those communities which showed an age-specific dysentery-diarrhea rate in excess of 500, and a summer-winter ratio in excess of 2.0 are presently being considered for operations. These communities are concentrated largely in New Mexico, Arizona, Texas, and Kentucky. However, during the course of the program, localized centers in other States will be investigated for possible inclusion in the program in subsequent years.

In those States where CDC activities are existent, dysentery-diarrhea fly control projects are added as integrated functions of existing CDC supervisory and administrative personnel. In the States and localities not previously served by CDC activities, arrangements have been made to provide additional technical and/or administrative services as required.

Arrangements with all local communities have not yet been completed; but the general pattern is that the community provides for the sanitation activities, the insecticides, and spray labor, while Federal funds provide for technical and supervisory personnel, spray equipment, and vehicles as required. The unit cost for the local community is expected to be on the order of from $0.25 to $0.50 per capita per year. The principal variables affecting this unit cost range are existing level of community sanitation and length of fly breeding season and prevailing wage scales. Except for extreme conditions, local costs should tend toward the lower end of the unit cost range.

Because fly control is a relative newcomer in the field of insect vector control, present control measures as yet are adaptable only in reasonably compact communities. Costs become prohibitive if ventures are made into sparsely populated areas. This situation is somewhat at odds with the facts indicated by the analysis of the mortality data wherein it appears that the higher summer-winter death ratios occur in the rural areas and in the smaller communities. Accordingly, it is expected that the greatest effort of the program will be expended in communities of approximately 10,000 population. Communities of this size represent a reasonable marginal overlap between decreasing summer-winter death ratios and sharply rising unit costs of fly control operations.

As is the case with the other fly control programs operated by CDC, the prime effort will be directed at the elimination of fly-breeding sources and the general lifting of the level of environmental sanitation in the operational area. Within communities, however, past experience indicates that the greatest dysentery-diarrhea mortality and highest fly densities are usually found in the areas of lowest economic level. In such substandard areas, the maintenance of domestic livestock, inadequate facilities for storage of garbage, and the existence of insanitary privies occur more frequently than not. It is obvious, therefore, that a sound program of environmental sanitation is not only costly but considerable time will be required to bring about significant improvement. Since sanitation is a frame of mind and must come from within the people of the community, educational programs must be developed to cause the citizens of the community to realize that improved sanitary practices are something to be desired for their own personal well being.

In the past, some sanitation programs have died "a-borning" because those people in the well sanitated areas of the community have felt secure in their isolation from the "wrong side of the railroad tracks" and have failed to support the program either financially or in spirit. In the smaller communities especially, such isolation affords little protection from infectious flies. There is much evidence to show that under certain ecological conditions large numbers of flies may migrate considerable distances, a mile or more.

For these reasons – that is, the time required to bring about significant sanitary improvements and the migratory habits of flies – the early efforts of the program will be directed toward the immediate reduction of fly populations by chemical means. It is fully realized that the control of flies by chemical applications is but temporary in nature and is, therefore, a never-ending operation. It does, however, afford a means of obtaining an immediate reduction in high fly densities and may be regarded as a delaying action to hold the line while the sanitation measures are being introduced.

From past experience, chemical fly control serves another very useful purpose. Because of its spectacular nature, it focuses attention on "The Fly," a currently popular subject, and provides impetus to the sanitation program in that it promotes sanitation for a definite purpose and removes it from the realm of abstractions.

The new program is small in comparison with other operation programs of CDC; probably no more than a dozen projects can be activated this year with the limited funds available. In contrast to its size, the following results expected from the program appear prodigious:

(a) The focusing of attention on the areas of exceptionally high mortality rates of dysentery and diarrheal diseases.
(b) The acceleration of "general" sanitation programs by introduction of an additional motive, namely, fly control.
(c) The more rapid spread of technical information concerning fly control as a health measure, thus bringing relief to certain areas of high morbidity years in advance of the normal turn of events.
(d) The increase of fly control programs in areas of high dysentery-diarrhea morbidity and high fly density is expected to broaden the base of the findings of the work of Watt and Lindsay in Hidalgo County, Tex.
(e) The pilot projects introduced with Federal assistance and guidance are expected to induce similar practices in nearby communities having similar problems.
(f) The operation of fly control projects in numerous States under a variety of conditions should produce new techniques and advances in present fly control procedures.

Taken as a whole, the new dysentery-diarrhea fly control program may be regarded as another undertaking for CDC in the fields of epidemiology, entomology, and engineering.

Whereas the new program is starting out in a very small way, its implications for the future are very broad. The possible expansion in this field of endeavor will depend largely on the success attained during this coming season.

A Preliminary Report of Studies on the EFFECTS on FLY ABUNDANCE of IMPROVED MUNICIPAL GARBAGE COLLECTION and DISPOSAL

DALE R. LINDSAY, Scientist
and
DUFFY E. McBRAYER, Sanitary Engineer

During the summer of 1948 considerable attention was given by the authors to an investigation of the sources of fly production in municipalities. This investigation was prompted originally by the overemphasis then placed upon the chemical control of flies. Failures of the most widely used of the chemical insecticides, DDT, in maintaining satisfactory fly control provided an added incentive to the study. Fly breeding sources in municipalities in New Mexico, Texas, and Georgia were investigated.

CONDITIONS PROMOTING FLY BREEDING IN MUNICIPALITIES

Although no startling or unsuspected discoveries were made, it was determined that in most of the small cities the production of flies was due in large part to the improper handling of household garbage, both by individuals and by the municipalities, and of animal refuse by individuals. In the larger cities an additional fly production source was found in various organic wastes from such industries as canneries and other fruit and vegetable processing plants, and from meat packing plants. In the Southeastern States fly breeding conditions are enhanced by normal climatic conditions which provide moisture as well as a relatively

prolonged breeding season.

A condition common to all the areas investigated was the failure to enact or enforce ordinances to regulate or exclude livestock from municipalities. While fly breeding nuisances were thus maintained on much of the residential property, the systems of garbage collection and disposal were usually so deficient as to provide little incentive to the average citizen to clean up his premises. In the Southeastern States poor garbage collections were not improved by the prevalence of very large blocks with deep residential lots and without service alleys – a condition that required either increased operational man-hours of city garbage crews or individual cooperation in placing garbage cans along the street curb on collection days.

In spite of rapidly expanding sewerage systems in nearly all of the areas investigated, many small cities, and the peripheral residential areas of larger cities, had numerous pit and surface privies. Since these are frequently both producers of flies and potential reservoirs of human enteric diseases, they operate with other and more important fly production sources to create a distinct hazard to human health. This factor alone is sufficient justification for increased expenditures by municipalities in improving both garbage and sewerage facilities. Whereas the extension of sanitary sewerage involves a significant expense, both to the municipal government and to the property owner it serves, the improvement of a garbage collection and disposal system was believed possible in many instances without substantially increasing city costs. For this reason the study described below was undertaken. Although the study is still in progress certain preliminary results are noteworthy, and together with the above presentation of the conditions normally existing, should be of value in planning improvements in municipal and environmental sanitation.

OBJECTIVES OF THE STUDY

1. To determine the degree of fly control that could be attained through improved garbage collection and disposal in a municipality.
2. To determine by epidemiological methods the effects, if any, of such control upon human enteric diseases.
3. To determine some of the important cost-amortizing possibilities of improved sanitation so that monetary as well as welfare gains over a period of time may be pointed out to financially handicapped cities.

SELECTION OF STUDY AREAS

In order to attain the above objectives it was essential that this study be conducted in the general area in southern Georgia where a study of the effects of fly control measures upon the morbidity rates of human diarrheal diseases was already under way. Two comparable municipalities, each with populations of 15,000 to 18,000, were selected. At the time the studies were being planned, both cities had deficient garbage-collection schedules averaging one collection weekly in residential areas; both cities had numerous livestock enclosures and pit or surface privies. The only difference in city sanitation was in the method of garbage and refuse disposal. In City I an area fill, located about 1½ miles north of the main residential area, was used. The garbage placed in the fill was poorly compacted and covered once or twice a week with a layer of dirt of highly variable thickness. Although this method served to bury the refuse, it imposed very little restriction upon fly emergence from the already heavily maggot-infested garbage. In City II garbage and other refuse were piled on an open dump, located at the northwest corner of the city. Some control of fly breeding was attempted at this dump through burning of combustible refuse. Approximately 150 city-owned hogs were penned on the dump and served to increase the surface area of fly breeding material through their foraging activities. In both cities the weekly collection schedules, frequently interrupted by weather or by equipment breakdown,

In a sanitary land fill, each day's collection of garbage should be compacted in a cell with a 2 foot cover of dirt.

favored excessive amounts of fly breeding in the garbage. Emergence rates of flies from such garbage, collected from both fixed and random sites, reached a figure of 1,500 flies per day per garbage can and consisted of many species with *Phaenicia pallescens* (Shannon) predominating at 95.0 percent and *Musca domestica* Linn. included at 1.5 percent.

The county in which City II was situated had participated during 1948 in the Malaria Control Program, and thus the residents of City II could request a free residual application of DDT in their homes. Records for the 1948 season revealed that not more than 15 percent of the homes in this city were thus sprayed and the authors felt that the over-all effects of this spraying upon the city's fly population would be negligible. This feeling was supported by high fly indices found in the city during 1948 and was further substantiated by records during the 1949 season when little, if any, effect upon house flies out-of-doors was noted. City I had enjoyed no such services, and only DDT applications by individual householders were expected.

On the dual basis of the willingness of city officials to cooperate and the fact that results would not be obscured by the effects of widely used chemical insecticides, City I was selected as the site of intensified sanitational measures during fiscal year 1950, for comparison with normal procedures in City II.

METHODS USED

The following arrangements for increased sanitational services were made with the officials of City I in order to provide suitable conditions for the study.

1. Garbage collections were increased from one per week to three per week. Three-times-per-week garbage collection effectively curtails fly breeding in garbage. In twice-weekly collection schedules, maggots of the genus *Phaenicia* can reach maturity during the intervals between collections and enter the adjacent soil to form puparia prior to emergence as adult flies.
2. The area fill method was discontinued and a daily-operated sanitary land fill was started on the same site. In order to shrink the operational costs of hauling garbage the authors recommended that this operation be moved into or nearer the city, but city officials demurred on the basis of nonavailability of suitable city-owned land. In addition a smaller machine (1 cubic yard capacity) than previously used (2 cubic yard capacity) was put into service on the sanitary land fill to further reduce maintenance costs. In addition to reduced operational cost the initial cost of this machine was approximately one-half that of its predecessor.
3. In order to obtain suitable cost figures on the comparative costs of back-yard versus street-curb collection of garbage, the cooperation of the householder in the latter was simulated by city crews who placed the cans along the street-curb in advance of the truck.
4. Enforcement of city ordinances regulating the holding of garbage, industrial refuse, domestic livestock, and the like was promised by city officials.

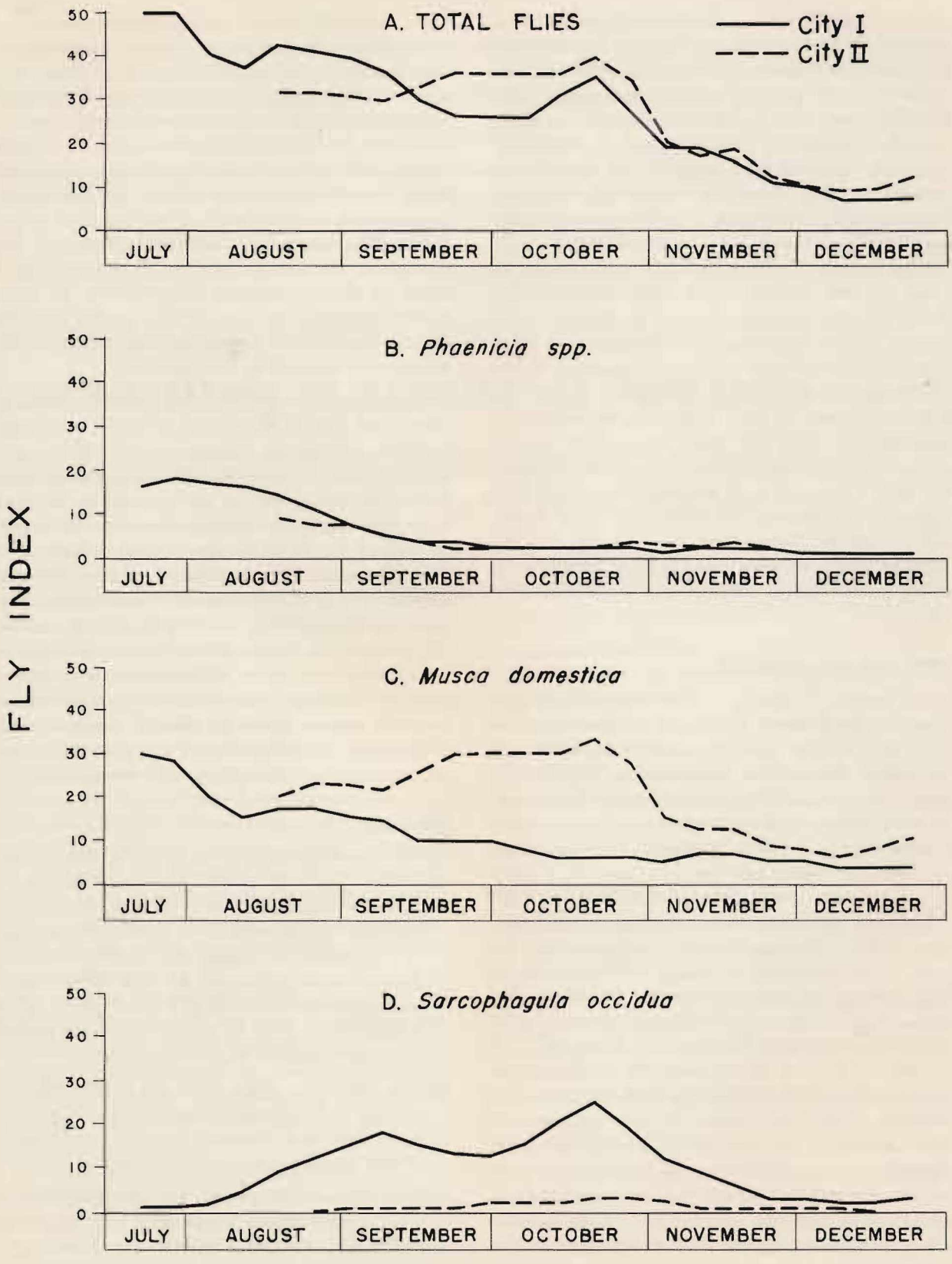
PROGRESS TOWARD OBJECTIVES

The intensified sanitational program was begun in City I early in August 1949. By mid-August the accelerated garbage collection schedule was operating fully, and after one week of operation the fly maggots had almost completely disappeared in garbage containers and in garbage brought to the sanitary land fill. Since the pupation period of *Phaenicia* spp. may last as long as 2 weeks under field conditions the curtailment of adult fly populations of this genus was not great until late in August.

During this same period breeding conditions became favorable at a peanut processing plant centrally located in City I and the resultant emergence of adult house flies, *M. domestica*, caused a temporary rise in the index of this species. In view of the other studies in progress it was decided to use a mist sprayer in applying DDT in the immediate three-block vicinity of the peanut mill in order to be able to observe results of the intensified garbage program. This was done on a weekly basis for 5 weeks and constituted the only use of a chemical insecticide in the study of City I.

Although operation of a sanitary land fill was begun on schedule, and immediately accomplished the desired prevention of fly breeding with relative ease, the most efficient operation of the equipment was not attained until January 1950. Cost records during the remainder of the study should be sufficient for purposes of determining operational costs.

Meanwhile officials of City II, the comparison



FSA-PHS CDC-ATLANTA, GA. APRIL 1950

city, had decided to undertake a fly control program of their own design. Between the dates of July 15 and September 1, 1949, they effected a 3-times-per-week garbage collection schedule, but did not change their method of disposal. In addition they purchased and dispersed, partially by knapsack sprayers and partially by mist-blower machine, 5,000 gallons of a commercially prepared 5 percent DDT solution in oil. Garbage can spraying with this material took place in City II throughout the summer of 1949; city-wide applications by a mist-blower machine were made twice weekly during the two periods of July 8 to August 1 and August 20 to September 11. Because of these changes, which do not permit a comparison of the 3-times-per-week garbage collection system of City I with one of less frequency, an additional municipality, City III, has been added to the study. City III, situated in the same general area and with a population of approximately 6,000, has twice-weekly garbage collection, an open dump, and animal enclosures and privies proportionate to those found in Cities I and II. City III has been under entomological surveillance since February 1950.

PRELIMINARY RESULTS

The results of the intensified sanitational program in City I during the first 5 months of operation, as compared with the intensified sanitational and added insecticidal operations in City II, are shown in the chart. While consideration of the total species index, part A, appears to show equal results from both of the programs, a breakdown of the total fly counts into species, parts B, C, and D, reveal interesting features. In part B the *Phaenicia* spp., breeding primarily in garbage, have indices of approximately equal magnitude in both cities indicating a similar effectiveness in both garbage collection programs. In part C the index of the common house fly, *M. domestica*, is about three times as high in City II as in City I during the months of maximum effect (September and October) until curtailed in both cities by cool weather. The significance of this is apparent from inspection records which reveal that high fly indices were obtained in the northwest section of City II nearest the dump and are undoubtedly a reflection of migration from the open dump into the city. Such house fly breeding as reflected in the index for City I was determined by surveys to be occurring in improperly maintained animal enclosures, primarily where cows, horses, and mules were stabled. In part D, the index of *Sarcophagula occidua* Fabr. began rising in City I about the time that the garbage collection was intensified and remained high until curtailed by cool weather. Since the breeding of this species is most often encountered in the excrement of carnivorous animals, including man, the garbage program would be expected to have little effect upon its abundance. The index for this species remained low throughout the season in City II, probably as a result of the insecticides dispersed by the mist-blower machine. In other towns in the area the authors have found space-spraying methods very effective in controlling this species.

It appears, therefore, that the difference between City I and City II which may be ascribed to sanitational methods is limited to the effects of a sanitary land fill, even though improperly operated during the period shown, as compared to an open dump where sporadic efforts were made by City II to control the flies by insecticidal means. This does not mean, however, that there were no measurable effects of the intensified sanitational program. On the contrary, as concerned with garbage collection alone, these effects were spectacular in both areas. Both cities still have much to accomplish in obtaining the maximum sanitational effect upon fly control primarily through enactment and enforcement of ordinances to prevent sanitational malpractices in the keeping of livestock.

SUMMARY

1. The general results of an investigation into the primary sources of fly production in municipalities located in New Mexico, Texas, and Georgia are presented. A preliminary account of the effort to apply the garbage handling improvements indicated by this investigation as fly control measures in a municipality in the Southeast, is given in order to point out some of the basic features of such measures.
2. The objectives of the study are to determine the effects of the possible improvements in garbage collection and disposal upon fly abundance, human disease, and operational costs.
3. The methods employed and the progress in effecting the improvements are given, as are the preliminary results obtained. Fly breeding in garbage, both in cans and at the sanitary land fill, virtually was eliminated by the accelerated

collection and disposal system. House fly breeding remained a problem but originated primarily from animal enclosures, pointing out the need for enforced ordinances to regulate livestock within the city. The difference in house fly indices between Cities I and II is due primarily to the house fly breeding in the open dump at City II. The spraying program in City II is believed to have been primarily effective in the control of the excrement-breeding fly, *Sarcophagula occidua.*

4. In addition to cost data the results of the study will yield data on the effects upon fly densities and human disease in: (a) City I with 3 garbage collections per week and an efficient sanitary land fill; (b) City II with 3 garbage collections per week but with inadequate disposal; and (c) City III with twice-weekly garbage collection subject to some variation and inadequate garbage disposal.

CONSIDERATIONS IN SAMPLING FLY POPULATIONS

JUDSON U. McGUIRE, JR., Assistant Sanitarian (R)
and
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In recent years many programs for the control of house-frequenting flies have been undertaken. As might be expected in such developmental programs, greater emphasis has been placed upon the methodology of control operations than upon the measurement of the fly populations. Various devices have been used in evaluating the effectiveness of the fly control measures, such as baited fly traps, tanglefoot tapes and sheets, and time counts over uniform attractants. The fly grill developed by Scudder* has been perhaps the most widely used of these devices in programs sponsored by the Communicable Disease Center. However, the application of the grill has varied between programs, and this lack of uniformity in the methods of taking and interpreting fly population samples has prevented really accurate direct comparison among programs.

The need for a standard method of sampling fly populations is indicated, not only to facilitate the evaluation of fly control operations but to make possible the correlation of the results with standard epidemiological measurements. A reliable sampling method should have the following characteristics:

1. It should be repeatable, and objective.
2. It should be useful in guiding control operations.
3. It should be economical to apply.
4. It should supply data which are suitable for statistical analyses.
5. It should be applicable over wide geographic areas.
6. It should be adaptable to a wide range of conditions.

The following discussion of the methods established and in use by the authors and their coworkers on an experimental study in south Georgia is presented for consideration by other programs.

PREREQUISITES TO SAMPLING

The following steps were followed in establishing the sampling program on the experimental project under discussion.

1. Maps of the cities were obtained and brought up to date. Besides large office maps, small notebook-size reductions were made for field use.
2. All blocks in the cities were numbered for easy reference.
3. The cities were divided into sub-areas of about 10 blocks, or a 10 percent sample, except that in the high-income residential districts, sub-areas of 20 blocks, or a 5 percent sample, were used. Sub-areas which included industries with

*Scudder, H. I.: A New Technique for sampling the density of housefly populations. Pub. Health Rep. 62:681-686 (1947).

Placing fly grill over attractant to sample fly population.

high production of organic wastes never exceeded 10 blocks.

SURVEY PROCEDURES

A. The Stationary Block Survey. In each sub-area, the block with the highest fly potential was selected as a so-called STATIONARY SURVEY BLOCK. The stationary survey block was not changed during the survey unless another block in the sub-area showed, over a period of three consecutive weeks, a higher weekly average than the one selected. All the stationary survey blocks thus selected were inspected once a week, using the grill method. Although 10 grill counts were recorded, the average of the 5 highest counts was used as the index for each stationary survey block.

The stationary survey block method had several advantages and disadvantages. The advantages were: (1) the speed and low cost with which the surveys could be made; (2) continuous data on the same blocks throughout the season at weekly intervals; and (3) with a low index, the indication that a minimum number of flies was present in the whole area. The disadvantages were: (1) the dubious economy of re-treatment throughout a sub-area on the basis of the data from a single stationary survey block; (2) the possibility of a bias having been introduced into the data by the way in which the blocks were selected and by the methods of spraying used.

B. The Visual Survey. In order to prevent unnecessary expenditures of time and materials in re-treating a sub-area on the basis of the data from a single stationary survey block, a visual or reconnaissance survey was used in conjunction with the stationary block survey. It was found advisable to precede the stationary block survey with the visual survey by 2 to 3 days, thus using the stationary block survey as a check on the effectiveness of the control measures as indicated by the visual survey. Without using the grill, but based on experience with it, estimates of grill readings of each concentration of flies encountered in each block were made rapidly by the inspectors. The average of the five highest of these estimated grill counts for each block was used as the index figure for the block. The control operations section was then furnished with a map upon which the block index figures were shown. All blocks whose index figures exceeded three flies were designated to receive treatment. Treatment was applied to the designated blocks in relation to the index figure. Blocks with the highest indices were treated first. This type of survey proved very useful in providing data for directing the effective application of insecticides, but its standardization requires further study.

C. The Random Sample and the Statistical Treatment of the Data. Data obtained from stationary block surveys were subject to bias in that the blocks were inspected at different times, in different places, and by different persons. The visual survey enabled broad coverage to guide fly control operations. Its continued use holds promise as a tool for entomological evaluation and epidemiological investigations, but its development as a meaningful measure requires further study of the sources of bias and the magnitude of sampling error.

The methods of analytical statistics are based upon the theory of probability. In order to use these methods validly, data must be collected in such a manner as to allow the application of the mathematical model upon which the probability theory is based. A random sample will be defined, for the present subject under discussion, as a sample in which each variate is selected with known proba-

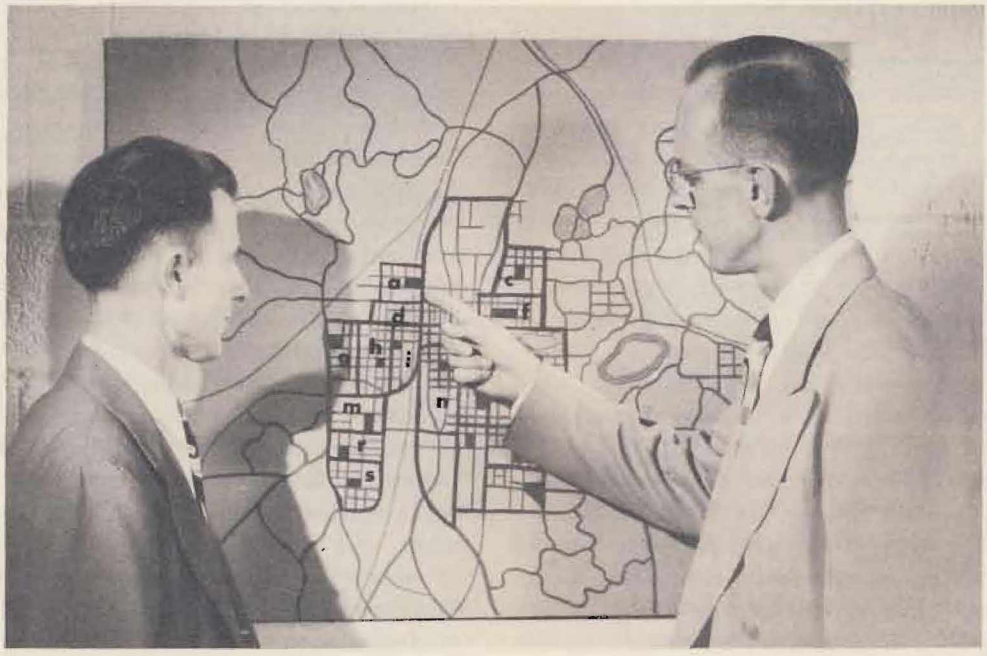
bility. If only the blocks with high fly counts are selected for sampling, any statistic derived from the data is representative of only a portion of the blocks, and no valid assumptions may be drawn with respect to the flies in the whole city unless one knows the relationship between the selected high blocks and all blocks in the area. This relationship depends on the local characteristics of each area and cannot be assumed invariant from one locality to another. It is therefore quite apparent that in order to insure generalization of inferences from one area to another, some form of random sample must be taken in all projects where fly indices are to be compared with epidemiological or other experimental data.

The random sample also serves as a check on change in the bias of stationary high count blocks. Bias is a conscious or unconscious allocation of greater probability of occurrence to some of the values of a variate. A mean calculated from biased data will not be an estimate of the true mean of the population being sampled. If, for example, only the stationary survey blocks are sprayed, a reduction in the city-wide fly index may not correspond to actual reduction of flies in the entire city, but only to the decrease of fly abundance in these blocks. A random sample, as used in this study, included one block out of each sub-area in the city at each sampling period, and was not biased in this particular respect, since the stationary survey block in each sub-area had only a 1-9 chance of being included in the weekly sample. The random sample also gave a similar check on the accurate selection of the highest block in the area.

The random sample therefore served a double purpose: It provided data by which to stabilize and check the operational surveys, and it supplied a valid statistical sample. This sample was drawn by selecting at random, with replacement, a block in each of the 10-block or 20-block areas in the city. The block thus drawn was sometimes surveyed by the same man who inspected the stationary survey block or by a different man altogether. For operational purposes the random survey was unsatisfactory if used alone. From this work it appears quite possible that with the use of a standardized visual survey as the principal guide to fly control operations the sampling procedure may be limited to the visual and random surveys.

DETERMINING THE NUMBER OF INSPECTORS NEEDED

Both the number and the accessibility of alleys in a town were of great importance in determining the number of inspectors needed. In cities with few or no alleys, such as were encountered in this study, the maximum number of blocks which could



In setting up a fly sampling program, one of the most important steps is to obtain maps of the town and divide them into sub-areas, indicating a stationary survey block in each sub-area.

be surveyed per week by each inspector was approximately 125 for both the stationary and random surveys and approximately 275 for the visual type of survey. These figures were attained only under conditions permitting a full work day, and when travel time each day to and from the work area was 30 minutes or less. Based upon previous survey experience in other areas, where each city block was traversed by an alley, it has been determined that the stationary and random block surveys required one inspector for each 150 blocks to be inspected each week on each of the two types of survey. Time studies of the few blocks traversed by alleys, in the area of the experimental study under discussion, indicated a visual survey time per block of 4 to 5 minutes. Assuming that all of a city's blocks were so traversed by alleys and that 450 working minutes were available each day of a 5-day week, one inspector could conduct a visual survey through 450 to 560 blocks per week. Variations in these figures will result when working time is decreased by weather or other factors or when maximum efficiency is lost for any reason.

FACTORS WHICH AFFECT VARIABILITY OF GRILL COUNTS

Various factors, which have been found in our experience to cause the greatest variability in grill counts of flies, are discussed in the following paragraphs.

Inspectors. In order to measure the accuracy of the different inspectors in estimating flies on the grill, certain tests were run. These tests involved the use of grills, to which dead flies had been fastened by means of an adhesive, and on which the fly distribution patterns were arranged to simulate field occurrence. In the lower density ranges, i.e., up to 50 flies, it appears that all the inspectors tested were reasonably accurate. The greatest errors of estimate were found to be in the vicinity of 175 to 250 flies. This readily may be explained by the fact that in this range of counts there were too many flies for the inspector to count on all parts of the grill; yet in this range, distributions of flies on the grill were quite uneven. Since the inspector counted the flies on a portion of the grill in order to estimate the total flies on the whole grill, an additional error, due to the uneven distribution of the flies on the grill, was introduced. This error reached a maximum in this interval. Below 50 flies the spread of the different readings was very small, but above 50 flies it was so great that special training of the inspectors in estimating large numbers of flies was indicated. The amount of time to be spent in training inspectors to use the grill will vary with the objectives of a fly control program. The authors recommend a minimum of 4 weeks' training wherever possible and frequent checks throughout the season. A time limit of 20 seconds should be used in all tests since it closely simulates the length of time in which flies can be expected to remain quiescent on the grill in the field.

Temperature. One of the early observations made by the inspectors was that the fly index on cold days was reduced to zero. This was to be expected since flies are poikilothermic. In determining the temperature threshold of fly activity from the grill records, it was found that the lowest air temperature at which countable flies were encountered was considerably lower than the observed temperatures at which flies would be immobilized. Field observations revealed that attractants in the sun had an environmental temperature about 24° F. warmer than the air temperature in the shade usually recorded. Counts made during those portions of the day when the air temperature is too low for normal fly activity will only reflect the number of attractants in the sun and the flies that were on or near them.

A preliminary laboratory test was conducted in 1949 in order to estimate more closely the threshold temperatures of various degrees of fly activity. Five house flies each were placed in large test tubes, 29 millimeters by 19 centimeters, which had been fitted with cork stoppers. A mercury bulb thermometer inserted through a perforation in each cork stopper allowed readings of the air temperature inside the test tube and within one-half inch of the flies. The test tubes of flies were cooled in a refrigerator for 30 minutes until the air temperature within each tube was approximately 27° F. After this cooling period, which immobilized the flies, the test tubes were brought into the laboratory where the temperature was 80° F. As the temperature within each test tube rose, the reading at the initiation of each level of activity was recorded. Fifty replications of this test were made, and a record was kept of the temperatures at which the following activities were initiated. The average temperatures at which the flies responded were: The first movement was made at 55° F.; at 62° F. they righted themselves; at 70° F. they crawled; and at 74° F. they became quite

active. From this information, and from field observation data, the authors concluded that grill counts taken when the air temperature is less than 70° F. should not be used in the calculation of fly indices. This 70° F. temperature is only a preliminary threshold selection, however, since further investigations as to the length of time the fly is subjected to a gradual increase in temperature are needed, and the final results may indicate a different threshold of activity.

Moisture. Moisture is usually an important limiting factor only in areas of low rainfall. In Texas, New Mexico, and Arizona the limiting factor for fly production during the summer appears to be the availability of moisture. Data from Albuquerque, N. Mex., show that hog pens and some other animal enclosures with a constant supply of moisture were the main fly attractants (30 percent), and accounted for most of the high counts. In the southern region of the United States, from east Texas to the Atlantic, excessive moisture may sometimes inhibit rather than increase fly production; this may be due to the drowning of the larval stages and the mortality in the adult fly population caused by heavy rains.

Shade. Shade is apparently of least consequence among the factors selected, but is of importance in determining the distribution of flies in a block at different times of the day. Early in the morning or during a relatively cool day (70° to 80° F.) the majority of high counts are found in the sun. As the air temperature reaches the vicinity of 90° F., the flies will tend to move from the sun into the shade.

METHODS EMPLOYED IN ORDER TO MINIMIZE THE EXPERIMENTAL ERROR INHERENT IN THE USE OF THE GRILL

Knowing that there is a certain inevitable variation in the ability of the different inspectors to count and estimate fly numbers, it was believed essential that a schedule for the systematic rotation of the inspectors from one inspection area to another be followed. In this manner the bias introduced by the individual inspectors will be equal in all cities or sections of a large city.

Of great importance in surveying small cities or sections of a large city, since they usually are surveyed during part of a working day, was the rotation of the time of day in which the inspection was made. It is quite obvious that the ideal procedure would be to have the inspections made at the same time of the day each week in all cities. Since this was not possible, the authors systematically rotated the inspection time in all inspection areas so that variability caused by the hourly fluctuation in numbers of flies was introduced to an equal extent in all areas when data from several inspection periods were combined.

In cities where fly inspections could not be completed in one day, the different parts of the city were inspected on different days in order to have one full inspection each week. Care was taken that no section was always inspected at the same time of the day. Blocks for each day's inspection were selected at random, without replacement, thus getting a full weekly sample from the town. The random selection of the stationary survey blocks to be inspected each day greatly increased the probability that all parts of the town were inspected at all the different times of the day.

SUMMARY

1. Data from the sampling of fly populations served a two-fold purpose: They provided the operating personnel with information needed to determine when and where control measures were needed; and they were compared with epidemiological data on diarrheal disease.
2. A three-point survey was believed necessary to best serve both purposes:
   a. A visual survey was of great value in directing control operations and holds promise, once fully tested, of serving all inspectional purposes.
   b. A stationary block survey was of value in supplying continuous data from the most fly-productive block in each sub-area.
   c. A random survey was needed in order to permit comparison of fly indices with epidemiological data and served as a check on the bias introduced in the stationary block survey.
3. The most important variables influencing fly density readings by the grill or other methods were individual inspector capacities, temperature, moisture, and shade.
4. Comparability of fly inspection data was improved by the rotation of (1) the inspectors, (2) the order of inspection of the small cities or of the sections of the larger cities, and (3) the order of the inspections of the blocks within the cities.

Basic Sanitation

PRODUCTION NO: CDC 4-090, Released 1950

DATA

Motion Picture; 16mm., Sound, Color;
Time: 9 Minutes; Length: 316 Feet

PURPOSE

To visualize how basic sanitation can help to eliminate flies in a community fly control program.

AUDIENCE

Professional and subprofessional public health personnel and others who will utilize or teach others to utilize basic sanitation in fly control.

CONTENT

1. **Introduction.** Flies will breed in open garbage cans, in open garbage dumps, and around open pit privies; in fact, any place where there is filth, warmth, moisture and sufficient time for the breeding and development stages to take place. Basic sanitation – the elimination of all these fly breed-sources – is the foundation of all successful fly control operations.

2. A community program in basic sanitation backed by local ordinances should provide for or enforce (a) the storage of all garbage refuse in covered and well-cared-for garbage cans kept on concrete bases, or in case of industrial refuse, in concrete bins; (b) collection of combined garbage and rubbish in closed collection units (daily in business sections and at least biweekly in residential areas); and (c) disposal of the collected waste in sanitary land fills or incinerators.

3. In rural areas, basic sanitation procedures parallel those of urban areas except that the disposal of garbage is the problem of each individual family. Screens on windows and doors aid in excluding flies from homes. Garbage should be wrapped in newspaper and stored in flyproof garbage cans until it can be burned or buried. On stock farms, manure should be spread thinly on the field at least twice a week or kept in fly-tight storage bins. Sanitary pit privies keep flies away from human excreta.

4. Recapitulation emphasizes that basic sanitation is the foundation of all successful fly control operations.

COMMENTS

Two other motion pictures of the Community Fly Control Series are "Spraying Equipment and Procedures": No. 4-091, Part I "Residual Spraying," and 4-110, Part II, "Space Spraying."

AVAILABILITY: Short-term free loan upon request to:
Medical Director in Charge
Communicable Disease Center
605 Volunteer Building, Atlanta 3, Ga.

U. S. GOVERNMENT PRINTING OFFICE : O—1950

Some Recent Manuscripts Relating to DIARRHEA and DYSENTERY by CDC Personnel

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Dow, R. P. and Maier, P. P.: A conical net for collecting flies. Pub. Health Rep. 64(19): 604-607 (1949).

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MANUSCRIPTS CLEARED FOR PUBLICATION AND/OR PRESENTATION

Baum, M. D.: Colorado's meat inspection program.

Coffey, J. H. and Maier, P. P.: Fly control techniques.

Edwards, P. R. and Hermann, G. J.: A new *Salmonella* type: *Salmonella allandale.*

Gilbertson, W. E.: Sharpening the focus of sanitation measures.

Simmons, S. W.: The resistance of flies to DDT.

Upholt, W. M.: The role of chemicals in fly control.

The printing of this publication has been approved by the Director of the Bureau of the Budget, January 19, 1950.