AN APPROACH TO THE STUDY OF INTERACTIVE PHENOMENA AMONG MICROORGANISMS INDIGENOUS TO MAN[1]

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The importance of interactions between species in the mixed microbic populations in nature is recognized, but the subject is poorly understood. The need for studies in this field has been noted by Appleton (1940), Woods (1947), and Lucas (1949). Some of the manifest difficulties that beset an adequate approach to it are mentioned by Topley and Fielden (1922) and Annear and Hayes (1950). Such an approach might be facilitated by means of a rapid screening method, sufficiently sensitive and preferably at least roughly quantitative, which would permit disclosure and preliminary classification of interactive phenomena among a wide range of microbic species. This report describes a method which seems to meet these criteria and presents data thus far obtained by its use.

The problem in hand may be delimited in terms of interactions of microorganisms as a phase of microbic ecology, with special reference to human hygiene. The microorganisms of most immediate interest are in consequence those indigenous to man, as they interact with one another and with pathogens and transient saprophytes. Our approach thus excludes phenomena in soil and sewage and differs from studies whose primary purpose is the discovery of useful antibiotic substances.

A moderately extensive literature bearing on aspects of this problem need not be reviewed here in detail. To save space, references are given only to early and recent papers.

Most studies have been concerned with inhibitory effects and have dealt mainly with the search for possible therapeutic agents or a particular disease, e.g., diphtheria. Included are (a) a group of reports on antibiotic substances produced by lactic streptococci and acting upon other streptococci, pneumococci, staphylococci, the diphtheria bacillus, and other organisms (Oxford, 1944; Hirsch and Wheater, 1951); (b) the so-called colicines, produced by strains of *Escherichia coli* and acting upon other strains of the same species or upon other enteric bacteria (Gratia, 1925; Blackford *et al.*, 1952); (c) both inhibitory and stimulatory effects produced by certain micrococci or staphylococci in relation to the diphtheria bacillus, streptococci, enteric pathogens, and other bacteria (Dujardin-Beaumetz, 1932; Halbert *et al.*, 1953); and (d) inhibitory effects produced by pneumococci, greening streptococci, and occasionally by hemolytic streptococci and lactobacilli mainly against *Corynebacterium diphtheriae* and certain staphylococci (McLeod and Gordon, 1922; Wheater *et al.*, 1952). The last mentioned group of phenomena has been attributed to hydrogen peroxide produced by the inhibitor species and is thought to encompass the analogous inhibitory properties found in whole saliva by Dold and Weigmann (1934) and many later workers. In addition two reports deal with effects produced by *E. coli*, but evidently distinct from colicines: one by Wiedling (1945) on an inactivator of growth of *Penicillium notatum* distinct from penicillinase; and another by Paine (1952) on a relationship between *E. coli* and *Candida krusei*.

Among examples of cooperative or probiotic microbial associations may be mentioned the classical satellitism of *Hemophilus influenzae* by *Micrococcus aureus* (Grassberger, 1897), and various fermentation effects described as "synergism" (Holman and Meekison, 1926) or "symbiosis" (Castellani, 1926) such as the production of gas from mannite by a mixture of *Proteus morganii* and *Shigella dysenteriae* Flexner.

The foregoing probably constitutes a very incomplete listing of either antibiotic or probiotic effects relevant to the indigenous microorganisms. The passing mention accorded by Evans *et al.* (1950) to inhibition of *Propionibacterium acnes* by anaerobic micrococci suggests the

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existence of other buried observations of this kind.

MATERIALS AND METHODS

Except as noted all cultures were typical morphologically, culturally, biochemically, and, where tested, serologically. Most of the cultures were obtained from the collection of the Department of Microbiology, Columbia University. The two strains of *Streptococcus pyogenes* in this group were both Group A; strain H Gay was Type 30. Of the two "viridans" strains, "L" was a typical bile insoluble greening form, presumably *S. mitis*; "S" was indifferent on blood and in its growth characteristics resembled an enterococcus. The strain of *Neisseria catarrhalis* in this group grew poorly in the absence of blood, serum, or ascitic fluid.

Escherichia coli, strain S, was obtained from the Department of Microbiology, School of Medicine, Washington University, *E. coli*, strain B, from Dr. A. D. Hershey, Carnegie Institute, Cold Spring Harbor, N. Y., and *Lactobacillus casei* from the Department of Bacteriology, St. Louis University.

The following were isolated in this laboratory either from saliva or from lesions of the mouth: *Micrococcus albus*, strain BTH (hemolytic, coagulase and mannite negative, resistant to 1,000 units penicillin in broth); *M. albus*, strain BC, and *M. aureus*, strain O'B; *Streptococcus mitis*, strains JRR, DG, and TR (from saliva); *S. salivarius*; *Candida albicans* and *C. albicans*, strain 67; *Neisseria catarrhalis*, strain EW (good growth on nutrient agar); and an oral lactobacillus (Hadley Type I).

S. mitis, strains MP10, CS15, and SK2, were isolated from separate guinea pig fusospirochetal passage exudates, derived originally from human gingival scrapings in the 10th, 15th, and 2nd passage, respectively. Strain MP10 has been described previously as S24 (Rosebury *et al.*, 1950).

Media. Broth cultures for use in the drop procedure given below were grown at 37 C for 16 to 18 hours, except for lactobacilli which were incubated 40 to 42 hours. The various strains of micrococci, *Sarcina lutea*, *Streptococcus faecalis*, *B. subtilis*, *E. coli*, and *C. albicans* were grown in extract broth (nutrient broth, Difco); lactobacilli in tomato juice broth, pH 6.1, containing 0.1 per cent agar (modified from Dewar and Parfitt, 1951); and all other strains in heart infusion broth (Difco), enriched with 5 per cent rabbit serum.

In preliminary tests of the cross-titration procedure detailed below, a wide range of media was employed, including extract (nutrient) agar, infusion agar (blood agar base, Difco), and infusion agar containing 5 per cent of ascitic fluid, horse or rabbit serum, defibrinated rabbit blood or two per cent of fresh potato juice (Thompson and Johnson, 1951). Addition of glucose was avoided except for the few tests conducted with lactobacilli for which 0.1 per cent glucose was added to infusion agar to permit adequate growth. The data given in table 3 were obtained by the use of infusion agar containing 5 per cent of either rabbit serum ("SI") or defibrinated rabbit blood ("BI"). In the tests with *E. coli* and *C. albicans*, media used other than those previously noted were extract or infusion agar with one per cent added glucose and 0.002 per cent bromcresol green, and, in addition, the chemically defined Fildes (1938) medium containing 1.5 per cent agar and the yeast extract-lactate medium of Douglas (1950), and modifications of both as noted below.

Plates were poured on the day preceding their use, incubated at 37 C overnight, and dried, open and inverted, at 37 C for two hours immediately before use. During the drying interval the indicated tenfold dilutions were prepared in 9 ml blanks of extract broth containing 0.2 per cent finely divided calcium oxalate. The rack containing the dilution tubes was kept in a 0 C water bath throughout the interval of preparation and use. Calcium oxalate, precipitated from potassium oxalate solution with calcium chloride, was used as an innocuous agent to disclose dried drops, otherwise often invisible, principally to facilitate placement of overlapping drops as mentioned below.

The cross-titration procedure. On each plate, 16 pairs of drops were placed as a cross-titration of four tenfold concentrations of each of two species. The drops were placed with a 27 gauge needle on a one ml tuberculin syringe. One syringe was used for each species by proceeding from the highest dilution downward and washing the syringe with each successive dilution before using it. For each species, pure spot cultures were prepared on all test media at appropriate dilutions for counting, to control independent growth on the media used, and as tests of purity.

The grid of 16 drop-pairs was arranged as follows: drops of the first ("effector") species were arranged in 4 columns increasing in concentration from left to right. After these drops had dried in air at room temperature, drops of the second ("test") species were arranged so that each overlapped the corresponding first drop, in 4 rows, increasing in concentration from bottom to top. The effector and test species thus were paired over a 1,000-fold range of concentration, usually from undiluted to 10^{-3}, but where indicated over a higher dilution range of one or both species. As shown in figure 1 and in figures 5–10, the most concentrated drops of both species appear in the upper right portion of the grid, the least concentrated drops of both in the lower left. Along each diagonal from lower left to upper right the ratio of the two concentrations is the same.

The present study, except for a few tests with lactobacilli, has not extended beyond microbic species that grow readily under aerobic conditions. Hence the plates have been incubated in air at 37 C for 16 to 18 hours before reading. With lactobacilli and in certain other instances as noted additional readings were made after further growth periods.

Reading the plates. The following notations, recorded on a grid as in figure 1, covered the range of phenomena thus far observed: × = stimulation of the test species by the effector species; ± = doubtful inhibition, suggested by fewer or smaller colonies (or both) of the test species within the area of overlap; + = definite inhibition of the test species indicated by distinct reduction of growth of the test species in the overlap; ++ = complete inhibition of the test species in the overlap without diffusion of inhibition; i.e., growth of the test species not appreciably impaired beyond the edge of the effector drop; +++ = complete inhibition as in the preceding, accompanied by diffusion of the effect, with growth of the test species only at the outer edge of its drop; ++++ = complete absence of growth of the test species. In the *E. coli-C. albicans* system incomplete inhibition with diffusion was seen, as noted below.

RESULTS

(1) *Survey of the findings in qualitative terms.* Table 1 summarizes all the findings obtained thus far in terms of definite (+ = + or more as defined above) or doubtful (±) inhibition, stimulation (×) or no observed effect (0). Distinct stimulation of growth has been observed under only two sets of circumstances: (a) of *N. catarrhalis* by most of the effector species, often accompanied on the same plate (but in different drops) by inhibition; and (b) of *E. coli* by *C. albicans*. Both phenomena were observed only on media on which the test species failed to grow alone or grew poorly. Stimulation of *N. catarrhalis* has not been studied further; the coli-albicans system is considered below.

The data on inhibitory phenomena in table 1 permit a provisional separation of three systems: (a) complete and diffusible inhibition on media not containing blood cells or potato juice, found to be antagonized partially or wholly on blood or potato agar—in which the effector species were streptococci or a pneumococcus, the test species micrococci, *C. diphtheriae* or *N. catarrhalis* (the "streptococcus-effector" system); (b) inhibition of *C. albicans* by *E. coli*; and (c) mutual inhibition of streptococci by other species of the same genus. The two latter systems differed from (a) in that inhibition apparently was not antagonized by blood agar, while the coli-albicans system differed from both (c) and (a) in that the inhibitory effect was characteristically less complete and usually nondiffusing, and in another respect to be noted. Systems (a) and (b) have been selected for particular study, principally with a view to development of the quantitative aspects of the method.

Table 1 also indicates that a strain of *Lactobacillus casei* was found to be (weakly) inhibitory to two strains of *C. diphtheriae*, that *Sarcina lutea* was inhibited (strongly) by *C. albicans*, and that doubtful or ambiguous inhibitory phenomena were observed with *E. coli* as effector and some of the test species of the streptococcus-effector system. These observations and most of the negative findings given are based on single plates and have not been studied further. The observed difference between *L. casei* and the oral lactobacillus may not be significant, but the difference between 4 strains of *S. mitis* obtained directly from human saliva (which were among the most active inhibitors studied) and 3 strains of the same species isolated from guinea pig fusospirochetal exudates, but originally derived from the human mouth (which were inactive in

TABLE 1

Inhibitory and stimulatory effects observed in studies of paired species of microorganisms by the drop-plate method

TEST CULTURES	NUMBER OF STRAINS	EFFECTOR CULTURES												
		Streptococcus mitis		*S. salivarius*	*S. faecalis*	*S. pyogenes*		*Diplococcus pneumoniae*, Type I	*Micrococcus albus*	*M. aureus*	Oral lactobacillus (Hadley Type I)	*Lactobacillus casei*	*Escherichia coli*	*Candida albicans*
		Oral or Stock	G. P. F-S*			H Gay	15A							
		4	3	1	2	1	1	1	2	1	1	1	3	1
Micrococcus albus	3	+†	0	+†	+†	0	+†	+†			0		±	
M. aureus	4	+	0		+†			+†					±	
Sarcina lutea	1	+			+									+
M. lysodeikticus	1	+			+									0
Corynebacterium diphtheriae, strain Gravis	1	+†	0		+†			+†	0	0	0	+		
C. diphtheriae, strain Mitis	1	+	0		+				0	0	0	+		
C. diphtheriae, strain PW8	1	+†	0	+†	+†		+†	+†					±	
Neisseria catarrhalis	2	+†×	×	+†	+†×		+†	+†	×				±×	×
Bacillus subtilis	1	0	0		0									
Escherichia coli	1				0									×
Candida albicans	2				0								+‡	
Streptococcus mitis, strain JRR	1				+‡		+‡							
S. pyogenes, strain 15A	1	+‡			+‡									
S. faecalis	1	+‡					+‡							

* Isolated from guinea pig fusospirochetal passage exudates.
† Inhibition antagonized by blood agar.
‡ Inhibition not antagonized by blood agar.
\+ Inhibition of growth of test species.
± Doubtful inhibition of growth of test species.
× Stimulation of growth of test species.
0 No observed effect of either species on the other.

these tests), seems worth pointing out although no explanation for it can be offered.

(2) *Analysis of the drop-plate data.* (a) *Characteristics of an individual plate.* Figure 1 presents an analysis of a representative plate in the streptococcus-effector system, the plate in question being illustrated in figure 15. Inhibition of the test species is recorded as ranging from ± to ++++. Assuming that the slope of inhibition at its limit (+ or more) may be expressed by a straight line, the line (broken in the figure) is drawn between two points, each placed midway between two limiting drop-pairs. Since the titration interval was tenfold, the true limiting slope is estimated to lie at a higher dilution by 0.5 interval, arbitrarily on the abscissa or effector-concentration scale, and the solid line is drawn accordingly. The slope in this instance = 4/3 = 1.33. The drop concentrations as determined from counts are given opposite the center of the upper right square, at which for this plate both cultures were used undiluted. Using a sliding logarithmic scale the intercept of test species concentration = 1.0×10^4 provides the value for effector organisms of 1.5×10^3, log = 3.2. The two values characterizing inhibition on this plate are thus S (slope) = 1.33; $C/10^4$ (inoculum concentration of effector species inhibiting 10^4 test organisms) = (log) 3.2. The 10^4 intercept was chosen as falling most fre-

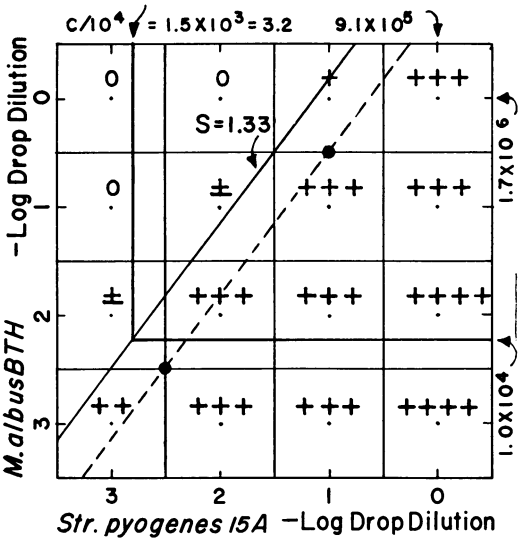


Figure 1. Analysis of a representative cross-titration plate. The plate is shown in figure 15. For explanation of the placement of slope lines and C/10^4 intercepts, see the text.

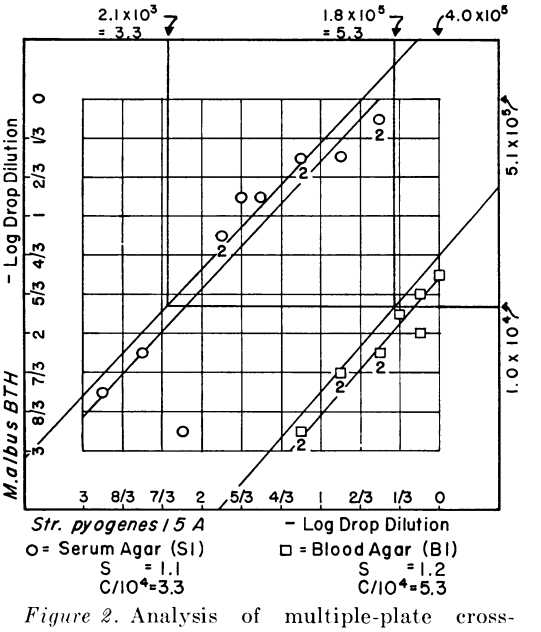


Figure 2. Analysis of multiple-plate cross-titration of one pair of species on two media. For explanation, see text.

quently within the grid; and the C/10^4 value was selected in preference to a reverse relationship (e.g., 10^4/C, yielding an inhibited concentration of test species) because since the slopes are found to be modally >1 as noted below, the intercept by the C/10^4 procedure is less likely to require extrapolation. These computations have been made with the aid of a slide calculator especially constructed for this purpose.

(b) *Validation of linearity of the slope.* To determine whether a straight line adequately expresses the slope at limiting inhibition in this (streptococcus-effector) system, two sets of experiments were conducted, one of which is represented in figure 2. The other gave comparable results. For this purpose the cultures were diluted at intervals of $\sqrt[3]{10}$ (2 ml added to blanks of 2.3 ml) over the customary 1,000-fold range of concentration of each. Using these dilutions, a 4-fold grid of drop-pairs was placed on each of 7 plates in duplicate for each of two pairs of cultures on each of two media (SI and BI), the grids overlapping at the 10^{-1} and 10^{-2} dilutions. Figure 2 shows the findings with one pair of cultures on the two media. Each point shown is again a midpoint between two limiting drop-pairs. The numeral 2 under a point indicates that the point was the same on the duplicate plates or in the overlaps. The shorter line for each set of points was fitted by the method of least squares. The longer lines, placed as previously 0.5 dilution interval to the left on the abscissa, were used as true slopes for determining the intercepts. The distribution of points in both experiments suggests that a straight line satisfactorily characterizes the slope, which is given in terms of Cartesian coordinates as computed from the least squares data. The values obtained in these experiments are considered below.

(c) *Data on slope in general.* A striking difference between the streptococcus-effector system and the coli-albicans system, in addition to those already noted, was found in the range of limiting slopes of inhibition. The observed slopes cover the positive range from ∞ to 0, with the intermediate steps for single plates of 4, 2, 1.33, 1, 0.75, and 0.25—7 classes grouped symmetrically around 1 as geometric mean. The 0 (horizontal) slope has been seen only twice in more than 300 plates on which inhibition appeared, and in line with the reasoning given below it is regarded as an artifact. In the coli-candida system, 54 of 62 plates on which inhibition appeared showed the vertical (∞) slope. Of the remainder, the pattern of 5 could be interpreted as deviating by 1 drop-pair from the vertical; 2 were irregular and 1 showed inhibition throughout the plate. By con-

trast there were only 3 vertical slopes among 256 plates in the streptococcus-effector system on which inhibition appeared. The remainder, with certain exceptions to be indicated, ranged from 4 to 0.25 through the 7 possible steps. This range would appear to indicate a relationship, dependent on relative concentration, between the inhibitory activity of effector organisms on the one hand and an appreciable opposing activity of the test organisms. Such a mechanism therefore would seem to be characteristic of the streptococcus-effector system. On the other hand, if S = ∞ be accepted as characteristic of the coli-candida system, it would follow that the test species in this instance opposed inappreciable resistance to inhibition over the tested range of concentrations, or conversely that inhibition in this system may be measured simply in terms of limiting effector species concentration. By the same reasoning the rare horizontal pattern (S = 0) would seem to be meaningless.

(d) *Distribution of slopes in the streptococcus-effector system.* The cross-titration procedure is intended as a roughly quantitative screening method. As such its value would be increased if it were possible to use not only those plates on which a given slope in the range S = 4 to 0.25 was fully represented (by 4 drop-pairs or points) but also as many other plates as possible on which by chance the number of limiting points was less than 4. Such plates might be used if a suitable basis could be found for assuming points outside the grid. Analysis of the distribution of slopes in the streptococcus-effector system suggests a basis for the required assumptions. It can be shown that the full (4 point) S = 1 can occur in only one position on the plate, whereas S = 1.33 and 0.75 can each occur in 3, S = 2 and 0.5 in 4, and S = 4 and 0.25 in 5 positions. Data on the frequency of 4 point slopes among 130 plates are analyzed from this viewpoint in table 2. It appears that S = 1, 1.33 and 2 occurred more than twice as often as would be expected on the basis of chance alone, while the other slopes occurred less often. Analysis of these data by the chi-square method indicates a probability considerably less than 0.0001 that the observed slopes were due to chance alone. The modal slope accordingly would be expected to lie between 1 and 1.33. In the absence of clear evidence to the contrary, and in the effort to minimize classification bias, S = 1 is provisionally accepted as the mode. The following procedure has been adopted therefore, subject to modification when additional data become available. As illustrated in figure 3, plates with 3 or 2 limiting points are augmented with 1 or 2 points assumed outside the grid in such a way as to make the slope approximate most closely to 1. Where the 3 or 2 limiting points are arranged diagonally, S is accepted as = 1; otherwise plates with 3 limiting points fall into S = 4, 1.33, 0.75, or 0.25; those with 2 points only into S = 1.33 or 0.75. Plates with patterns on which 1 or more points are aberrant, including both vertical and horizontal patterns, are discarded as irregular.

Data for 321 plates in the streptococcus-effector system are assembled on this basis in figure 4.

TABLE 2

Analysis of slopes of 130 plates in the streptococcus-effector system, all determined by 4 drop-pairs or points

SLOPE	NUMBER FOUND	PER CENT FOUND	PROBABILITY OF CHANCE OCCURRENCE	PER CENT EXPECTED BY CHANCE	RATIO FOUND/EXPECTED
4	7	5.4	5	20	0.27
2	45	34.6	4	16	2.16
1.33	38	29.2	3	12	2.43
1	12	9.2	1	4	2.30
0.75	7	5.4	3	12	0.45
0.50	11	8.5	4	16	0.53
0.25	10	7.7	5	20	0.39

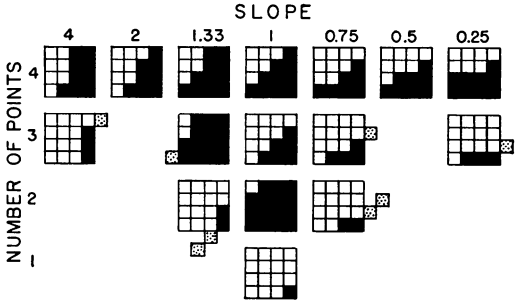


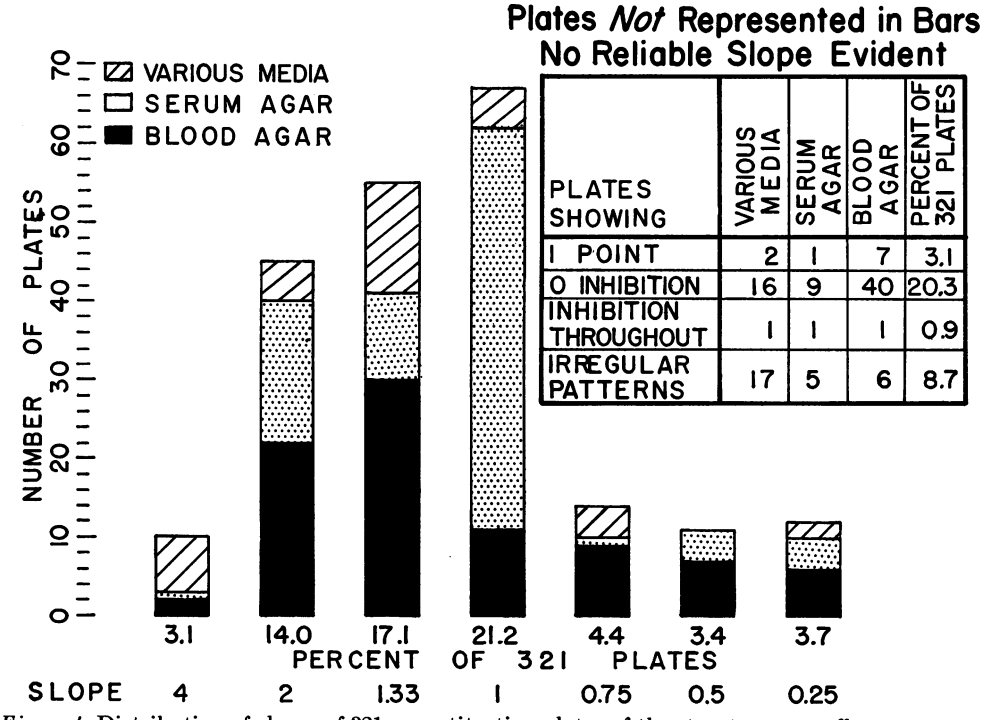
Figure 3. Representative patterns of inhibition on cross-titration plates classified in terms of limiting slope. Each black square represents a drop-pair (point) showing + inhibition or more. On the plates with 3 such points or less, the stippled squares represent inhibition assumed outside the grid for estimation of slope as explained in the text.

The bars are based on plates with 2, 3, or 4 limiting points. The total distribution has been weighted evidently toward S = 1 but otherwise conforms with the pattern of the data in table 2. An apparent difference in distribution of slopes for serum infusion and blood infusion-agar may be reserved for later consideration.

Plates showing only 1 limiting point (in the lower right hand square of the grid) or + or more inhibition in all 16 squares might be discarded, but the presence of plates in this series on which no inhibition appeared in any square suggested that all such plates be utilized for screening purposes. The 65 negative plates listed in the inset table in figure 4 were all prepared with pairs of species that showed inhibition on other plates; 40 of these were blood plates, and it seemed desirable that numerical estimates characterizing these plates be available for assessment of the inhibition-antagonizing activity of blood cells. Accordingly all such plates (1 point, no inhibition, and inhibition throughout) were assessed as S = 1, the slope used for estimation of $C/10^4$ values passing 0.5 interval to the left of the point when available or 0.5 interval to the left of an assumed slope of 1 just outside the plate on negative plates. The $C/10^4$ value for such negative plates is estimated as minimal.

In computing the geometric means of slopes, the arithmetic means of $C/10^4$ values, and the standard deviations of the latter, the values for a given plate have been weighted by the number of limiting points per plate from 1 to 4. The weighting value 1 has been used for negative plates. Where one or more negative plates occurred in a group the mean $C/10^4$ value is given as minimal. Such values appear to have comparative significance. Mean slopes that include such negative plate data, on the other hand, are obviously not comparable with those based on observed points.

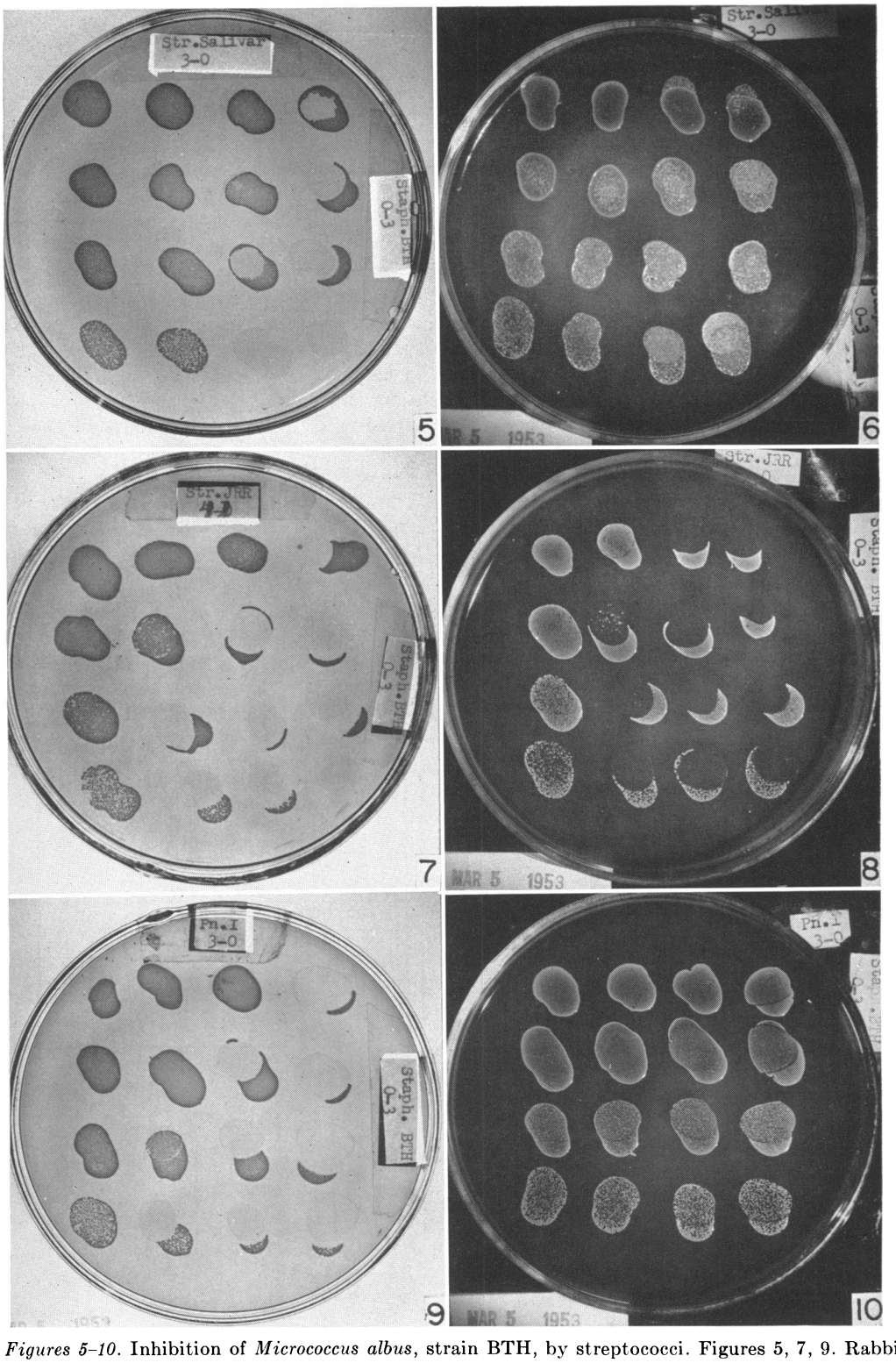
(3) *The streptococcus-effector system.* (a) *Individual cross-titration plates.* Representative cross-titration plates in this series are shown in figures 5–10 and 15. Figures 5, 7, 9, and 15 are serum infusion plates photographed by transmitted light and show different patterns of inhibition of the more opaque (darker) test



Plates *Not* Represented in Bars
No Reliable Slope Evident

PLATES SHOWING	VARIOUS MEDIA	SERUM AGAR	BLOOD AGAR	PERCENT OF 321 PLATES
1 POINT	2	1	7	3.1
0 INHIBITION	16	9	40	20.3
INHIBITION THROUGHOUT	1	1	1	0.9
IRREGULAR PATTERNS	17	5	6	8.7

Figure 4. Distribution of slopes of 321 cross-titration plates of the streptococcus-effector system. The bars include only plates that showed two or more limiting drop-pairs or points; the remaining plates are listed in the inset table.



Figures 5–10. Inhibition of *Micrococcus albus*, strain BTH, by streptococci. Figures 5, 7, 9. Rabbit serum infusion agar, photographed by transmitted light. Figures 6, 8, 10. Rabbit blood infusion agar, photographed by reflected light. Concentrations of the streptococci (effector species) increase in columns from left to right by 10-fold intervals; concentrations of *Micrococcus albus* (test species) increase in rows similarly from bottom to top.

species. Figures 6, 8, and 10 are blood plates photographed by reflected light. It is apparent that on blood agar more marked antagonism of inhibition occurred with *S. salivarius* and *D. pneumoniae* than with *S. mitis*. Inhibition of the test species by *S. mitis* on blood agar is clearly shown, but the inhibitory activity does not extend beyond the margin of the effector drop (i.e., does not exceed ++ as recorded). Such lack of diffusion of inhibition was characteristic of blood agar cultures.

Quantitative data on inhibition of 3 test species by 4 species of streptococci and a pneumococcus on the two media, obtained by the cross-titration method, are assembled in table 3. The species are arranged in order of effector potency and of test species sensitivity as measured by the $C/10^4$ values on serum infusion agar. All slope (S) values are weighted geometric means. Standard deviations are given for $C/10^4$ values only. It will be seen that in certain instances (e.g., with *S. faecalis*) these values are relatively high. Since the $C/10^4$ values are derived by means of slope, variation of the former in part reflects variation of slope. It has not seemed useful thus far to attempt to assess independent variation of the S values, nor at the present stage of this work to undertake the statistical evaluation of significance of differences. Only the broadest indications in table 3 may be suggested.

It is evident that the $C/10^4$ values are uniformly higher for blood agar than the corresponding values for serum agar, indicating antagonism of inhibition by blood cells. The degree of such antagonism, however, (aside from the diffusion effects noted above, which are not measured by the procedure used) shows marked and relatively consistent differences, evidently related to effector species more than to test species. With the strain of pneumococcus tested all blood agar plates were negative, and the $C/10^4$ values, given as minima, are all correspondingly high. With *S. mitis*, strain JRR, on the other hand, antagonism by blood cells was much less marked. The other effector species lie between these extremes.

S. mitis, strain JRR, appears in general as the most potent inhibitor in this group although with *C. diphtheriae*, strain PW 8, both *S. pyogenes*, strain 15A, and *S. salivarius* seemed slightly more actively inhibitory. The three test species show only slight differences in sensitivity among themselves. The average $C/10^4$ values for the test species on serum agar, it may be observed, are influenced and perhaps distorted by the relatively high and variable values for *S. faecalis* versus *C. diphtheriae* and *N. catarrhalis*.

Quantitative data for the various media used in assembling table 1 were generally consistent with those in table 3, with indications that another strain of *S. mitis* (strain DG) was a more active inhibitor than strain JRR, and with no clear indication of differences attributable to the medium, except that potato juice agar, which was tried only in a few instances, gave results similar to blood infusion agar.

Data for slope in table 3 are inconsistent and of uncertain significance. There is a suggestion that the slope tends to be greater on blood than on serum agar. This trend is suggested also in figure 4. In 3 of the 5 instances in table 3 in which this tendency is not shown, the blood S values are assumed from negative plates. It is noted below that for one of the remaining exceptions (*S. pyogenes*, strain 15A, versus *M. albus*, strain BTH) separate data showed the more usual greater slope for blood agar.

(b) *Comparative data from multiple plate titrations.* Data obtained in the two sets of experiments described in (1, b) above, based on 7-plate titrations at dilution intervals of $\sqrt[3]{10}$ are compared in table 4 with data from individual plates for the same species-pairs. In the 7-plate series the slopes were fitted by least squares and their values obtained from the least squares equations; for the individual plates they are geometric means, weighted in terms of number of

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Figures 5, 6. Streptococcus salivarius. Inhibition of *Micrococcus albus* discernible on the blood plate is not shown clearly in the photograph.

Figures 7, 8. Streptococcus mitis, strain JRR. Inhibition of *Micrococcus albus* shows clearly on both plates. Note diffusion of inhibition in figure 7, with complete absence of growth of *M. albus* (++++ inhibition) in the lower right drop-pair. In figure 8, no diffusion of inhibition appears (maximum, ++ inhibition).

Figures 9, 10. Diplococcus pneumoniae, Type I. The blood plate in this instance (figure 10) showed no inhibition of *Micrococcus albus*.

TABLE 3

Mean values for inhibition of growth of Micrococcus albus, Corynebacterium diphtheriae, and Neisseria catarrhalis by four species of streptococci and Diplococcus pneumoniae as determined on rabbit serum and rabbit blood-infusion agar

EFFECTOR CULTURES		TEST CULTURES									TOTAL PLATES	AVERAGES: EFFECTOR CULTURES	
		Micrococcus albus, strain BTH			*Corynebacterium diphtheriae*, strain PW8			*Neisseria catarrhalis*					
		P	S	C/10^4	P	S	C/10^4	P	S	C/10^4		S	C/10^4
Streptococcus mitis, strain JRR	SI	8	1.3	2.7 ± 0.5	6	1.0	3.0 ± 0.3	8	0.9	2.4 ± 0.7	22	1.1	2.7 ± 0.6
	BI	6	3.2	3.3 ± 0.2	3	1.3	3.7 ± 0.5	6	1.2	3.3 ± 1.5	15	2.1	3.4 ± 0.8
Streptococcus pyogenes, strain 15A	SI	11	1.3	3.5 ± 0.7	3	0.3	2.7 ± 1.0	8	1.1	3.3 ± 0.9	22	1.0	3.3 ± 0.9
	BI	5	0.6	6.3 ± 0.7	4	(1.0)	>8.3 ± 0.2	6	0.6	6.6 ± 1.0	15	(0.6)	>6.8 ± 1.0
Streptococcus salivarius	SI	4	2.0	3.8 ± 0.0	4	0.4	2.7 ± 0.8	8	1.0	4.0 ± 0.2	16	1.0	3.6 ± 0.7
	BI	4	3.3	4.6 ± 0.1	4	1.0	5.4 ± 0.5	3	1.1	4.7 ± 0.4	11	1.7	4.8 ± 0.5
Diplococcus pneumoniae, Type I	SI	6	1.7	4.3 ± 0.3	8	1.1	3.8 ± 0.9	7	1.0	4.4 ± 0.5	21	1.2	4.1 ± 0.7
	BI	5	(1.0)	>7.4 ± 0.0	7	(1.0)	>8.2 ± 0.2	3	(1.0)	>7.8 ± 0.0	15	—	>7.8 ± 0.4
Streptococcus faecalis	SI	7	1.3	2.9 ± 0.8	10	0.7	5.0 ± 0.3	8	0.9	5.4 ± 2.3	25	1.0	4.2 ± 2.2
	BI	9	1.5	4.3 ± 1.3	10	(1.0)	>8.2 ± 0.4	8	1.0	>8.1 ± 0.1	27	1.5	>5.8 ± 2.2
Total plates and averages: test cultures	SI	37	1.4	3.4 ± 0.8	31	0.7	3.5 ± 1.4	39	1.0	3.7 ± 1.4	190		
	BI	29	1.9	>4.5 ± 1.3	28	1.2	>6.3 ± 2.1	26	0.9	>5.7 ± 2.1			

P = number of plates; S = slope as estimated at limit of inhibition (geometric means); C/10^4 = log drop concentration of effector cultures at intercept of 10^4 test organisms; SI = rabbit serum infusion agar; BI = rabbit blood infusion agar; ± values are standard deviations. > = more than; these values include 1 or more plates on which no inhibition appeared. Where all the data for a pair of species are based on negative plates, the slope is given as (1.0); these data were not used in estimating group means.

TABLE 4

Comparison of slope and C/10⁴ data obtained as means of individual plates and by multiple-plate titration

		MEANS OF INDIVIDUAL PLATES			7-PLATE TITRATION		
		Number of points*	S (Geometric mean)	C/10⁴	Number of points	S (By least squares)	C/10⁴ (By least squares)
S. mitis, strain JRR/*M. albus*, strain BTH	SI	29	1.3	2.7	8	1.7	1.8
	BI	24	3.2	3.3	22	4.2	2.9
S. pyogenes, strain 15A/*M. albus*, strain BTH	SI	41	1.3	3.5	24	1.1	3.3
	BI	7	0.6	6.3	20	1.2	5.3

* Number of limiting drop-pairs.

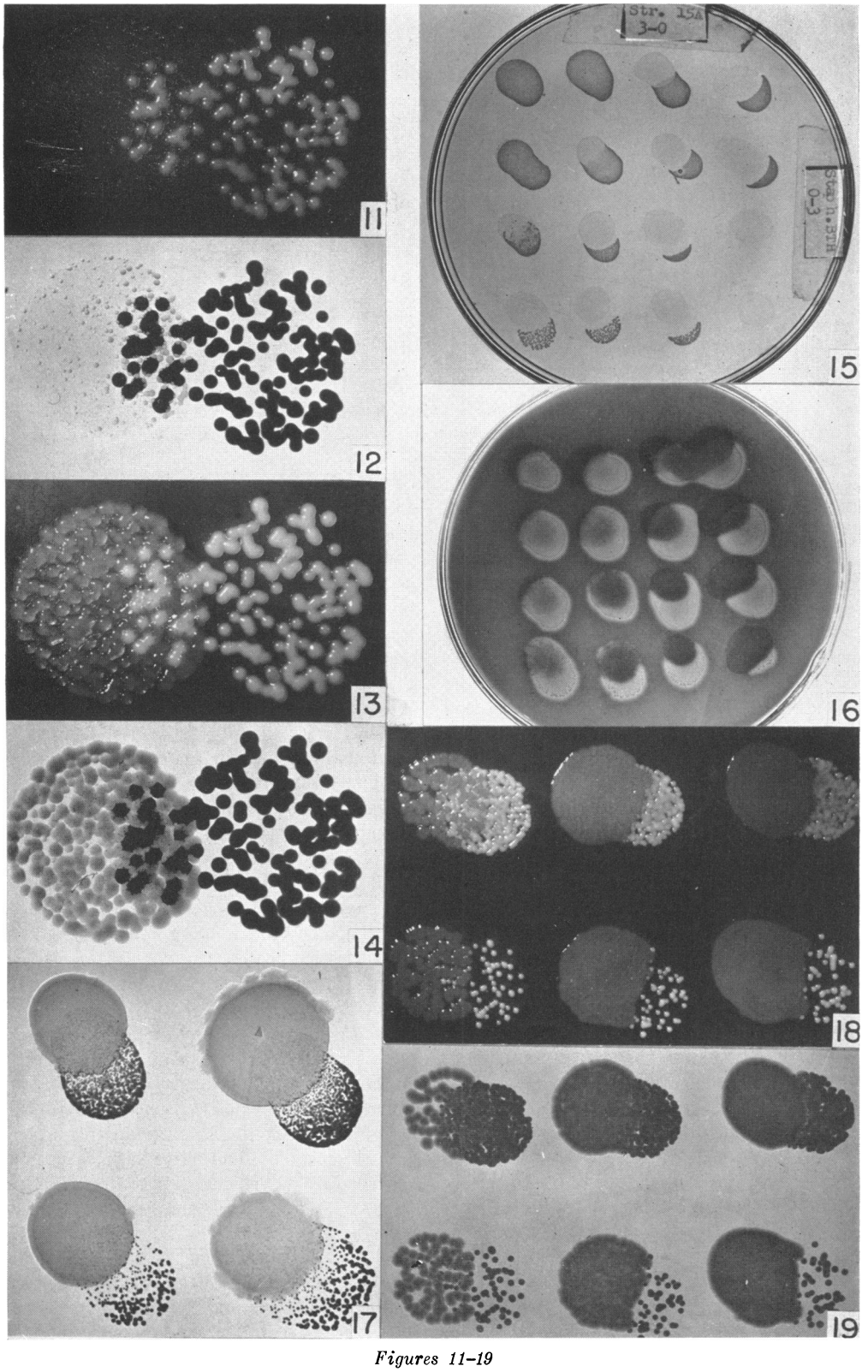
SI, rabbit serum infusion agar; BI, rabbit blood infusion agar.

The data in the left half of the table are given in table 3; those for the *Streptococcus pyogenes/Micrococcus albus* 7-plate titration are shown in figure 2.

points per plate. Of the individual plate data, with the exception of those for *S. pyogenes* vs *M. albus* on blood agar, the number of points per plate was not less than 3. That the S value in this exceptional instance is the only one in the table which is lower than the corresponding value for serum agar may depend on inadequate data. The low C/10⁴ value in the 7-plate series for *S. mitis* vs *M. albus* on serum agar also may have been influenced by the fact that the line fell close to the edge of the grid and had few points. With the exception noted, however, the least-squares data are seen to confirm the individual plate data with respect to (a) higher C/10⁴ values on blood agar; (b) a larger difference between C/10⁴ values for serum and blood agar with the hemolytic streptococcus; and (c) the tendency to a greater slope on blood agar.

(3) *The coli-candida system.* (a) *Stimulation of E. coli.* The chance finding that *E. coli*, on a medium unsuitable for separate growth, shows delayed but eventually luxuriant growth in the presence of *C. albicans* is illustrated in figures 11–14. This phenomenon occurred on Douglas lactate agar (yeast extract (Difco), 0.5 per cent; lactate, 1 per cent; agar (Difco), 1.5 per cent), made up with lactic acid (General Chemical Co., reagent grade), neutralized with either NaOH or NH_4OH. On this medium, in repeated trials, *E. coli* failed to grow alone during incubation periods up to 7 days. In the presence of *C. albicans*, however, while colonies of *E. coli* were generally absent after 16 to 18 hours at 37 C, growth was apparent 24 hours later with distinct satellitism of *C. albicans* (figures 11, 12) and was luxuriant after another 24 hours (figures 13, 14). Attempts to define the mechanism of stimulation were unsuccessful. Both species grew independently on Fildes agar (0.28 per cent sodium lactate plus salts and 1.5 per cent agar), on 0.5 per cent yeast extract agar, and on various modifications of both the Douglas and Fildes media intended to test each ingredient. When a second lot of lactic acid (Mallinckrodt reagent) was substituted for the first, *E. coli* was found to grow independently on the Douglas medium itself but now failed to grow at lactate concentrations of 3 per cent or higher. Further attempts to explain these findings have not been made.

(b) *Inhibition of C. albicans.* Inhibition of *C. albicans* by *E. coli*, as described above, occurred on all media tested, being found on 62 of 72 cross-titration plates. On the richer media the phenomenon appeared as in figures 18 and 19, with *C. albicans* colonies reduced in size or absent under *E. coli* colonies but with no diffusion of the inhibitory activity. Evidence of diffusion, accompanied, however, by incomplete inhibition (figure 17) appeared on the simpler media (Douglas, Fildes, and variants) and irregularly on extract agar. The limiting pattern of inhibition, as previously noted, was preponderantly vertical ($S = \infty$), suggesting dependence on a particular concentration of *E. coli* but independence of *C. albicans* concentration in the tested range. The plates therefore have been characterized by the inhibitory *E. coli* concentration, estimated as before at 0.5 dilution interval more dilute than the observed inhibition of + or more. Logs of



Figures 11–19

TABLE 5

Logs of inoculum concentration per drop of Escherichia coli (3 strains) estimated as inhibitory to Candida albicans (2 strains)

EFFECTOR CULTURES	TEST CULTURES	MEDIA				
		Yeast-lactate agar and variants	Fildes agar and variants	Extract, infusion or serum infusion agar	Blood or potato juice infusion agar	Extract or infusion agar plus 1% glucose
E. coli communior	*C. albicans*			(4) 2.5	(3) 2.2	(1) > 4.8
	C. albicans, strain 67			(1) 2.8	(1) 2.8	(1) > 4.8
E. coli, strain S	*C. albicans*	(24) 4.0	(15) 4.0	(6) > 3.8	(1) 2.8	(6) > 4.6
	C. albicans, strain 67			(1) 2.8	(1) 2.8	(1) 2.8
E. coli, strain B	*C. albicans*			(1) 4.5	(1) 3.0	(1) 4.5
	C. albicans, strain 67			(1) > 4.5	(1) 2.5	(1) 4.5
		(39) 4.0 ± 0.5		(14) >3.4 ± >0.8	(8) 2.6 ± 0.5	(11) >4.5 ± >0.7

Numerals in parentheses = numbers of plates tested.
Logs of *Candida albicans* inoculum concentrations per drop tested ranged from 0.9 to 5.0.
> = more than; these values include 1 or more plates on which no inhibition appeared.

average inhibitory concentrations of 3 strains of *E. coli* for 2 strains of *C. albicans* are given in table 5, arranged according to the groups of media used. The averages for each group of media, given with their standard deviations, indicate that inhibition is not antagonized by blood or potato juice but may be somewhat enhanced by them. The slight antagonism of inhibition on the glucose media hardly appears significant.

In view of the incomplete character of this inhibitory effect, particularly on the richer media, parallel tests of the phenomenon were made in broth mixture. Aliquots of 18 hour heart infusion broth cultures of *E. coli*, strain S, and *C. albicans* were inoculated separately and as a mixture in heart infusion broth, and viable counts were prepared of all three cultures after 6, 24, and 48 hours at 37 C. Bromcresol green glucose extract agar was used to distinguish *E. coli*, colonies of which selectively take up the dye. Infusion agar containing one per cent glucose and 0.33 mg per cent aureomycin (50

Figures 11–14. Stimulation of growth of *E. coli*, strain B (left), by *Candida albicans*. The same drop-pair is shown in the 4 figures, photographed in figures 11 and 13 by reflected light and in figures 12 and 14 by transmitted light. Douglas lactate agar after 48 hours' (figures 11, 12) and 72 hours' (figures 13, 14) incubation. *Escherichia coli* showed no growth independently on this medium during the same interval.

Figure 15. Cross-titration of *Streptococcus pyogenes*, strain 15A, and *Micrococcus albus*, strain BTH, on rabbit serum infusion agar. Analysis of this plate is presented in figure 1.

Figure 16. Cross-titration of *Streptococcus mitis*, strain JRR, and *S. pyogenes*, strain 15A, on rabbit blood agar, showing mutual inhibition of each species by the other, varying with relative concentration.

Figure 17. Four drop-pairs of *Escherichia coli*, strain S (left), and *Candida albicans* on Fildes agar containing 0.5 per cent yeast extract, showing partial inhibition of *C. albicans* with diffusion of inhibitory activity beyond the *E. coli* drop.

Figures 18, 19. Escherichia coli, strain S (left side of each drop-pair), and *Candida albicans*, strain 67, on bromcresol green infusion agar, photographed by reflected (figure 18) and transmitted (figure 19) light, showing from right to left, ++, +, and 0 inhibition of *C. albicans* without diffusion of inhibitory activity. (*E. coli* inoculum concentration decreases from right to left, *C. albicans* concentration decreases from above down.)

mg per plate = 15 ml, Hesseltine *et al.*, 1952), on which *E. coli* was completely inhibited, was used for estimation of *C. albicans*. The counts of *E. coli* were equivalent in the pure and mixed cultures, while those of *C. albicans* were 12 to 15 per cent lower in the mixture at all three tested intervals.

(4) *Streptococci versus streptococci.* Figure 16 illustrates what appears to be a phenomenon distinct from those described above: mutual inhibition of a *mitis* strain of streptococcus, strain JRR, and either *S. pyogenes*, strain 15A, or *S. faecalis*. Cross titration plates of the two pairs were prepared in the usual way on rabbit serum and rabbit blood-infusion agar in duplicate. The serum agar plates showed only slight and irregular inhibition by *S. mitis* of the other streptococcus; mutual inhibition appeared only on the blood plates. Figure 16 is representative of the 4 plates on which this phenomenon appeared. Here the greening streptococcus occupies the usual position of effector, the hemolytic streptococcus that of test species. In the upper left area, where the test species is most concentrated and the effector most dilute, inhibition of the greening streptococcus appears with little or no effect on the hemolytic form. In the lower right, where the ratio of concentrations is reversed, inhibition of the hemolytic streptococcus is evident. In the lower left area, where both species are at their lowest concentrations, mutual inhibition is shown by interruption of hemolysis and a moth-eaten appearance of the greening streptococcus. In the upper right area, where the concentration of both species is highest, there is no appreciable effect of either species on the other. These two strains of streptococci appear to be approximately equal both in activity and in sensitivity on the two plates. *S. faecalis* seemed slightly less sensitive and distinctly less active in relation to *S. mitis* than did the hemolytic streptococcus.

DISCUSSION

The principal purpose of this report is the presentation of a simple, sensitive, and roughly quantitative screening method for the disclosure and preliminary classification of interactive phenomena among microorganisms cultivable on agar surfaces, intended as an approach to the problem of microbic ecology as a phase of human hygiene. The usefulness of such a method must be measured ultimately by the data it reveals. The data thus far presented are of interest in themselves; but the emphasis at the present stage of this investigation has been on development of the method, the data being regarded essentially as examples both of its potentialities and of the complexities of analysis the method may entail.

The pattern of 16 overlapping pairs of drops as used, providing a cross-titration on one plate of two species each at 4 progressive tenfold dilutions, is intended to yield the broadest practicable range of inoculum concentrations. This procedure may be expected to offer relatively high sensitivity in disclosing interactive phenomena that depend on different absolute or relative concentrations of the two species. The variety of phenomena observed on a single plate on which two species of streptococci were paired (figure 16) is an indication of the sensitivity of the method. Since the inoculum concentration per drop can be estimated from separate drop counts, moreover, the findings can be expressed quantitatively. These considerations suggest distinct advantages of the method for its intended screening purpose over procedures involving mixtures in broth, cross streaking, or drops of one species placed on plates seeded uniformly with another.

Despite the technical simplicity of the method, however, the complexities intrinsic to the study of interactive phenomena among diverse microbic populations remain and are obviously not fully resolved. Studies by other methods of the mechanisms of the phenomena disclosed doubtless will be required for this purpose. It may be pointed out that only the inoculum concentrations can be estimated from the drop counts, the true interacting concentrations which result during growth within each drop being undetermined. With the exception of lactobacilli, which have been used only in a few pilot experiments, all species thus far tested grow within the 16 to 18 hour incubation period. Unequal growth rates, although they have not as yet been taken into account in this work, doubtless bear on the findings presented and may be expected to become increasingly significant when pairs of species having more widely divergent growth rates are tested.

In view both of the tenfold dilution interval employed and the use of inoculum concentrations rather than true reacting concentrations —doubt-

less augmented by uncontrolled variation of the reacting species and of their interactions—the quantitative data cannot be expected to have a high order of precision. They appear nevertheless to be valid as first approximations, both on theoretical grounds and in view of the findings that the inhibitor concentration values, which in the streptococcus-effector system were in turn based on estimated slopes, were reproducible within limits that seem satisfactory.

The slopes on the cross-titration grid, as estimated from the limiting patterns of inhibition on each plate and from the multiple-plate titrations, are interpreted as indicating whether or not the inhibitory activity of the effector species is appreciably opposed by activity of the test species. In the coli-candida system, in which a vertical limiting pattern was characteristically found, represented as slope = ∞, only the effector (*E. coli*) concentration seems significant in the reaction. In the streptococcus-effector system, on the other hand, the observed range of finite slopes is interpreted as indicating that both species play an active part in the interaction. It would seem that the balance of inhibitory activity and resistance would shift in favor of the former as the slope increases toward infinity, and *vice versa*. Evidence in support of this view, however, has not been found. Indeed the suggestion in the data that slopes in this system tend to be greater on blood agar than on serum agar, despite clear evidence that blood cells antagonize inhibitory activity, would seem to be in conflict with it. Blood cells, however, may affect the interaction not only by antagonizing inhibition but in other ways as well, e.g., perhaps by disproportionately stimulating growth or significant metabolic activity of the effector species. Such considerations again suggest the complexity of the phenomena under study.

Even though the deeper significance of the reported data be in question, however, the data themselves, both qualitative and quantitative, have made possible a separation of three interactive systems that appear to be mutually distinct, suggesting that separate mechanisms are involved. The coli-candida interaction and that found between two species of streptococci may justifiably be treated as separate systems in view of the biological relationships of the species concerned as well as the distinctive characteristics of the phenomena. The streptococcus-effector system, on the other hand, shows evidence of heterogeneity, in degree both of inhibitory activity and of the antagonistic effect of blood. Again it is curious that although such differences might have been expected to reflect the diversity of the test species, they seem more related to the inhibitors, all of which are biologically related.

Inhibitory phenomena evidently corresponding with those described in the present report under the streptococcus-effector system have been reported by previous workers as dependent upon hydrogen peroxide produced by the inhibitor, opposed by catalase in the test species or in red blood cells or potato juice. As early as 1915, Colebrook reported inhibition of growth of meningococci, *N. catarrhalis*, and certain other gram negative cocci by a pneumococcus and by streptococci. In 1922, McLeod and Gordon found that pneumococci inhibited the growth of staphylococci and certain other bacteria and attributed the effect to hydrogen peroxide. More recent workers have emphasized the role of greening streptococci (or generally of salivary streptococci) principally in inhibiting *C. diphtheriae*, also staphylococci (Mühlenbach, 1939; Thompson and Johnson, 1951; Annear, 1951). Hemolytic streptococci have been found inhibitory to *C. diphtheriae* by Besta and Kuhn (1934), Weigmann and Holzl (1940), and irregularly by Thompson and Shibuya (1946) and Sherwood *et al.* (1949). Thompson and Shibuya also noted that 1 of 17 strains of streptococci "with salivarius properties" and 2 of 4 "with enterococcus properties" inhibited *C. diphtheriae*. The present writers have seen no report of inhibition of staphylococci by *S. salivarius* or *S. faecalis*. Inhibition of *Neisseria* species by pneumococci and streptococci has been reported in the early literature (Colebrook, 1915; Gordon, 1916) and more recently by *S. lactis* (Mattick *et al.*, 1947), but in the latter instance a distinct mechanism appears to be involved.

Evidence that inhibition of *C. diphtheriae* and staphylococci by streptococci may be due to hydrogen peroxide has been presented by Bethge *et al.* (1947), Hegemann (1950), Thompson and Johnson (1951), and Berger (1952). This evidence consists principally in that inhibitory activity (a) was found to be proportional to capacity to produce hydrogen peroxide; (b) did not occur under anaerobic conditions; and

(c) was antagonized by catalase producing bacteria or other catalase containing materials, e.g., red blood cells and fresh potato juice. Su (1948) accepted antagonism of inhibition by blood agar as evidence that peroxide was the inhibitory agent. On the other hand, Annear (1951) interpreted his finding that lysed blood failed to influence inhibition of a staphylococcus or a diphtheroid by a viridans streptococcus as failing to support the hydrogen peroxide hypothesis.

The data presented herewith on the streptococcus-effector system may be compatible with the view that hydrogen peroxide is the inhibiting agent since in all instances blood cells antagonized the inhibition in some degree. The fact, however, that the test species used are all known to produce catalase (Porter, 1946; Molland, 1947) as well as differences in the degree of antagonism by blood cells and their apparent lack of simple relationship to activity of the effector species, suggests that other mechanisms, perhaps in addition to hydrogen peroxide-catalase, may be operative.

Whether the phenomenon observed in this work of mutual inhibition by different species of streptococci is also dependent on hydrogen peroxide production cannot be established from the available data, even though the effect occurred preferentially on blood agar, since some of the other streptococcal inhibitory effects described above may occur also on this medium. It is generally accepted that both pneumococci (McLeod and Gordon, 1922; Annear and Dorman, 1952) and hemolytic streptococci (Fuller and Maxted, 1939) may be autosterilized by this mechanism. On the other hand, Sherwood *et al.* (1949) and Murray and Pearce (1949) have described inhibitory effects against hemolytic streptococci and pneumococci due to hyaluronidase produced by other hemolytic streptococci and other bacteria; and both groups have also noted other antibiotic effects between strains of hemolytic streptococci which appear to depend on a distinct substance called by the former workers "streptostasin". Oxford (1944) reported inhibition of *Streptococcus lactis* and *S. cremoris* by a protein, "diplococcin", isolated from *S. cremoris*. Mattick *et al.* (1947) obtained in crystalline form a distinct substance, "nisin", from culture filtrates of *S. lactis* which inhibited *S. cremoris*, *S. lactis* itself, other streptococci including those of group A, pneumococci, and species of *Neisseria* and *Corynebacterium*. The activity was not affected in media containing blood. It is apparent that streptococci may produce a variety of antibiotic substances other than hydrogen peroxide.

The only report we have seen dealing with an inhibitory phenomenon that seems comparable with the coli-candida effect described above is that of Paine (1952) which appeared while these studies were in progress. Paine reported partial suppression of growth in yeast extract broth of a strain of *C. krusei* by *E. coli*. He also noted that on a synthetic, vitamin-free agar medium growth of *C. krusei* was more luxuriant in the neighborhood of growing intestinal bacteria—a relationship opposite to that described herein. He observed similar effects, of both inhibition and stimulation, with a strain of *Saccharomyces cerevisiae*.

The possible significance of inhibition of *Candida* by *E. coli*, in relation to overgrowth of the former after suppression of enteric bacteria by antibiotic therapy, has been discussed by Paine. As for the effects induced by streptococci, these appear to have been interpreted only in terms of resistance to exogenous infection, notably diphtheria, or of the possible production of therapeutically useful substances. In line with the orientation of the present study toward human hygiene, an additional possible phenomenon may be suggested, namely that the inhibitory activity of these streptococci, particularly of *S. mitis*, may help to account for the common predominance of this species in the healthy mouth and throat. It seems of interest to consider that this species may participate more frequently in a beneficial rather than a harmful role in human hygiene.

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SUMMARY

A simple, sensitive and roughly quantitative screening method had been described for the

study and initial classification of interactive phenomena among pairs of microorganisms cultivable on agar media. A cross-titration over a 1,000-fold range of concentration of each species is performed on each plate with 16 pairs of overlapping drops of inoculum. The method has been applied in a preliminary survey of aerobic microorganisms characteristic of human mucous membranes, as part of an ecological study in relation to human hygiene.

Stimulation of growth of *Neisseria catarrhalis* by several other species, and of *Escherichia coli* by *Candida albicans*, occurred in both instances on media inadequate for independent growth of the stimulated form.

Inhibition of growth was observed (a) by *Candida albicans* of *Sarcina lutea;* (b) by *Streptococcus mitis, Streptococcus faecalis, Streptococcus salivarius*, 1 of 2 strains of *Streptococcus pyogenes*, group A, *Diplococcus pneumoniae*, type I, and 1 of 2 strains of lactobacilli, of 1 or more of the following: several strains of micrococci, *Sarcina lutea, Corynebacterium diphtheriae*, and *Neisseria catarrhalis;* (c) by *Escherichia coli* of *C. albicans;* and (d) mutual inhibition, dependent on relative concentration, between *S. mitis* and either *S. pyogenes* or *S. faecalis.*

Data for selected species included under (b) above (the "streptococcus-effector system") and for the coli-candida interaction have been analyzed with a view principally to development of the quantitative aspects of the method. In this analysis, the limiting pattern of inhibition on each plate was defined as a straight line on a log-log grid. Evidence supporting such linearity has been presented. In the coli-candida system the slope of this line was characteristically $= \infty$, suggesting inhibition dependent only on *E. coli* concentration in the tested range. In the streptococcus effector system a range of finite slopes has been defined pointing to a relationship dependent on both species in the tested pair. The slope of each plate was used to obtain a value, at an arbitrarily fixed intercept, for limiting inoculum concentration of inhibitor species.

The findings have been compared with those of previous workers, and suggestions offered regarding their possible significance and the mechanism of some of the phenomena.

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