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AN APPROACH TO THE MENSURATION OF DISINFECTANT PENETRABILITY

By

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To my Mother and Father

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INTRODUCTION

> Logic is an intellectual treadmill which takes the mind round and round in the same course, perpetually restraining it from new avenues of adventure, and leading it nowhere. Robert C. Miller

In the evaluation of substances used for or recommended for disinfection processes, a large number of testing procedures have been developed in the past half century. A review of such developments is beyond the scope of this effort; suffice it to say that methods have been devised which are adequate for the determination of the efficacy of a compound in killing test organisms suspended in an aqueous medium or under special limited conditions. The use of such tests will immediately inform the investigator if a compound is without value as a disinfectant, inasmuch as it may be assumed that a compound failing to kill microorganisms under the rather idealized conditions of the Phenol Coefficient test (as an example) cannot be expected to be a satisfactory disinfectant under the more rigorous conditions obtaining in practical situations.

Compounds which have been shown to be of high germicidal activity by the Phenol Coefficient test may often be demonstrated to be ineffective or of considerably lessened activity when organic compounds are added to the test system (1, 2). This illustrates the fact that the value of a disinfecting substance cannot be predicted by one test alone. Salle and Catlin (3) recommend that a "profile" evaluation of a disinfectant be made employing an array of tests. This array is designed to demonstrate a germicide's performance in the absence of bacteriostasis, its speed of action, tissue toxicity, inactivation by organic matter and its penetrability.[1] This viewpoint is admirable, although it may be somewhat exaggerated by the inclusion of tissue toxicity tests (a protean task in execution!). The chief current trend in chemical disinfection is toward environmental sanitation (4) and the clinical use of disinfectants for wound treatment has largely been supplanted by use of the sulfa drugs and the antibiotics (4). It is, however, the last

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[1] "Penetrability" is used in the antithetical sense of the dictionary definition and, as such, is equivalent in meaning to "the penetrative power of a substance" or "the diffusibility of a substance." This definition is warranted by reason of usage and convenience.

criterion—that of penetrability—upon which I wish to place major emphasis.

Present practices in sanitation emphasize the importance of the rigorous cleaning of surfaces prior to any attempts at disinfection. This emphasis is fully justified and the following remarks pertain only to the provision of an adequate margin of safety in the disinfection of the residual debris on surfaces subjected to treatment with chemical disinfectants after adequate cleaning of the surface.

It should be obvious that unless a chemical disinfectant is capable of penetrating an organic or proteinaceous matrix to reach organisms embedded therein, it will be of doubtful value as an environmental or clinical disinfectant in situations where organisms are so protected. For those disinfectants whose mode of action in killing microbes is other than by purely surface activity phenomena, the cell wall and membrane of the organism to be killed acts as a barrier to the penetration of the disinfectant, and it is apparent that such compounds as do kill must have the power to penetrate these barriers to a greater or lesser extent in addition to possessing characteristics which cause them to be lethal once within the cytoplasm. Speed of

disinfection tests can give us information concerning the sum of two effects; the rate of disinfection will be a function of the extrinsic mobility of the disinfectant across the cell wall or membrane barrier and of the intrinsic rate of reaction of the disinfectant with such centers of activity within the organism as may be responsible for the ultimate killing action. Failure to give a high rate of kill may, of course, be attributable to the influence of either factor in the particular test system. It is certain that, in a speed of disinfection trial, if a compound can accomplish the destruction of life of the organisms in the reaction vessel in a very short time interval, then the time necessary for the compound to have penetrated the organisms in sufficient concentration to be lethal in subsequent reactions with the cytoplasm of the organisms must be somewhat less than the total time involved. This time for penetration of the compound to the adequate concentration cannot be greater than the total time interval involved in the test. If this time interval is accepted as the maximum value for penetration to have occurred within, then speed of disinfection tests can be considered to measure the over-all efficacy of a compound with the penetrability of the compound considered as one of the limitations of the rate of

kill. These tests, however, cannot divulge information about the exact rate of penetration and, thus, where penetrability alone is to be measured, other means of measuring penetration of colloid barriers must be resorted to.

At this point, the term penetrability must be defined and I choose to do so as follows. The penetrability of a chemical disinfectant is the ability of a disinfectant at some original concentration in the environment to enter a colloid barrier and result in a concentration sufficient to kill organisms at this site in some minimum time interval. This may be alternately taken to mean that penetrability is a function of the rate at which the disinfectant will traverse a linear distance through a colloidal barrier which may or may not have the capacity to enter into chemical or physical combination with the penetrating substance. This is clearly a statement of the rate of diffusion of a substance with the added qualification that the stationary phase of the system may enter into chemical or physical combination with the migrating substance. It then follows that when one considers penetrability, one must consider the nature of both the diffusing substance and the stationary phase, the concentration of the diffusing substance and of the elements of the

stationary phase susceptible of combination with this substance. The conditions of temperature, pressure, pH (due to the effect on ionic species of the components of both phases) and the presence of additional ions present in either phase will likewise affect the rate and extent of the penetration.

If adequate information concerning the diffusion coefficients of substances used as disinfectants versus the appropriate stationary phases likely to be encountered in application of disinfectants were available, it would be a matter of simple calculation to predict penetrability. Unfortunately, this condition does not exist for any combination of either practical or theoretical interest, as a brief examination of the International Critical Tables (5) will disclose. Therefore, empirical methods for the determination of penetrability must be employed.

Many empirical tests (to be described more completely in the succeeding section) have been devised for the ostensible determination of penetrability employing "nonreproducible" stationary phases. An example of one such technic is a modified agar cup procedure wherein the disinfectant is sealed in a sac made of a length of chicken intestine tied at the amputated ends and the whole embedded in a bacteria-seeded nutrient agar

plate, diffusion of the disinfectant through the tissue layers of the intestine resulting in a greater or lesser zone of inhibition of the bacterial growth in the surrounding medium. Such tests, employing tissues as barriers to the penetration of a disinfectant, fail in simplicity of execution and comparative value because of the essential nonreproducibility of the stationary phase. This type of test is inadequate in yet another respect, as the investigator's choice of primary stationary phases is distinctly limited and experimental variation of the concentration and condition of this phase is not flexible.

If, then, a tissue barrier be omitted and only a bacteria-seeded nutrient agar gel phase utilized, a serious objection still remains in that growth of the organisms used as biological indicators is proceeding in a logarithmic order while the diffusion of the disinfectant cannot exceed that of a first order reaction rate. Hence, the advancing germicidal concentration of the disinfectant encounters increasingly greater numbers of bacteria in varying physiological states. This factor is most evident when different organisms are tested against the same compound at identical concentrations. The differing diameters of the zones of inhibition obviously cannot be due to a varying diffusion rate

(a constant for identical conditions), superficially then this would appear to be caused by differing susceptibilities of the organisms to the compound. Actually the phenomenon may be related almost entirely to the different growth rates of the organisms and partly to different susceptibilities of the organisms to the disinfectant. This criticism should likewise extend to those attempts to determine the antibiotic spectra by the same rationale. A further limitation of this method is that the investigation is limited, by the requirement that growth of the indicator organism must take place in the suspending menstruum, to the physiological range of temperature, pH and salt concentration, and the elimination of bacteriostasis is quite difficult when not impossible.

The problem thus presented is to evolve some technic for measuring the rates of diffusion of disinfectant compounds in reproducible systems of the experimenter's design, where none of the above criticisms pertain. Further, it would be desirable to have available a second technic utilizing a completely different <u>modus operandi</u> whereby checking of results could be possible.

This has been the author's objective, but the degree to which he has been successful in attaining this end must rest upon the acceptance and extension of his labors by independent investigators.

HISTORICAL REVIEW

The Development of Methods for Measuring the Penetrative Powers of Disinfectants

The methodologies which have been developed for measuring the penetrative powers of disinfectants may be considered under four headings, organized as to the type of barrier primarily involved in the test. These classes are: (1) those in which the organisms to be killed are protected by a barrier in the form of adherent particles of organic matter; (2) those in which the barrier is a "natural" tissue (e.g., an epidermal tissue); (3) those in which the barrier is an artificial colloidal matrix such as an agarified nutrient solution; and (4) those in which the cell wall or membrane of a particular organism serves as the only barrier to entrance of the disinfectant.

Under the first heading, this methodology was introduced by Vincent (6) in 1897. This investigator studied the action of disinfectants in use at that time upon fecal material and demonstrated that chemical agents were largely ineffective in the disinfection of such matter. The enclosure of bacteria in the organic matter of the feces served as an effective protective

device against the action of the chemical agents employed. This same methodology was later employed by Mallmann and Chandler (7) in an investigation designed to find a disinfectant which could destroy all the bacteria present in feces while not adversely affecting coccidia. In this study, it was recognized that the finely divided particles of the avian feces served as a barrier to penetration of the disinfectant. As a resultant of this experimental procedure, it was found that Iodine Suspensoid (Merck) was the only disinfectant of the many that were tested which would rapidly sterilize the avian feces under the test conditions. A high degree of penetrability was ascribed to this preparation, about which more will be said below.

Johns (8) described a method for determining sanitizing efficiencies of compounds which employed an organism-seeded skim milk dried in a film on sterile glass slides. These prepared slides were dipped in the solution to be tested, rinsed, placed in sterile Petri dishes and covered with a nutrient agar. The resulting diminution in numbers of colonies appearing expressed the efficiency of the test compound in penetrating the milk solid particles and in killing the embedded organisms.

The second category of methods, where natural barriers have been used in demonstrating penetrability of disinfectants, mainly has been used for the study of compounds designed for use as antiseptics in presurgical preparations. Seelig and Gould (9) implanted capsules containing iodine or alcohol in living animal skin tissue to determine the osmotic behavior of these compounds in penetrating the living tissue. Nyiri and Jannitte (10) placed various iodine preparations on the sound skin of dogs and rabbits to demonstrate the penetration of the skin by iodine. Only iodide-containing preparations appeared to penetrate to any degree. Karns (11) and Karns, Cretcher and Beal (12) used guinea pig skin as the barrier to demonstrate that iodide-containing and aqueous preparations of iodine were superior in penetrability to tinctures and noniodide-containing preparations of iodine. Essentially the same results were obtained by Biskind (13) employing frog epidermis. Anderson and Mallmann (14) studied the penetration of living rabbit skin, using a modification of the tissue irritant determination technic devised by Etchells and Fabian (15), and the penetration of rabbit skin removed from sacrificed animals. The "dead" skin in the latter test was employed as a barrier between the test disinfectant

and a saline suspension of bacteria. The rate of bacterial death in the latter instance correlated with the results obtained in the tests employing the "living" skin and with the aforementioned results of Karns et al. on iodine preparations. In the same publication, Anderson and Mallmann examined the same series of disinfecting agents for their ability to penetrate excised adult chicken ceca. The washed ceca were filled with the compound to be tested, the open end tied off with thread and each cecum suspended in a broth culture of the test organism. The rapidity with which death of the organisms in the culture medium ensued indicated the rate of penetration through the tissue of the cecum by the disinfectant. Again, as in the previously cited publications, the iodide-containing iodine preparation was superior to other iodine preparations, and iodine in the concentration employed superior to all other disinfectants studied.

The third class of methodologies can be considered to be an inversion of the classical auxanographic technics developed by Beijerinck (16) in 1889 for the study of nutritional responses of microbes to organic compounds. In an auxanographic technic, as the name implies, the test writes its own answer—a putative inhibitor of bacterial growth placed on a bacteria-seeded

nutrient agar plate indicates its activity by the presence or absence of a zone of inhibition at the site of placement. In this manner, Kendall and Edwards (17) prepared agar cylinders seeded with bacteria. After contact with a solution of a disinfectant a core was removed and the extent of inhibition of growth in this core represented the penetration of the disinfectant. In 1918, Carnot and Dumont (18) ascertained diffusion of disinfectants in the horizontal plane by pouring seeded agar plates and placing the disinfectant in a perforated glass cup embedded in the center of the plate. The penetrability of the disinfectant was represented as the diameter of the subsequent zone of inhibition. This technic, with minor modification, was later recommended by Ruehle and Brewer (19) for determining the comparative penetrability of disinfectants and antiseptics. This test is generally referred to as the "FDA agar-cup procedure" and is probably the most widely used test for penetrative powers of disinfectants—in those rare instances when penetrability is considered in the evaluation of a disinfectant. This testing procedure has since been adapted for specialized applications. Anderson and Mallmann (14) demonstrated that volatile substances could not be assayed by the FDA procedure because of

distillation of the disinfectant from the center cup to the surface of the plate with consequent erroneous impressions of high activity. This objection was met by covering the agar surface with a layer of paraffin. To accomplish the measurement of the rates of diffusion of iodine preparations, the bacteria in the agar were replaced with starch-iodide solution and the extent of the blue zone was recorded at frequent intervals. Where organisms were seeded in the agar, and a series of disinfecting agents tested by this adapted technic, the rates of diffusion were not in themselves determinable, only the final extent of the zones of inhibition were recorded. This technic was further modified by Anderson and Mallmann, so that the disinfectant was enclosed in a sac made of a section of chicken intestine and the latter buried in the nutrient agar. The presence of a zone of inhibition about the section of intestine indicated penetration of the tissue, the authors disclaiming any importance ascribable to the size of the zone of inhibition. This method, which has been discussed at length in the Introduction, is actually more closely related to the second group of methodologies than to this third group, in which it has been included because of its developmental history.

The fourth category of methodological approaches to the problem of penetrability comprises those methods in which some particular—usually large—microorganism has been examined. In 1924, Chandler (20) reported that aqueous solutions of iodine were of high vermicidal activity. This author stated his belief that nematode eggs and encapsulate larvae were resistant to most forms of disinfectants because these disinfectants ". . . immediately coagulate the albuminous coatings of nematode eggs and larvae forming, thereby, an impervious membrane which prevents the further penetration of the chemical . . ." Chandler believed that iodine did not do this and thus was capable of penetrating the vitals of the organisms and causing their death.

In 1930, Knaysi (21, 23, 24, 25) and Knaysi and Gordon (22) published a series of papers dealing with the kinetic aspects of disinfection. Knaysi and Gordon subscribed to the view that the death rate of bacteria obtained in studies on chemical disinfection was a function of the distribution of variation in resistance among the bacterial population. This view was at variance with those propounded by Chick (26) and by Rahn (27) who held the rate of death to be an expression of the rate of

chemical combination in monomolecular and polymolecular reactions. Knaysi stressed penetrability as the limiting factor in the rate of death and stated: "If a poison is to kill . . . a cell, it must first penetrate the system of membranes and come into contact with the protoplasm." He claimed to demonstrate, using Saccharomyces cerevisiae, that individual cells varied in their penetrability to iodine and that there were apparent regions of the cell wall and membranes possessing higher permeability to iodine than other areas. Knaysi also demonstrated that iodine was taken up from solution by yeast cells in accordance with an adsorption isotherm.

Fish (28), in 1931, demonstrated the extreme resistance of oocysts of Eimeria tenella to physical and chemical agents. Anderson and Mallmann (14) attributed this high resistance of the coccidial oocysts to the presence of a heavy cell wall which acted as a barrier to penetration of chemical agents. These investigators therefore used suspensions of the cysts of E. tenella for determining the penetrative power of chemical disinfectants. The rates of penetration of disinfectants were measured by the percentage inhibition of sporulation of the oocysts following exposure to the disinfectants. They found an iodine

hydrosol to be the only substance effective in killing the oocysts and related this to a supposed high penetrative power of the preparation. Anderson and Mallmann (14) also selected strongylid larvae as organisms suited for observing the penetration of disinfectants. This organism is protected by a waxy cuticle and was shown to be resistant to the action of most disinfectants except the halogens—chlorine and iodine. Chlorine was observed to digest the outer covering of the larvae and subsequently movements of the larvae ceased; iodine caused a rapid cessation of movements of the larvae, with no prior digestion of the cuticle observed.

Anderson and Mallmann (14) also described a speed of disinfection test employing Micrococcus pyogenes var. aureus as the test organism. This test was a rate of disinfection determination in the absence of organic matter with sampling conducted in the shortest possible intervals. The test was reported to measure penetrability of disinfectants and gave similar results with the compounds tested as did the tests employing coccidial oocysts and strongylid larvae.

In 1947, Mallmann (29) upon addressing the National Association of Insecticide and Disinfectant Manufacturers, stressed

the importance of a disinfectant having the property of penetrance into organic barriers and emphasized the neglect of this character in the laboratory evaluation of prospective disinfectants. The difficulty of performing such evaluations was highlighted by his statement, "At present there are no good routine laboratory procedures (for determining penetrability) that yield satisfactory results." This author is in complete accord with Mallmann in that respect and would go so far as to state that in his opinion there do not appear to be any research procedures for the determination of the penetrative powers of disinfectants which have been established on a sound basis.

Colloidal Iodine

Amman (30), in 1910, observed that certain solutions of iodine exhibited a Tyndall effect and thus concluded that suspended iodine particles of fine size could exist. Bordier and Roy (31) obtained a fairly stable hydrosol of iodine in water by the dilution with water of a saturated alcoholic solution of iodine in the presence of gelatin as a stabilizer. In 1924, Chandler (20) reported the preparation of a "hyperactive" iodine preparation. Chandler and Miller (32) described the preparation

and physical properties of this "hyperactive" iodine and redesignated it as Colloidal Iodine. The preparation was made by acidification with hydrochloric acid of an ostensible sodium iodohypoiodite at 0 C in the presence of gum arabic as a protective stabilizing agent. Bennett (33) has described the details of preparation in full. This colloidal suspension of iodine has been marketed as Iodine Suspensoid (Merck) and as Iodine Vermicide (Merck).

The chief interests in the use of Colloidal Iodine as a disinfectant and as a therapeutic agent lie in that it contains neither iodides nor alcohols and still is available in concentrated form, in that it has been shown to be of low toxicity, in its value as a vermifuge, and in the fact that it is the only preparation to the author's knowledge for which the specific property of high penetrability has been claimed.

In 1924, Chandler (20) reported that "hyperactive" iodine in a castor oil vehicle was effective in the treatment in the field of ascarid and heterakid infections of poultry, ascarid and hookworm infections of foxes and ascarid infections of swine. The Merck preparations were used in subsequent studies. Chandler and Ferguson (34) demonstrated one hundred per cent efficiency

for the preparations in the treatment of tapeworms and roundworms of poultry. Alderman (35) confirmed these findings. Chandler (36) reported killing the stomach worms in 97 of 98 lambs subjected to treatment with Colloidal Iodine. Weisner (37) demonstrated that Colloidal Iodine was of value in the control of coccidiosis in poultry and rabbits, and Chandler (38) demonstrated the ability of this preparation to kill sporulated and unsporulated oocysts of Eimeria tenella and recommended its use as a disinfectant in poultry practice. Wilson (39) reported the destruction of Habronema spp. in the stomach and upper intestine of the horse, and the removal of large larvae of a Gasterophilus sp., although small larvae were not affected by the iodine therapy.

Stafseth (40) determined that Iodine Vermicide (Merck) was capable in vitro of killing ascarids and the proglottids and onchospheres of tapeworms. He demonstrated, however, that the scolices of the tapeworms infecting the chicken were neither dislodged nor killed by the treatment with Colloidal Iodine. Subsequent to the removal of the strobilae of the tapeworms by the Iodine Vermicide dosage, Stafseth demonstrated that the development of the parasites continued. Two to three weeks were

then necessary for the development of mature Raillietina cesticillus in chickens. It is apparent then, that the high efficiency of iodine preparations in apparently curing tapeworm infections in poultry, may have been only a concomitance of the technic of administering a check dose a few days after the initial therapeutic dose and adjudging the infection terminated if no ova nor proglottids were found in the feces. As Stafseth pointed out, Colloidal Iodine can still be recommended as a vermifuge where the objective is to break the cycle of development of the parasite for a limited time. At the present, however, Colloidal Iodine is not reported as the drug of choice for any of the worm or protozoan infections of animals or man.

As mentioned above, Mallmann and Chandler (7) reported the effectiveness of Colloidal Iodine in disinfecting chicken feces, accomplishing sterilization (sic) of the feces in ten minutes while no other disinfectant tested could accomplish this feat in more extended periods of time. Anderson (41) determined the Phenol Coefficient of Colloidal Iodine to be identical to that of Lugol's Solution and to Tincture of Iodine. Anderson and Mallmann (14) and Mallmann (29) ascribed the higher activity of the Colloidal Iodine to greater penetrative power of this

preparation. Anderson and Mallmann (14) compared the penetrability of the three iodine preparations by means of the paraffin-covered agar cup plate method and found that Colloidal Iodine in five per cent concentration gave a lesser zone of inhibition than did the Lugol's Solution or the Tincture of Iodine in like concentrations. Similar results were obtained using the technics employing chicken intestines or ceca as barriers. The order of effectiveness in these cases was: (1) Lugol's Solution, (2) Tincture of Iodine, and (3) Colloidal Iodine. Colloidal Iodine was most effective in the speed of disinfection test, in the inhibition of sporulation of coccidial oocysts and in the destruction of strongylid larvae. It was apparent that exceedingly disparate results were obtained by these two groups of methodologies. Anderson and Mallmann believed that the settling of the colloid particles in the first group of experiments cited may have prevented establishment of a uniform maximum concentation of iodine in the solution, and that the solvated preparations actually may have had a greater "diffusibility" than the colloidal preparation. This "diffusibility" they believed to be separate and distinct from penetrability—an argument this author believes untenable. It must be noted that in their experiments

the hydrogen ion concentrations of the preparations were not adjusted to like values nor recorded, inasmuch as these authors were studying the properties of the preparations as generally obtained and the pH of each preparation was of no intrinsic interest to them. Gershenfeld and Witlin (42) have demonstrated the acute dependence of the bactericidal efficiency of iodine upon the pH at which tests are conducted. The difference in activities of the three iodine preparations as determined by the speed of disinfection test, the inhibition of sporulation of coccidial oocysts and the killing of strongylid larvae may be a reflection of differing pH values in each preparation. That this is possible will be demonstrated below.

Colloidal Iodine has been demonstrated to be only one-fourth as toxic as Tincture of Iodine to rabbits when administered orally (43). Intravenous administration of either preparation resulted in death of rabbits with the same minimum dose. Chandler and Miller (32) also noted that concentrations of Colloidal Iodine sufficient for germicidal purposes did not corrode the skin and other tissues to the extent that alcoholic solutions did, nor did it stain the skin as intensely as did the latter.

Colloidal Iodine would thus seem to be more desirable than tinctures for wound dressing and for internal medication. The indications that it may possess a high degree of penetrability make it a good subject for studies on this subject and so a large portion of the experimental work to be reported below is therefore concerned with the examination of the penetrative power of this preparation.

MATERIALS AND METHODS

The Inhibition of Sporulation of Coccidial Oocysts

Eimeria tenella was propagated in six to eight week old cockerels. On the tenth day of infection (when oocyst production is at a maximum) the chicks were sacrificed and the ceca collected. Ceca showing visible lesions were homogenized in the Waring Blendor and the oocysts separated from tissue fragments by straining through four layers of clean cheesecloth. The oocysts were concentrated by flotation in a concentrated sugar solution followed by differential centrifugation in a refrigerated centrifuge. The final suspensions were diluted with distilled water so as to contain approximately 200,000 oocysts per ml and stored in the refrigerator at 4 C for future use.

Five-ml portions of iodine preparations at the desired concentrations and pH value were brought to 27 C in a water bath. The solutions were in graduated centrifuge tubes stoppered with rubber stoppers. After temperature equilibration, a one-ml portion of the oocyst suspension was added to each tube of disinfectant and immediately shaken by hand to insure

distribution of the tube contents. After incubation for the desired exposure period, four ml of a saturated sodium thiosulfate solution was rapidly pipetted into the centrifuge tube to neutralize the iodine. Control suspensions of the oocysts were treated in the same manner, with distilled water replacing the disinfectant. Following neutralization, the suspensions were centrifuged and the supernatant liquid decanted, the ten-ml volume was restored with distilled water and recentrifuged. The supernatant was again discarded to leave a volume of one ml of sediment and fluid in each tube. One ml of a 2.5 per cent solution of potassium dichromate was added and the sediment was suspended by trituration with a wooden applicator. The tubes were incubated in an inclined position for 48 hours at room temperature to permit sporulation of the oocysts. The suspensions were then examined under the microscope using 430X magnification, duplicate counts of each tube were made. The numbers of sporulated oocysts in a total of 100 were counted. The results were expressed as the percentage of inhibition of sporulation on the basis of the sporulation of the untreated controls.

The "Penetube" Technic

All glassware used in this portion of the studies was cleaned by soaking for at least 24 hours in acid-dichromate cleaning solution. The acid was removed by five rinses in tap water, a 24-hour soak in distilled water and four subsequent rinsings in distilled water. The glassware was then drained and air-dried.

All chemicals used were c.p. grade unless otherwise specified.

All reagents and solutions were prepared with distilled water which had been redistilled from an alkaline permanganate solution in a glass still.

Cultures of bacteria were checked for purity at frequent intervals by repeated streaking on a tryptone glucose yeast extract agar (TGE) and by direct microscopic examination of smears stained by the Gram technic (Hucker modification). The cultures were maintained on TGE slants in screw-capped vials.

Pyrex glass tubing of 5-mm outside diameter was cut in 12-cm lengths and one end ground flat on a fine emery wheel. After cleaning and drying, the unpolished end was

plugged with cotton. The tubes were distributed in clean test tubes and sterilized in the dry-air oven at 170 C for 2 hours.

The colloid system to be examined contained agar (Difco granulated) in a final concentration of 1.5 per cent. It was necessary to prepare the agar gel in a concentration such that, upon dilution of 16.2 ml with adjunct substances to 20.0 ml, the final concentration would be as stated above. Thus, 16.2 ml of this agar gel was distributed per 50-ml Ehrlenmeyer flask and autoclaved at 121 C for 20 minutes. The gauze plug of each flask was wrapped with "parafilm" and these flasks could then be stored in the refrigerator at 4 C for periods up to one month in length with no discernible loss of moisture. Prior to use, the agar gel was melted and cooled to 45 C and 2.0 ml of molar phosphate buffer solution added so that the final concentration of the buffer was tenth molar at the desired pH value. Either 0.8 ml of a protein solution, distilled water or a combination of these to the same volume was then added. Each flask was seeded with 1.0 ml of a suspension of bacteria in distilled water. This suspension was prepared by washing the bacteria from the surface of a 24-hour TGE agar slope culture with distilled water. The turbidity of the bacterial suspension was

adjusted to 15 per cent transmittance at 490 mu in a Cenco-Sheard-Sanford Photelometer. The seeded agar gel, now at a volume of 20.0 ml, was agitated to distribute the contents and was maintained at 45 C during subsequent manipulations.

To prepare columns of the agar gel within the tubes it was necessary to devise a method for setting the gel rapidly so that large numbers of tubes could be prepared without an agonizing wait between the filling of each succeeding tube. This was done by dipping a sterile tube into the molten agar gel and drawing a column of the liquid into the tube to a height of 5 to 7 cm. With the forefinger covering the plugged end of the tube, the tube was rotated over the surface of an ice cube for about four seconds so that the agar gel rapidly solidified in the upper end of the tube. The tube could then be laid in a Petri dish half to complete gelation while other tubes were being prepared. It was found convenient to insure stability of the ice cubes by placing them on another filled tray of ice cubes so that the tendency to slither about was inhibited by the partitions of the tray. If a meniscus depression appeared at the lower end of a tube due to drainage, the tube was touched to the surface of the molten agar at an acute angle and this depression filled.

Tubes bearing agar gel columns of differing constitution were color coded with wax-crayon marks at the end having the cotton plug. In almost all cases, this method resulted in the preparation of a uniform column of a bacteria-seeded agar gel with a bottom nearly perfectly flat whose cross-sectional area was 7.06 $mm^2 \pm 0.003\ mm^2$.

To clean, dry, 20-mm test tubes, 5.0 ml of disinfectant, buffer solution, salts or other adjuvant substances were added and brought to the desired temperature in a water bath. The final buffer concentration was half molar and the buffer solutions were made of appropriate mixtures of mono- and dipotassium phosphates.

The prepared tubes containing the agar gel columns were wiped dry with clean cheesecloth squares and placed in the test tubes containing the disinfecting solutions and these latter tubes stoppered with No. 2 rubber stoppers. Each test tube could accommodate as many as 7 tubes. There were always at least two tubes per test tube to insure against a tight seat between the bottom of the test tube and the circumference of the tube containing the agar gel column.

When volatile disinfectants which might be absorbed by the rubber stoppers (e.g., halogens) were tested, 25-ml glass-stoppered amber mixing cylinders replaced the test tubes and rubber stoppers.

At the end of the exposure period, the tubes were removed from the disinfectant by means of a forceps and the excess disinfectant removed by rinsing the tubes in sterile distilled water or in a solution of a suitable neutralizing agent. The column of agar gel was expressed from the tube into a test tube of an appropriate sterile nutrient broth containing a neutralizing agent to permit the development of viable organisms in the column. The extrusion of the column was accomplished by blowing at the plugged end with the mouth or by employing the cotton plug and an applicator as a piston and plunger. The broth tubes were then incubated at 37 C for 18 to 24 hours in a forced-draft incubator. The contents of each broth tube were then poured into a Petri dish and examined by illumination from beneath the plate. The clear zones of inhibition were measured with the aid of a steel divider and metric scale to the nearest tenth millimeter. The appearance of some typical zones of inhibition are demonstrated in Plate 1.

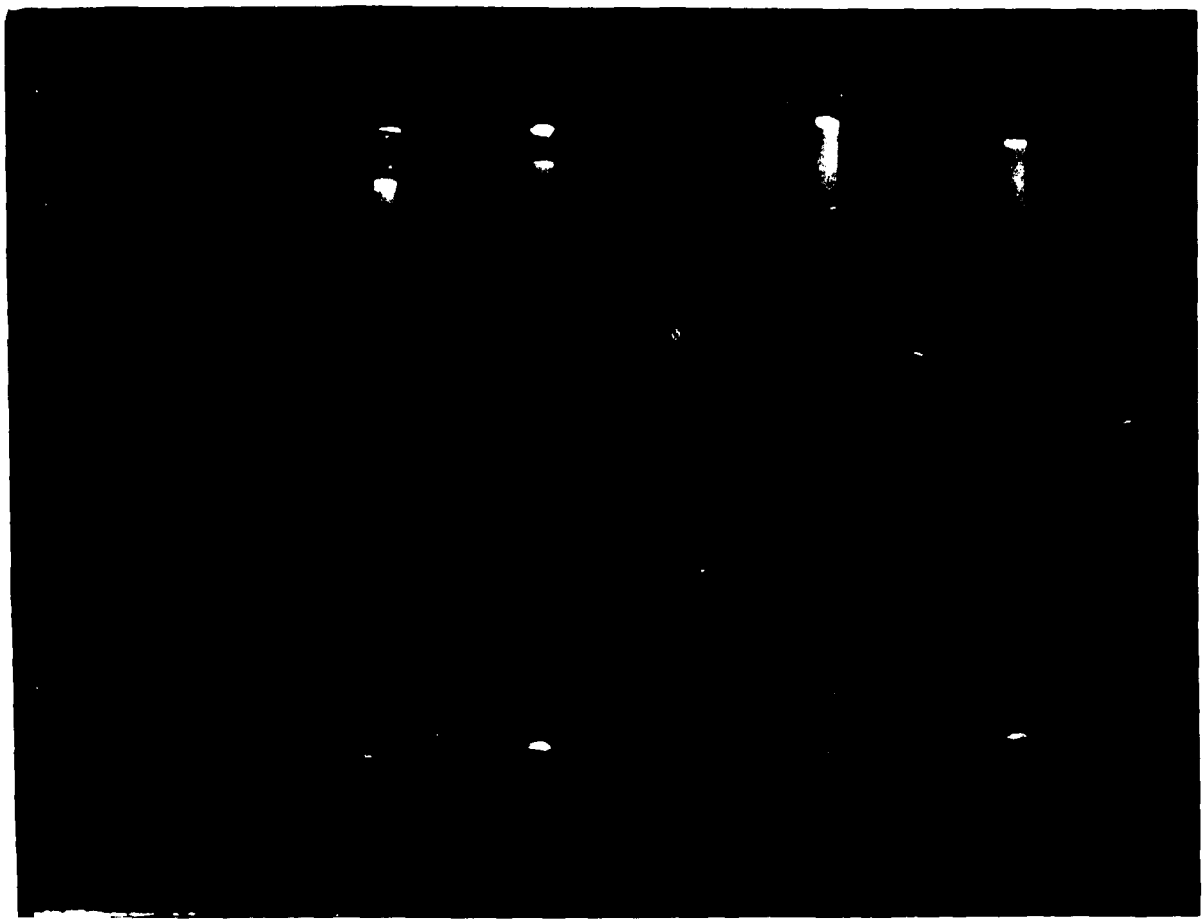


PLATE 1. The appearance of some typical zones of inhibition in agar gel columns following development.

A. Columns exposed to Colloidal Iodine for 24 hours at pH 5.0 and 27 C.

B. Columns exposed to phenol for 24 hours at pH 5.0 and 27 C.

The Disinfection of Chicken Feces

Chicken feces was collected from at least six cages containing normal adult birds of both sexes on a grain diet. Twice the weight of distilled water was added to the feces and the mixture homogenized for one minute in the Waring Blendor. The resulting suspension was strained through a double layer of cheesecloth and collected in screw-capped vials. With the aid of the microscope, the suspensions were seen to contain many particles of matter of varying sizes, some of which were as large as 20 micra in diameter, as well as numerous bacteria freely suspended in the fluid. Some crystals, probably of precipitated urates, were encountered.

To clean sterile screw-capped vials, 9.5 ml of the disinfectant plus phosphate buffer mixture were added. The final concentration of the buffer was half molar at the desired pH value. The tubes were placed in a water bath and brought to temperature equilibrium at 27.5 C ± 0.5 C.

The feces suspension was thoroughly agitated with a pipette and 0.5 ml added to each disinfectant-buffer tube and to a distilled water-buffer control tube. The contents were mixed by inversion of the tubes a number of times during the

course of the exposures. After the exposure interval, 1.0-ml samples were withdrawn and pipetted into 9.0 ml of an appropriate neutralizing agent to stop the action of the disinfectant. This tenfold dilution was then shaken for five minutes on a mechanical device at a rate of 200 strokes per minute through a one-inch excursion. Appropriate dilutions were then plated in duplicate with TGE agar. After incubation for 48 hours at 37 C, the plates were examined and the numbers of colonies recorded. Results were expressed as the numbers of organisms per ml of the reaction mixtures.

Special Vessels—Three Compartment Technics

Attempts were made to construct diffusion cells where a disinfectant solution could be separated from a bacterial suspension by a barrier solution. Collodion sacs were intussuscepted over the ends of glass tubing and suspended in a larger vessel containing the suspension of bacteria. The innermost compartment contained the disinfectant, the mid-compartment the barrier substance in the form of a solution or gel. Alternately, the position of the bacterial suspension and the disinfectant was reversed in the system. No results were obtained

with these cells as the membranes were exceedingly fragile and had a great tendency to break during trial runs.

Cells were constructed of thin rings of unglazed porcelain. A small ring was cemented to a cover glass with de Khotinsky cement and a larger ring then cemented in a concentric position. The center well was filled with the disinfectant and the annulus with the barrier substance. A cover glass was sealed to the top surfaces of the rings and the whole arrangement dropped in a suspension of bacteria in distilled water. This suspension was stirred by means of a magnetic stirring device and samples withdrawn at intervals for the enumeration of the viable bacteria. The fragility of the system and the difficulty involved in obtaining a perfect seal between the inner ring and the top cover glass obviated continued use of this technic and so the method was also abandoned and no results are recorded for experimental use of the method.

EXPERIMENTAL AND RESULTS

Experiments Using Oocysts of E. Tenella

The effect of hydrogen ion concentration on the disinfection of oocysts of E. tenella by iodine was determined. The pH of Iodine Suspensoid (Merck) in 5.0 per cent concentration was determined to be 2.0. A 5.0 per cent Lugol's Solution was adjusted to this pH with concentrated HCl. Other portions of each preparation were adjusted to pH 7.0 with 2.5 N NaOH. Exposure of the oocysts to aliquots of these preparations were made, as described above, for periods of 5, 10 and 20 minutes. The per cent of inhibition of sporulation of the oocysts as computed from control sporulations is given in Table 1. These data show the marked effect of hydrogen ion concentration on the kill of the coccidial oocysts. At the same pH values, there is very little difference in activity between the two iodine preparations. The colloidal preparation has a slightly higher activity but nowhere of the order of difference as determined by Anderson and Mallmann (14). This discrepancy is easily understood on the basis of the high acidity of the colloidal preparation as compared

TABLE 1

THE PENETRATION OF COCCIDIAL OOCYSTS BY 5 PER CENT IODINE PREPARATIONS AT 27.0 C

5% Iodine as	pH	Per Cent Inhibition of Sporulation*		
		5 min	10 min	20 min
Suspensoid (Merck)	2.0	76	96	100
Suspensoid (Merck)	7.0	43	89	99
Lugol's Solution	2.0	69	91	100
Lugol's Solution	7.0	41	79	97

* The iodine was neutralized with sodium thiosulfate immediately after the noted exposure period and sporulation of the oocysts accomplished in 2.5% potassium dichromate for 48 hours at "room" temperature.

to that of Lugol's Solution. Preparations of the latter usually are in the range of pH 5 to pH 6.

The preparation of suspensions of oocysts of E. tenella was quite tedious and the resulting suspensions gave quite variable sporulation percentages. These ranged as low as 6 per cent in some instances. No further experiments were conducted with this method.

Experiments with the "Penetube" Technic

Indicator organisms. Penetubes were prepared containing agar and M/10 phosphate buffer at pH 5.0. One series of penetubes contained M. pyogenes var. aureus (FDA 209) as the indicator organism and another set contained E. coli (laboratory strain) as the indicator organism. These penetubes were exposed for 24 hours at 27.0 C in tubes containing M/2 phosphate buffer at pH 5.0 and Roccal or phenol in varied concentrations. After exposure the tubes were rinsed and the columns developed. The columns which had been exposed to phenol were developed in FDA broth and those which had been exposed to Roccal were developed in FDA broth containing 0.1 per cent Tamol-N. After development, the zones of inhibition were

measured and the average value for each point plotted in Figure 1.

In Figure 1A, it can be seen that Roccal apparently penetrates further where M. aureus is the indicator than where E. coli has been used. This is more apparent than real. The explanation of this is that M. aureus is more sensitive to the disinfecting action of Roccal than is E. coli. In Figure 1B, it is evident that on exposure to phenol, M. aureus is only slightly more sensitive than is E. coli. These results are in accordance with the general expectation that M. aureus will display a greater sensitivity to the action of most disinfectants than will E. coli.

The shapes of the curves for either disinfectant are the same whether M. aureus or E. coli serve as indicators. This indicates that penetration is proceeding in identical fashion in each set of penetubes, independent of the indicator organism. The difference in sensitivities of the organisms to the disinfectants result in a different endpoint being reached in each case. These endpoints reflect the extent of the concentration gradient of the disinfectant in the range where each organism is killed. M. aureus appears to be more suitable for use as an indicator because of its low resistance.

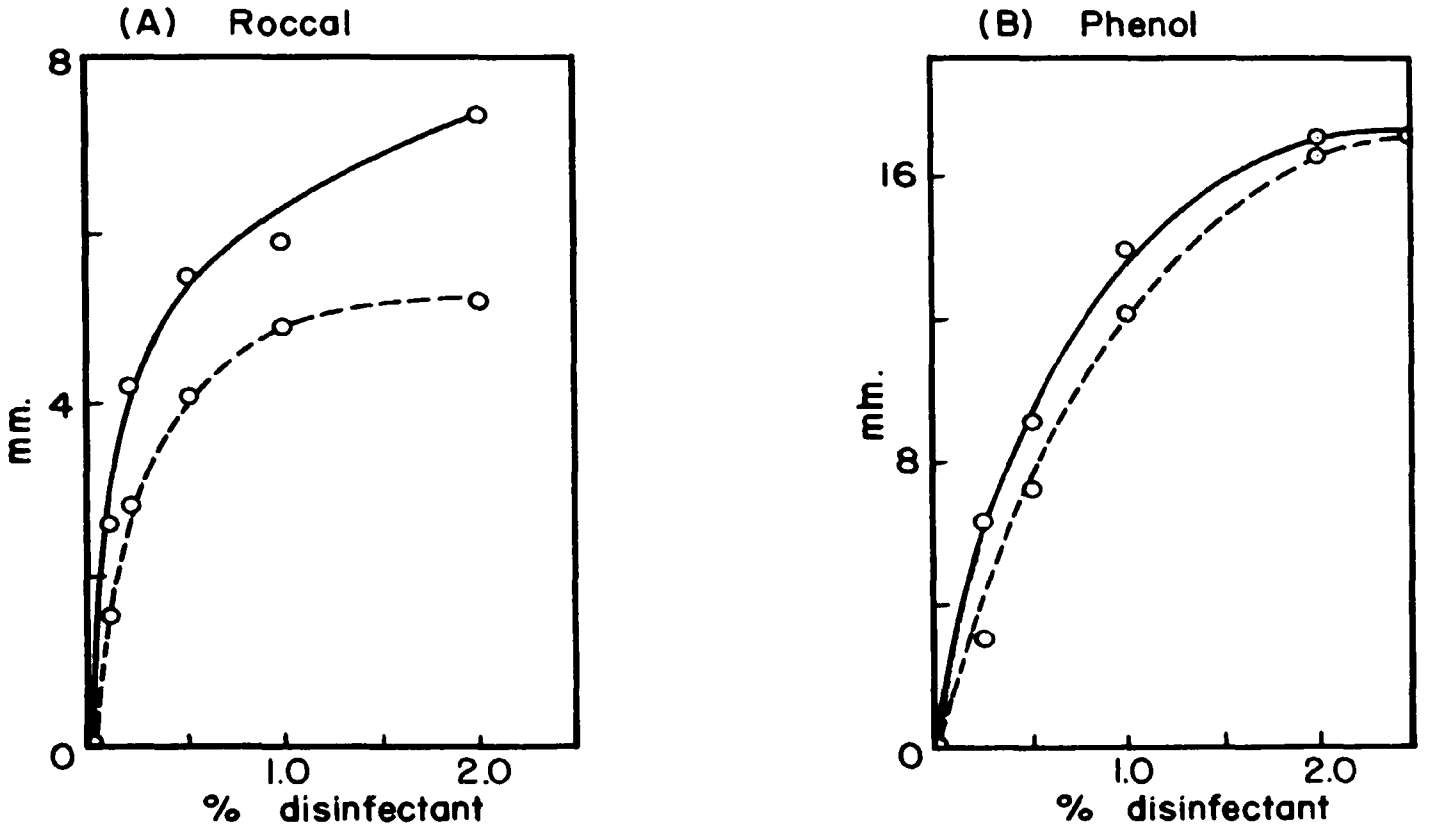


Figure 1-A,B. The penetration, at pH5.0 and 27°C., of Roccal and Phenol in increasing concentration with M. pyogenes (var. aureus)(——) or E. coli (----) as indicators in agar.

In additional experiments Mycobacterium pflei and Bacillus subtilis were used as indicator organisms. Because of the strict aerobic nature of both organisms, development was accomplished in Petri dishes containing the broth rather than in plugged test tubes. This treatment was necessary to provide adequate aeration and was found to be extremely inconvenient as a routine procedure. Under these conditions, columns containing B. subtilis gave identical zones of inhibition as columns containing M. aureus following exposure to iodine solutions. M. pflei did not give columns demonstrating sharp demarcations of the zones of inhibition, probably because of the failure to obtain uniform suspensions of this organism. No further experiments were conducted with these organisms.

All subsequent experiments reported below were made with M. aureus as the indicator organism.

The effect of pH variation. Penetubes were prepared containing agar, agar plus 1.0 per cent gelatine and agar plus 1.0 per cent human serum albumin. Each set also contained M/10 phosphate buffer at the pH value to which the tubes were to be exposed. The indicator organism was M. aureus.

Series of exposure tubes containing the disinfectants at the indicated final concentrations in M/2 phosphate buffer were prepared at pH values covering the range from pH 5 to pH 9. Duplicate penetubes were placed in each exposure tube and incubated at 27.0 C for 24 hours. After exposure the tubes were removed and rinsed and then the agar columns developed in the appropriate neutralizing broths. The lengths of the zones of inhibition were recorded and averaged representative data graphed in Figures 2 and 3.

Figure 2A illustrates the rapid loss of penetrative power of iodine with increasing pH. The depressing effect of the protein additives is well illustrated in the lower lines of the graph. Not illustrated is the extent of volatilization of the iodine at the different pH values. This volatility of iodine results in zones of inhibition being evident at the upper ends of the columns (see Plate 1A). At pH 5.0, the length of this zone in agar alone is 12.5 mm, decreasing rapidly to zero mm at pH 9.0. It is evident then, that if it were possible to prevent escape of iodine molecules from the solution to the gas phase, the extent of penetration at lower pH values would be accordingly increased.

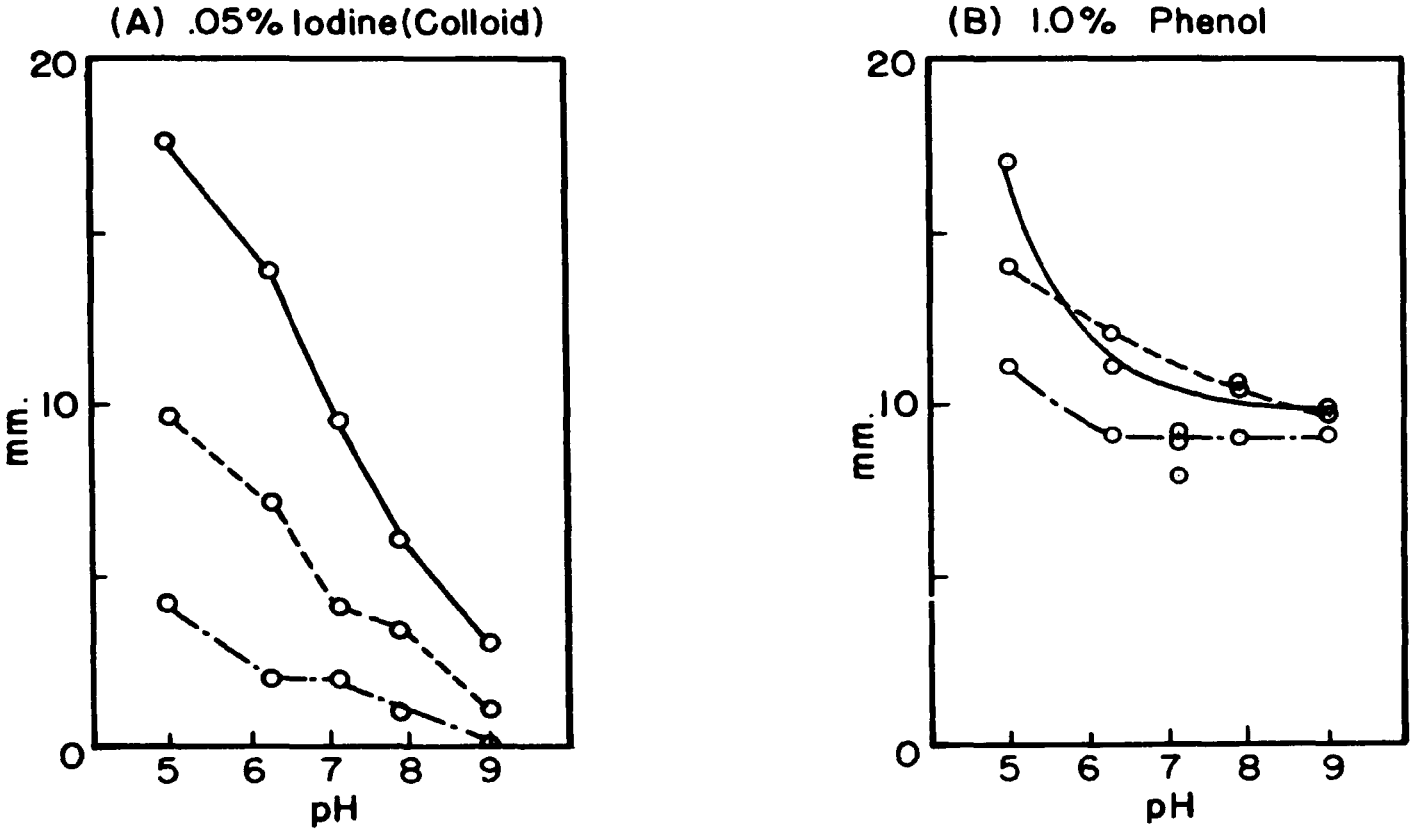
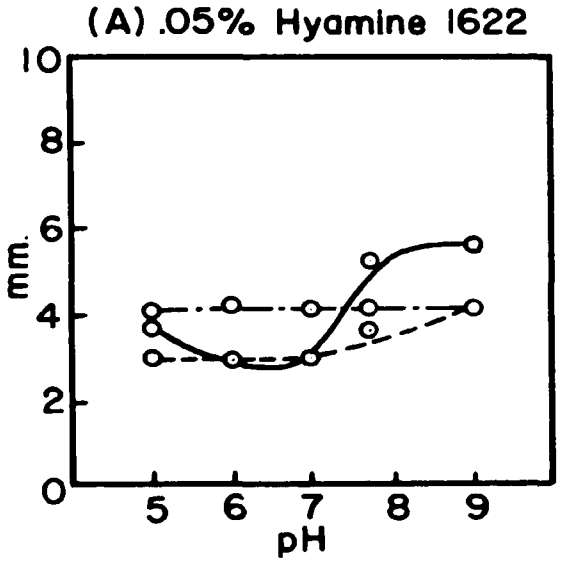


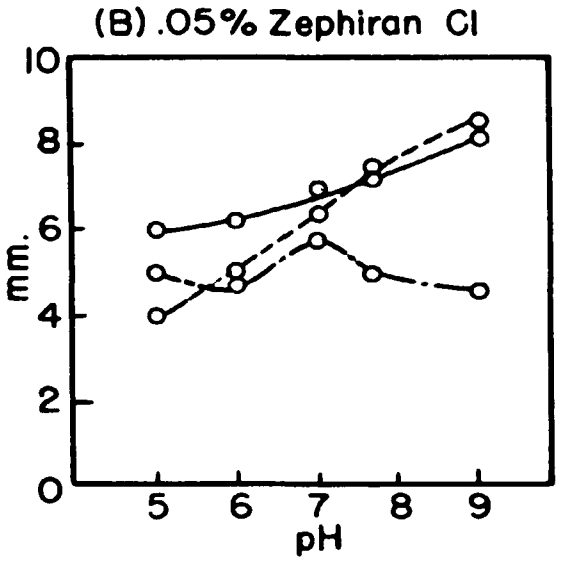
Figure 2-A,B. The penetration of Iodine and Phenol with respect to pH into agar(——), agar+ 1.0% gelatine(- - -), and agar + 1.0% human serum albumen(—·—).

Figure 2B represents the extent of penetration of 1.0 per cent phenol into the three gel phases. The greater extent of diffusion in agar alone at lower pH values (below pH 6.4) is evident. The diminishing penetration with increasing pH in the protein-containing gels is partly a reflection of this lower diffusion rate of phenol at higher pH values and partly to the influence of the hydrogen ion concentration on the reactivity of the protein with phenol. Comparison of these figures with those for iodine show that phenol penetration is not as adversely affected by increasing pH as is iodine, nor is the presence of protein as inhibitory as with iodine.

In Figure 3, the influence of pH on the penetration of the quaternary ammonium compounds Hyamine 1622, Zephiran Chloride and Roccal is graphed. In all cases the penetration of these compounds is greater with increasing pH. The dependence of penetration upon pH does not appear to be as great as in the cases of phenol and of iodine, nor is the extent of inhibition by protein as great.

The effect of temperature variation. Penetubes containing agar and agar plus 1.0 per cent human serum albumin were prepared with M. aureus as the indicator organism. The columns





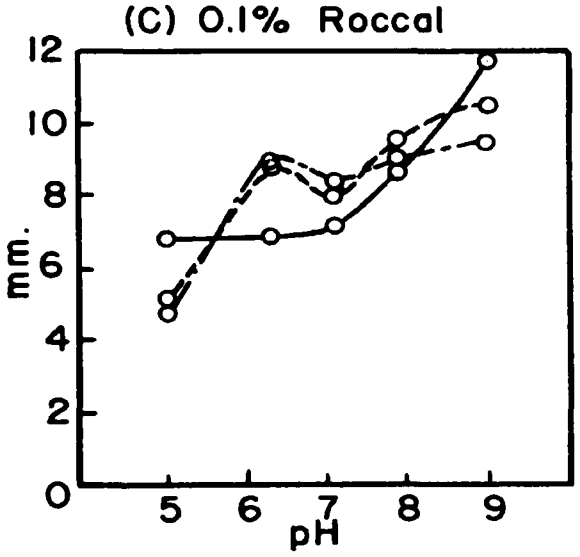


Figure 3-A,B,C. The penetration of quats with respect to pH into agar (—), agar+1.0% gelatine (---), and agar+1.0% human serum albumen(—·—).

were buffered at pH 5.0 and pH 9.0 in the usual manner and exposures were made to disinfectants at the same pH values in M/2 phosphate buffers. Exposure was for 24 hours in water baths at temperatures from 2.5 C to 37.0 C. Development and neutralization of the columns were accomplished as previously described for each disinfectant.

Figure 4 represents the extent of penetration of each disinfectant at pH 5.0. Figure 4A illustrates the linear increase in the penetration of 0.75 per cent phenol with increasing temperature. Roccal at 0.1 per cent concentration (Figure 4B) shows a similar linear increase of penetration with increasing temperature.

In Figure 4C and D the linear responses of Succinchlorimide containing 0.025 per cent available chlorine and of 0.05 per cent Iodine Suspensoid (Merck) are plotted. The zones of inhibition due to the volatility of the halogens are indicated as the "upper zones." A linear increase in the penetration in these latter zones is also noted.

The lesser slopes of the lines representing the penetration of each disinfectant in the presence of the albumin indicate that with increasing temperature the reaction of the

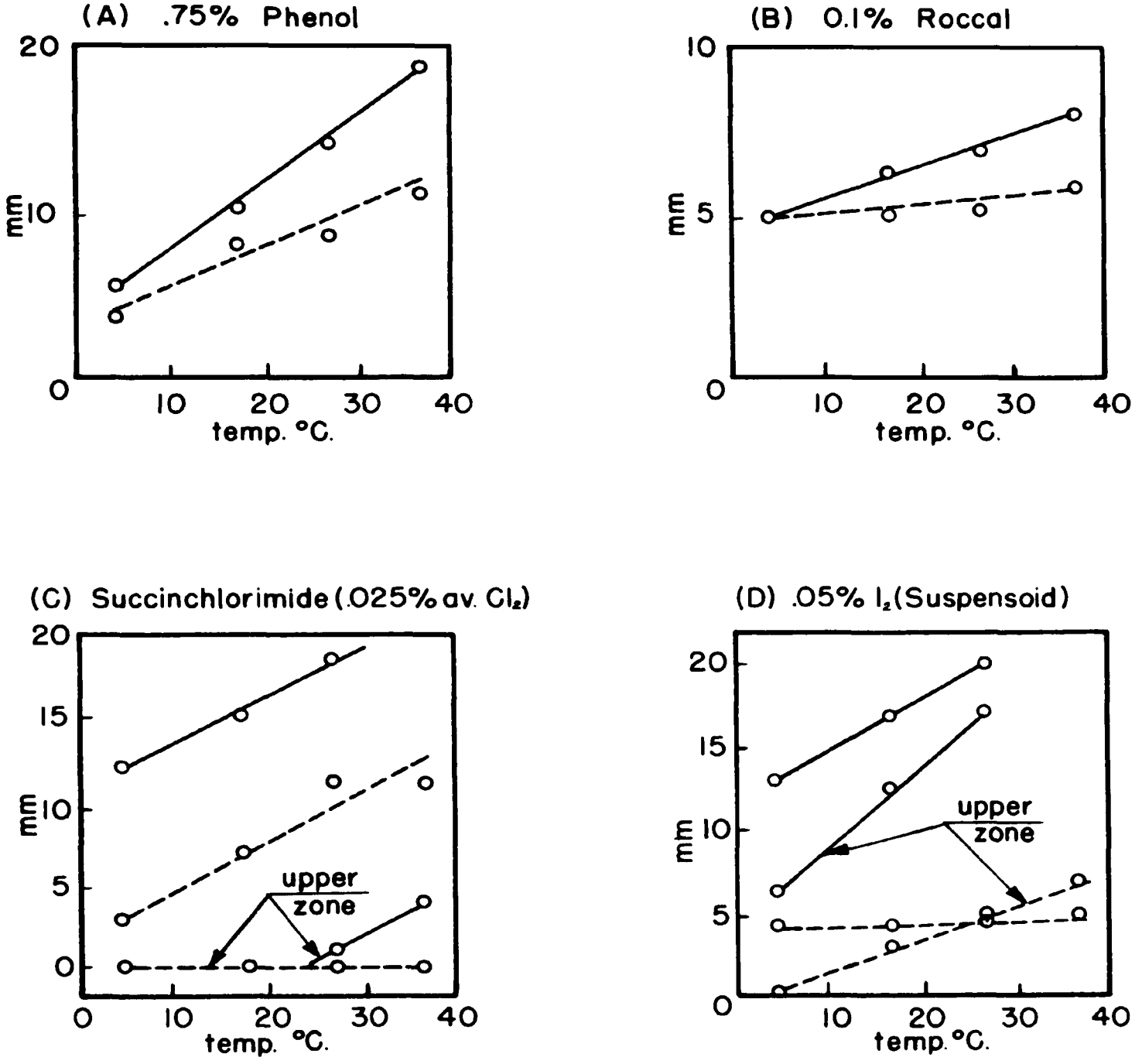


Figure 4 - A,B,C,D. The effect of temperature on the diffusion of various disinfectants at pH5.0 into agar(—) and agar+1.0% human serum albumen.(---).

disinfectant with the protein is increased, otherwise the slopes of the lines would be identical to those representing the diffusion in agar alone, although in such circumstances the position of the lines would still be at a lower position in the graphs. Where the lines representing the penetration in agar and in protein are almost parallel, as with Succinchlorimide, a high avidity of the disinfectant for the protein is indicated. The total reaction of the disinfectant with the protein must be nearly complete at the lower temperature and thus increases in temperature do not have a marked influence on the reaction rate.

At pH 9.0 the nature of the response of penetration of the disinfectants to temperature variation differs from the linear responses at the lower pH. As seen in Figure 5, none of the disinfectants tested gives a linear increase in penetration with respect to increasing temperature. The curves for phenol, Roccal, Zephiran Chloride and Hyamine 1622 are logarithmic. These same data yield straight lines when the logarithms of the penetrations are plotted against the temperature. All these resulting logarithmic plots have the same slope. No satisfactory explanation for this behavior has been propounded by the author. Iodine Suspensoid under these same conditions gives curves as

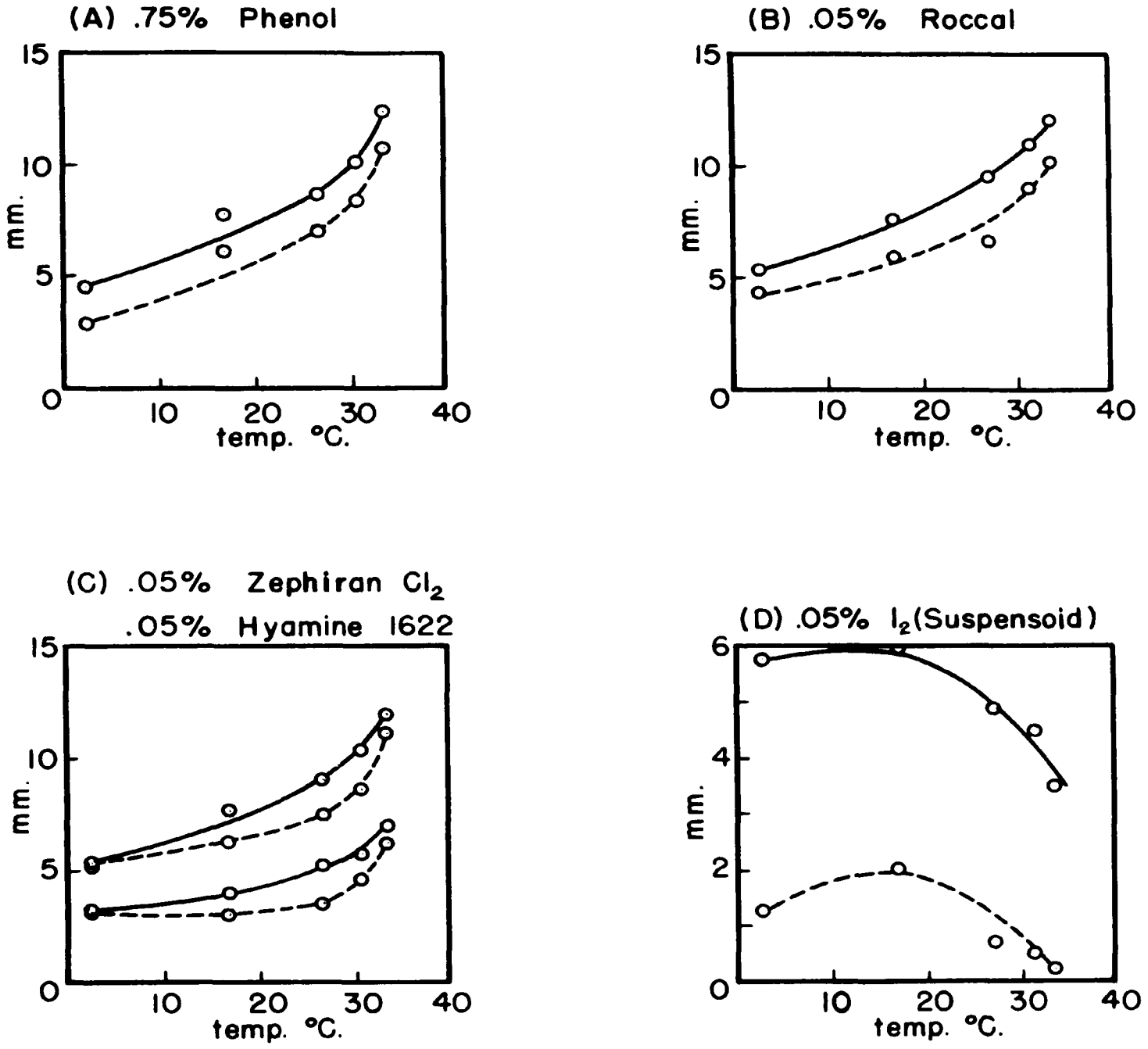


Figure 5 - A,B,C,D. The effect of temperature on the diffusion of various disinfectants at pH9.0 into agar (—) and agar + 1.0% human serum albumen. (---).

in Figure 5D. The penetration reaches a maximum value at approximately 15 C. This condition is likewise difficult to explain, inasmuch as no "upper zones" of inhibition occur at this pH value and so the decrease in penetration at high temperatures cannot be due to a loss of iodine through volatilization.

The effect of variation of disinfectant concentration. Penetubes were prepared in the same manner as in the preceding experiments. Duplicate tubes were exposed at 27.0 C for 24 hours to disinfectants in varying concentrations in M/2 phosphate buffer at the selected pH values. Neutralization and development of the columns were accomplished as indicated in the previous experiments. The protocol of each experiment is described below.

The penetration of iodine preparations into agar and agar-containing proteins was examined. Shown in Figure 6 is the penetration of Lugol's Solution and Iodine Tincture at increasing concentrations into agar plus 1.0 per cent gelatine and 1.0 per cent human serum albumin. These exposures were made at pH 5.0. Iodine Suspensoid (Merck) was examined in the same manner and the curves for penetration at increasing concentrations were similar to those of Figure 6. Typical curves for

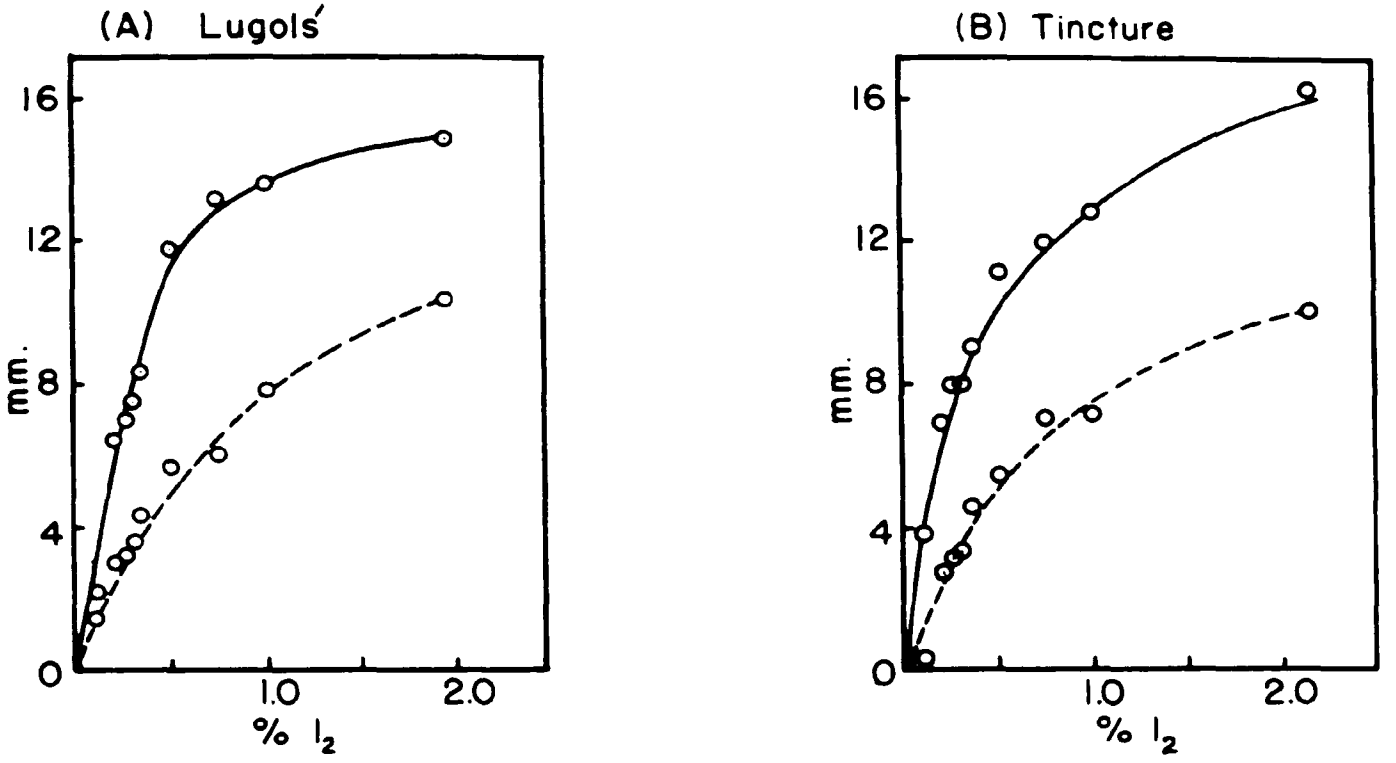


Figure 6-A,B. The penetration of Iodine preparations, with respect to increasing concentration, into agar + 1.0% gelatine (——) and agar+ 1.0% human serum albumen (----).

the Colloidal Iodine may be seen in Figure 12A, B and C. This entire family of curves appear to be cases of Hill's equation and will be discussed from this standpoint in a later section. The penetrating ability of the three iodine preparations, in descending order, is Lugol's Solution, Tincture of Iodine and Colloidal Iodine. This order is in agreement with the results obtained by Anderson and Mallmann (14) using the paraffin-covered agar plate method.

In another set of experiments, four preparations of Colloidal Iodine of varying particle size were prepared in accordance with the directions of Miller (44). These suspensoids (I - IV) were of increasing particle size, ranging in color from a brick red (I) through increasing dark shades of brown (II, III) to black (IV). These suspensoids were diluted to the same iodine concentrations as preparations of the Merck Suspensoid and all adjusted to pH 5.0 with M/2 phosphate buffer. Penetubes containing agar and agar plus 1.0 per cent gelatine were exposed in these hydrosols and after development in the usual manner the zones of inhibition recorded. These results are shown in Table 2. It can be seen that the zones of inhibition produced by all the preparations are nearly identical at identical

TABLE 2

THE EFFECT OF PARTICLE SIZE ON THE PENETRATION OF COLLOIDAL IODINE INTO AGAR PLUS 1.0 PER CENT GELATINE AT pH 5.0 AND 27.0 C FOR 24 HOURS

Preparation	mm Penetration with	
	0.29% I_2	0.03% I_2
Merck Suspensoid	15.1	5.2
	15.1	5.2
Suspensoid I	15.2	5.2
	15.2	5.2
Suspensoid II	15.8	5.3
	15.7	5.2
Suspensoid III	14.8	5.3
	15.2	5.1
Suspensoid IV	15.6	5.1
	15.6	5.2
Average	15.3	5.2

concentrations. The particle size of the suspensoids does not appear to be critical in the penetration of the iodine derived from these particles.

Chlorine compounds were investigated in the same manner as the iodine preparations, with the results obtained plotted in Figure 7. The shape of the curves representing the penetration brought about by increasing concentrations of available chlorine are the same as those found for the iodine preparations. A comparison of Figures 7A and B, representing the data for sodium hypochlorite (Roman Cleanser) at pH 5.0 and pH 9.0 respectively, shows the marked dependence of this compound upon hydrogen ion concentration. It is also apparent from the curves that the penetration of these chlorine compounds is most markedly limited by the presence of proteins and that chlorine compounds in general do not penetrate the test systems to the same extent as iodine.

Phenol penetration is shown in Figure 8. It can be seen that the penetration of phenol is not affected to the same degree by the hydrogen ion concentration nor by the presence of proteins as are the halogens. The curves obtained with phenol are of the same nature as those obtained with the halogens although

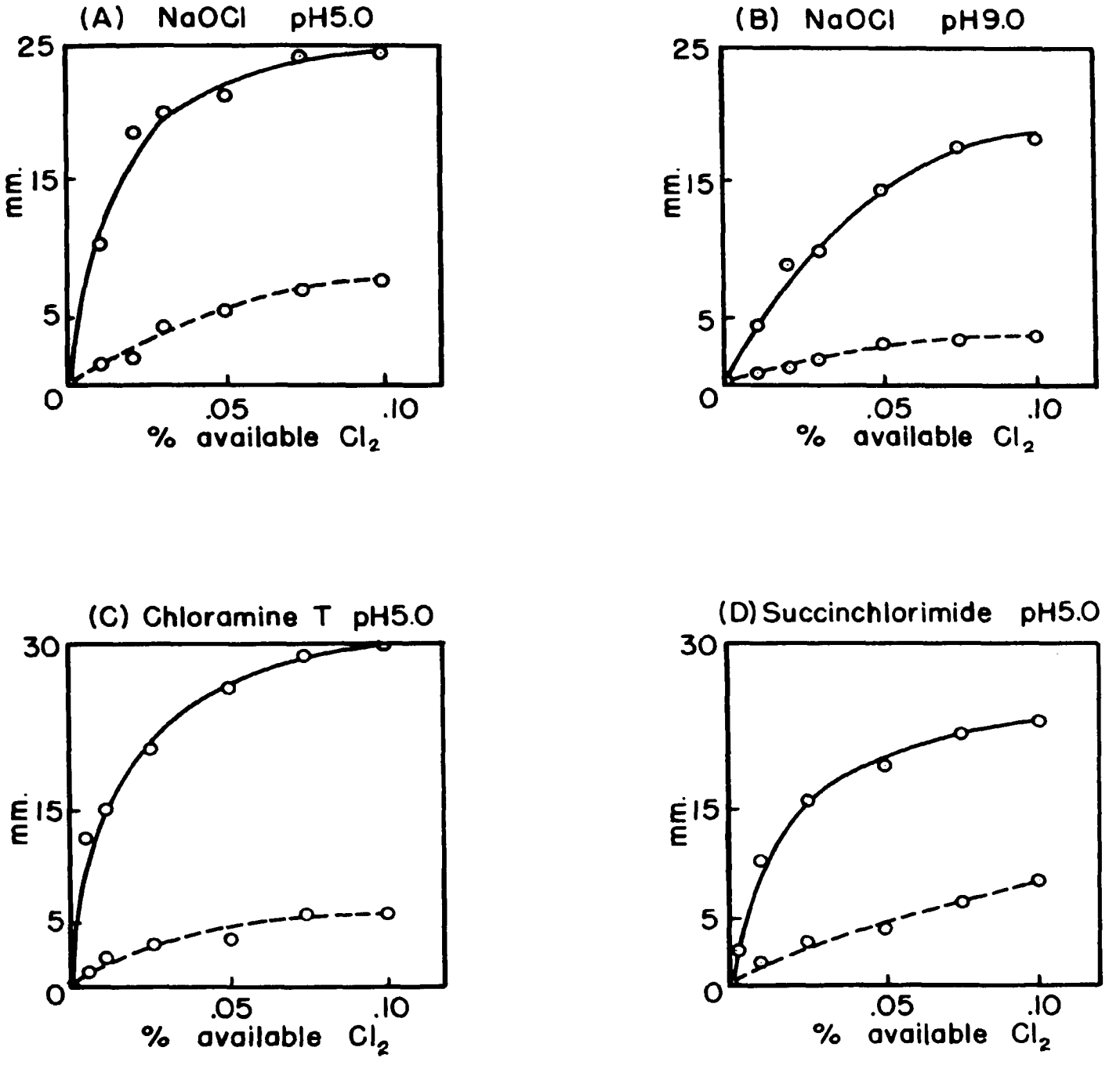


Figure 7 – A,B,C,D. The penetration of chlorine compounds, with respect to increasing concentration, into agar (——) and agar + 1.0% human serum albumen. (- - -).

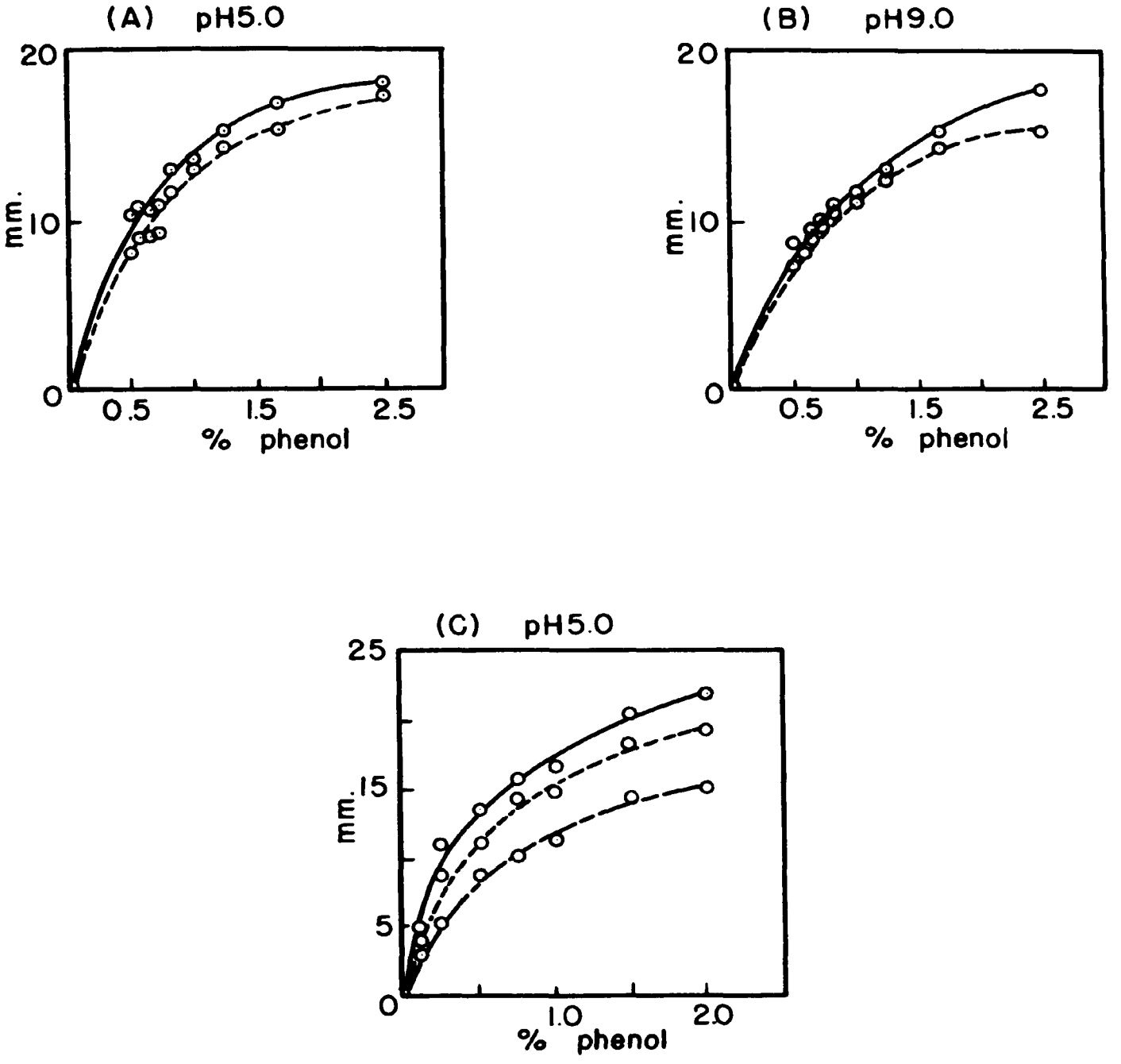


Figure 8 – A,B,C. The penetration of phenol, with respect to increasing concentration, into agar(—), agar + 1.0% gelatine (---), and agar + 1.0% human serum albumen. (—·—).

the initial slope is lower than in the curves relating the penetration of the halogens to the concentration thereof.

The penetration of Roccal at increasing concentrations into agar and agar plus 1.0 per cent human serum albumin is shown in Figure 9. The penetration of this compound at pH 9.0 is seen to be greater than at pH 5.0. The presence of the protein does not appear to inhibit the penetration to as great a degree as with the halogens.

Merthiolate was examined as an aqueous solution and as a tincture containing 35 per cent ethanol. The neutralization and development of columns exposed to this compound were accomplished in freshly prepared Fluid Thioglycollate Medium (Difco). In Figure 10A and B the results are plotted for two experiments run concurrently with the aqueous solution and the tincture. The curves obtained with the tincture have not been corrected for the effect of ethanol alone, which can be seen to account for approximately 5.0 mm of the penetration. The penetration of the tincture is not as greatly reduced by the presence of gelatine as the aqueous solution. Repeat experiments of this type gave highly variable results. In Figure 10C, another experiment with aqueous Merthiolate is recorded. It

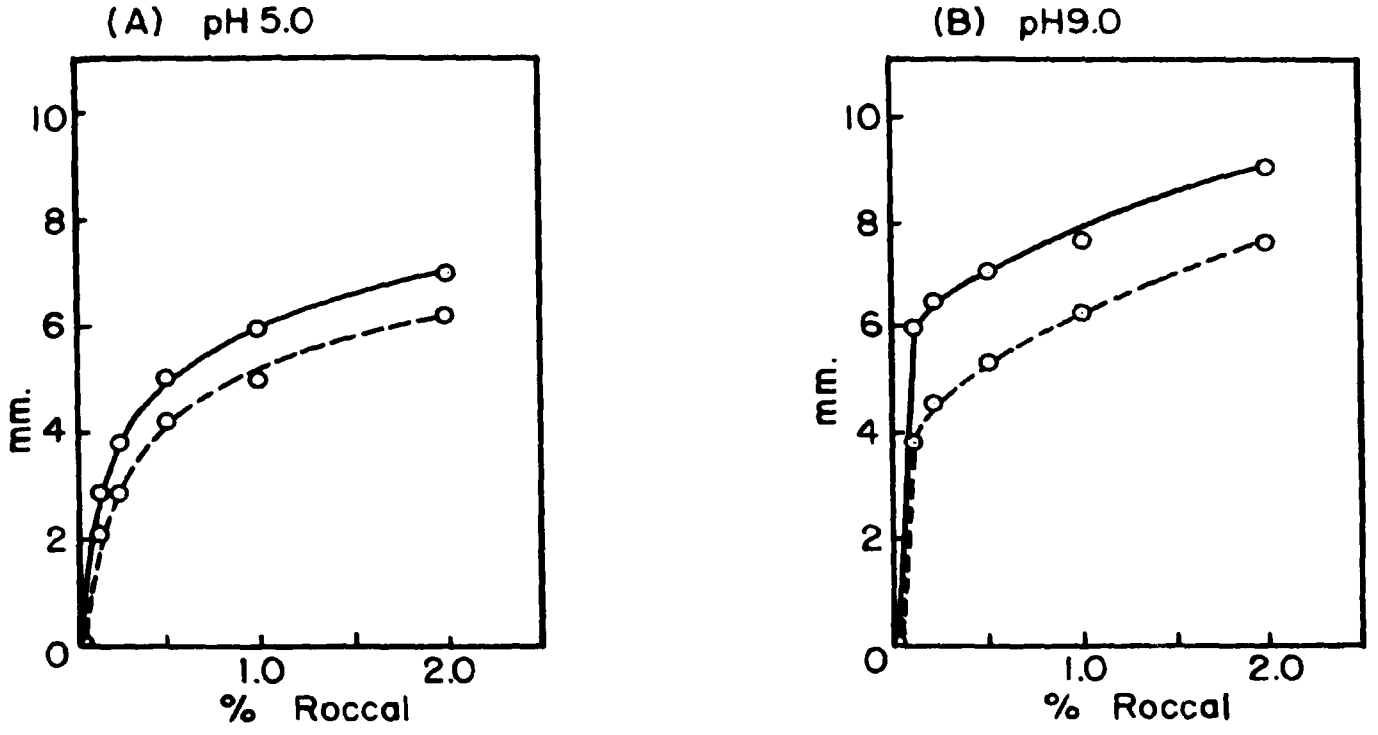


Figure 9-A,B. The penetration of Roccal, with respect to increasing concentration, into agar (——) and agar + 1.0% human serum albumen(----).

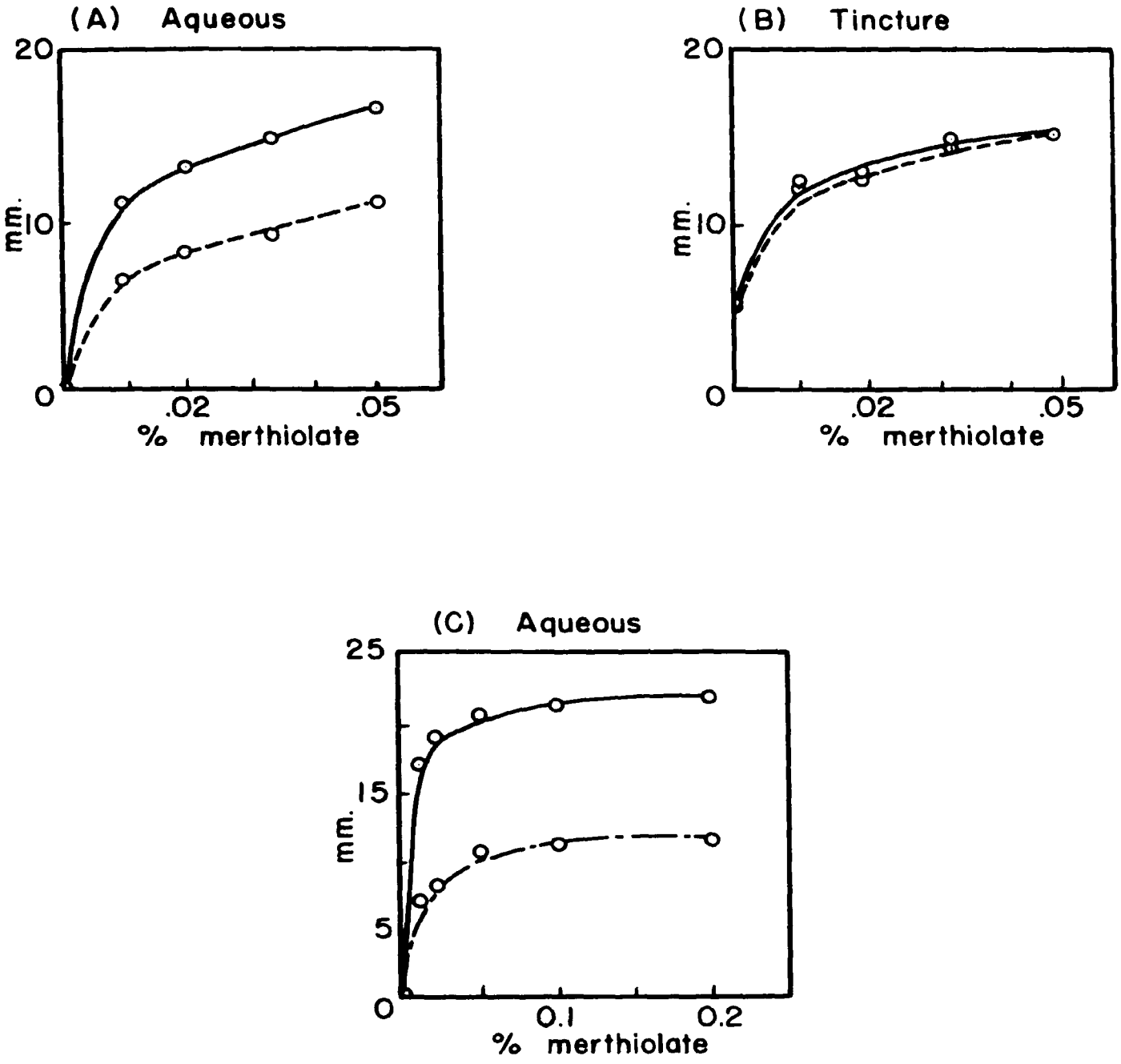


Figure 10 – A, B, C. The penetration of merthiolate at pH5.0, with respect to increasing concentration, into agar (——), agar + 1.0% gelatine, (– – –), and agar + 1.0% human serum albumen (– · –).

can be seen that the extent of penetration of the Merthiolate in this experiment is far greater than in the experiment graphed in Figure 10A. One wonders whether neutralization of the excess Merthiolate has been successful in all trials.

The variation of stationary phase components. Penetubes were prepared containing gelatine or human serum albumin at concentrations from zero to 1.0 per cent. The pH of these columns was set at pH 5.0 with M/10 phosphate buffer. The penetubes were exposed to 0.1 per cent Roccal, 1.0 per cent phenol and 0.05 per cent Iodine Suspensoid at pH 5.0 and 27.0 C for 24 hours. Neutralization and development were accomplished as before and the resulting zones of inhibition recorded. These results appear in Figure 11. For each of the disinfectants tested it can be seen that the albumin is more inhibitory to the penetration of the disinfectant than gelatine. Another salient feature of the curves is that they level off, demonstrating that above some concentration of the protein the rate of penetration of the disinfectants is nearly independent of the protein concentration. This observation makes logical the use of 1.0 per cent protein concentrations in the other experiments. Under these circumstances, minor errors in the addition of the protein will

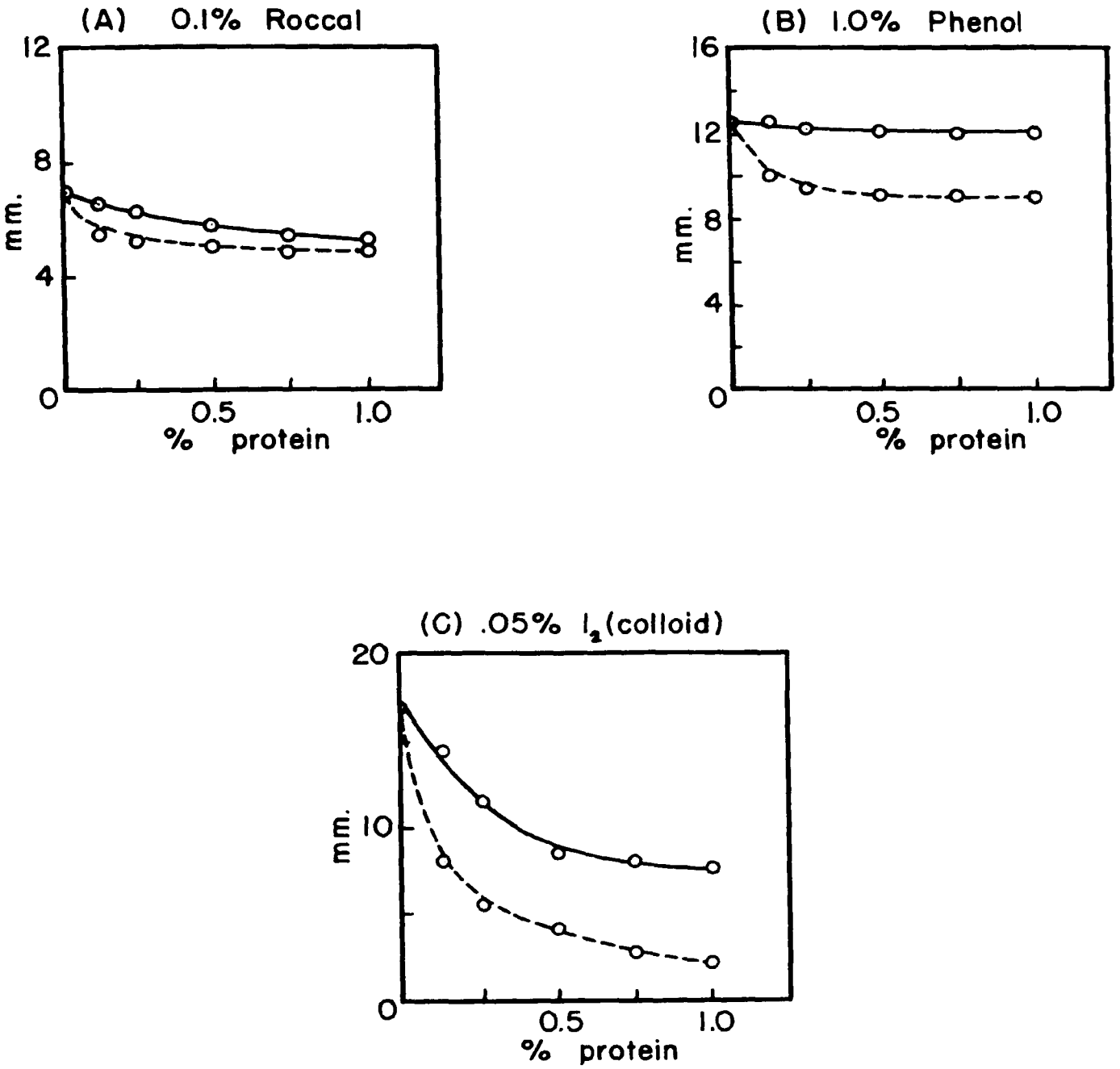


Figure II – A, B, C. The penetration of Roccal, Phenol, and Iodine at pH5.0 and 27°C. into an agar stationary phase containing increasing concentrations of gelatine (—) or human serum albumen (---).

not then cause large errors in the resulting observations. Once again, it is noted that penetration of Roccal and phenol is less adversely affected than iodine by the presence of protein.

The results plotted in Figure 12A and B demonstrate the effect of adding 0.5 per cent potassium iodide or sodium chloride to the stationary phase on the penetration of iodine into such gel systems. The penetration of Colloidal Iodine when sodium chloride is contained in the gel phase is identical to the penetration when no salt is added. The presence of potassium iodide in the gel phase enhances the penetration of the columns by iodine. This experiment was conducted at pH 5.0 and 27.0 C for 24 hours.

In a subsequent experiment an unbuffered solution of iodine in distilled water containing 0.028 per cent iodine was allowed to penetrate agar gel columns and columns with the addition of either 1.0 per cent potassium iodide or sodium chloride to the stationary phase. A duplicate set containing 1.0 per cent gelatine was also exposed. The exposure was made at 27.0 C for 24 hours and the results are tabulated in Table 3. These results are in agreement with those previously cited. It is apparent that the addition of potassium iodide has occasioned

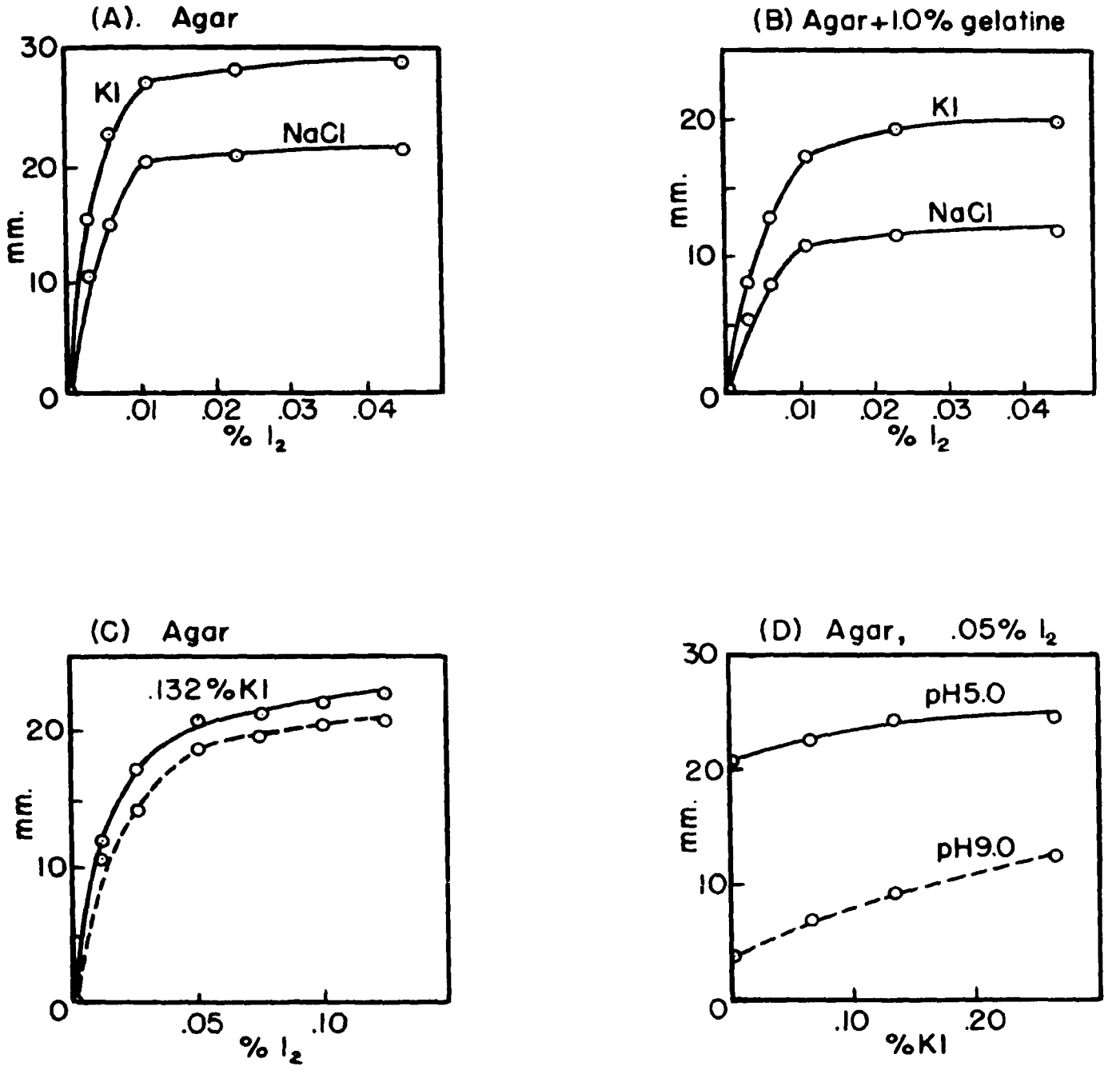


Figure 12-A,B,C,D. The effect of salts on the penetration of Colloidal Iodine. Explanations in text.

TABLE 3

THE PENETRATION OF 0.028 PER CENT IODINE IN AQUEOUS SOLUTION INTO AGAR COLUMNS WHERE THE GEL SYSTEM CONTAINS ADDITIVES AS INDICATED. M. AUREUS INDICATOR, 24 HOUR EXPOSURE AT 27.0 C

Salt Added	mm Penetration in	
	Agar	Agar + 1% Gelatine
None	33.1	22.0
1% KI	52.1	34.0
1% NaCl	34.2	22.1

a 55 per cent increase in the penetration of either gel system by the iodine. The sodium chloride addition gives no increase in penetration of iodine over that obtained in the control columns, hence the effect of the potassium iodide must be specific and not due to the effect of salt concentration. This effect is also seen to be the same for the agar system and the gelatine-containing system and thus it is logical to suspect that the increase in penetrability when potassium iodide is present is due to the formation of triiodide ion.

This possibility was further explored by adding 0.132 per cent potassium iodide to a series of exposure tubes containing increasing concentrations of Colloidal Iodine at pH 5.0. The exposure of agar columns in these tubes was conducted at 27.0 C for 24 hours. The penetration of the columns at each concentration is plotted in Figure 12C together with the results of exposure of replicate penetubes to controls containing no potassium iodide. It is apparent that the penetration of the agar gel is greater where potassium iodide is present (and consequently triiodide) than where free iodine alone is present.

This point is further documented in Figure 12D. The results plotted in this figure show the penetration of 0.05 per

cent Colloidal Iodine at pH 5.0 and 9.0 when increasing amounts of potassium iodide have been added to the disinfectant. The linear increase with increasing concentrations of the salt demonstrates the increasing penetration of the agar gel by increasing concentrations of triiodide ions. It should be noted that the addition of the potassium iodide breaks the hydrosol and that at pH 9.0 the hydrosol is not stable at a concentration of 0.05 per cent.

In other experiments, casein was added to the stationary phase. The insolubility of this protein at pH 5.0 prevented its addition as a solution to the melted gel. When casein was suspended in the agar gel and the mixture autoclaved, a dark brown gel eventually was formed in which the zones of inhibition could not readily be discerned.

In one experiment ammonium sulfate was added to the stationary phase. The penetration of phenol and Roccal was not affected by this addition. The penetration of Colloidal Iodine was affected to a great degree, an agar containing 0.5 per cent ammonium sulfate was penetrated 45.5 mm and a 1.0 per cent concentration of this compound resulted in 54.8 mm penetration. In agar alone the penetration of the columns amounted to only

17.0 mm with the same disinfectant exposure. The ammonium sulfate in neither concentration inhibited the growth of the inhibitor organism as demonstrated by exposure to a buffer solution. These results are interesting but are not readily explained. Addition of 1.0 per cent urea to the stationary phase seemed likewise to facilitate the penetration of this phase by iodine but urea was inhibitory to the indicator organism and so the exact zones of inhibition proved difficult to measure.

The rate of diffusion. Penetubes containing agar and agar plus 1.0 per cent gelatine were prepared at pH 5.0. These penetubes were exposed in large tubes stoppered with aluminum foil-covered No. 7 rubber stoppers containing a 0.028 per cent iodine solution at pH 5.0. The iodine solution was prepared from Iodine Suspensoid (Merck). At intervals up to 27 hours duplicate pairs of penetubes were removed and treated in the prescribed manner. The resulting zones of inhibition following each exposure period are plotted in Figure 13. After an initial rise, the penetration was linear with respect to time. The assumption that the penetration in 24 hours was at a constant rate is not far from the actual case and may be used as an expression of the rate of penetration. These results are considerably different

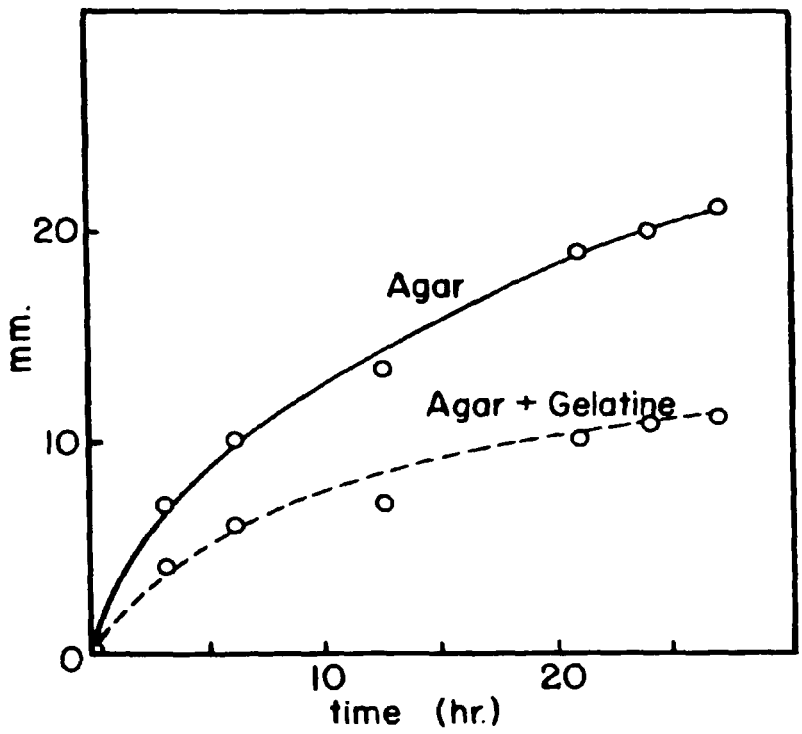


Figure 13. The rate of diffusion of .028% Iodine at pH5.0 and 27.0°C into agar and agar + 1.0% gelatine.

from the rate of penetration reported by Anderson and Mallmann (14) for 5 per cent Colloidal Iodine, but the difference in hydrogen ion concentration in these trials does not permit a direct comparison.

Experiments on the Disinfection of Chicken Feces

The variation of disinfectant concentration. Iodine preparations were adjusted to the desired pH value in M/2 phosphate buffer. The iodine content of each preparation was determined just prior to use by thiosulfate titration. The exposure period of the fecal suspension to the action of the disinfectant was 5.0 minutes, at which time the 1.0 ml samples were removed to 9.0 ml sterile 0.05 N sodium thiosulfate dilution blanks for neutralization and subsequent operations. Initial counts of bacteria in the fecal suspension were obtained from control treatments in the phosphate buffer. All counts are the average of the numbers obtained from duplicate plate counts. The logarithms of the numbers of organisms surviving after 5.0 minutes exposure are plotted at each concentration of iodine tested.

In Figure 14A, results obtained with Iodine Suspensoid (Merck) are plotted; in Figure 14B, the results obtained with

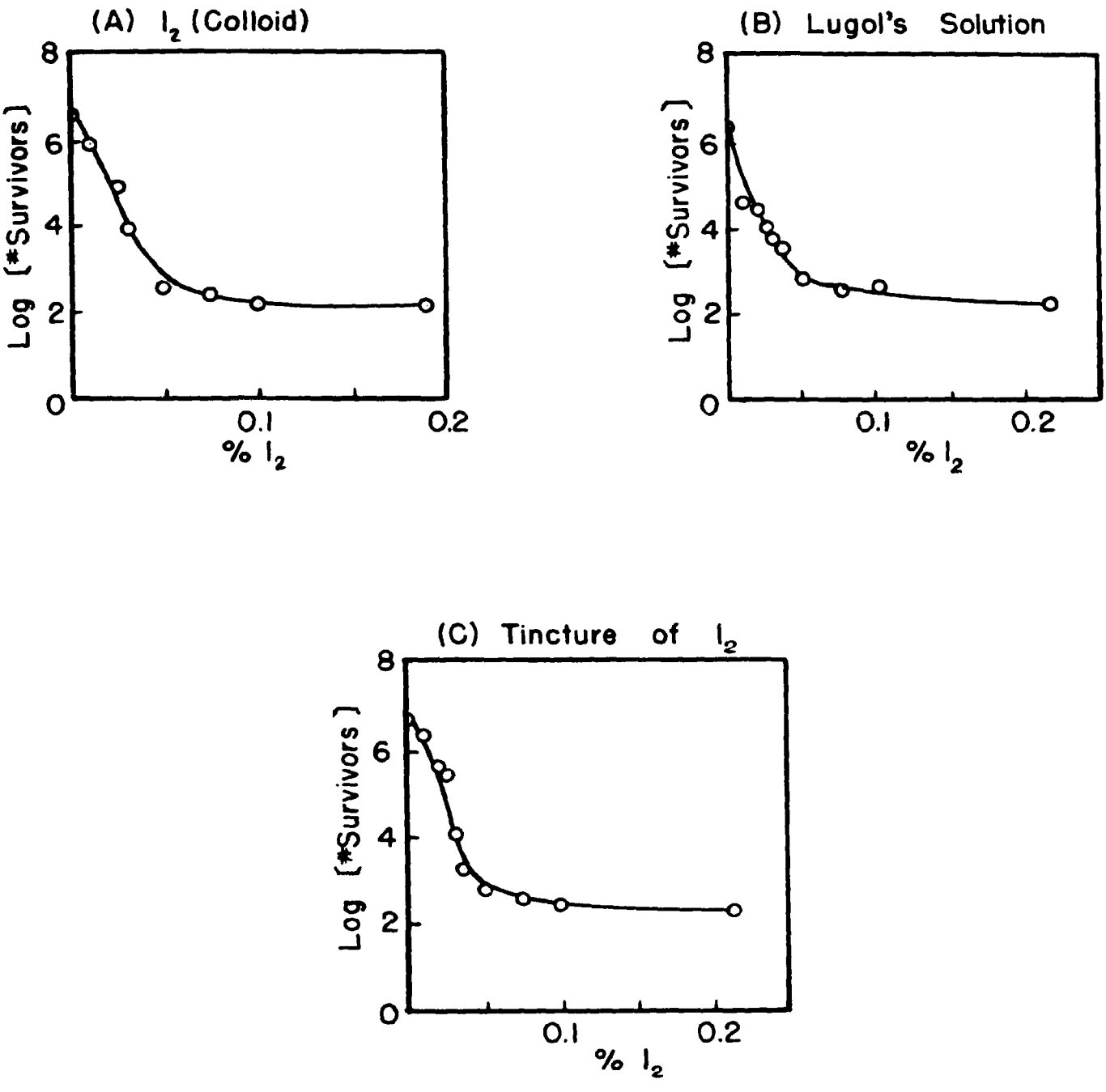


Figure 14-A,B,C. The reduction of bacterial population of chicken feces in 5.0 min. at pH 5.0, 27.5°C.

Lugol's Solution (U.S.P. XIII) are plotted and in Figure 14C, the data obtained with Iodine Tincture (U.S.P. XIII) are plotted. All dilutions were made with distilled water, so that the alcohol content of the tincture was practically nil and certainly not in the range of concentration of alcohol which is germicidal.

It can be seen from the graphs that the curves of bacterial reduction with respect to increasing iodine concentration are practically identical, so that, under these conditions, the activity of iodine is the same in all three preparations tested. The curves are all slightly sigmoid and it is of possible significance that the concentration at which the curves start to become asymptotic to the abscissa is at a concentration of 0.05 per cent iodine.

To determine further the properties of the Colloidal Iodine, a number of preparations of the hydrosol were made in accordance with the directions of Miller (44). The preparation of finest particle size ("fine" suspensoid)—a light brick red in appearance—was prepared for final use by centrifuging a hydrosol of small particle size and discarding the largest particles. The preparation of largest particle size ("coarse" suspensoid)—appearing almost black—was prepared by collecting

the sediment from the centrifugation of the coarsest hydrosol. Both preparations were composed of particles averaging 0.7 u in diameter, ranging in diameter from 0.2 u to 0.9 u. In the "fine" suspensoid these particles were suspended individually in the saturated solution of iodine, while the "coarse" suspensoid was composed of clumps of the particles. These suspensoids were used in experiments in the same manner as the previously mentioned iodine preparations with the results as illustrated in the curves of Figure 15. The resulting curves are superimposable on each other and very similar to the curves of Figure 14. They differ in shape from the curves in the latter figure by not demonstrating increasing slopes as concentration is increased at extremely low dilutions. This difference is probably due only to the lesser number of points examined in this region than were examined in the earlier experiments. Again, the point of diminishing returns is reached at a concentration of 0.05 per cent iodine concentration. Apparently the form of the solution or suspension of iodine is not critical for the disinfection of chicken feces under these conditions.

Chlorine compounds were examined under the same conditions as were the iodine preparations. The concentration of

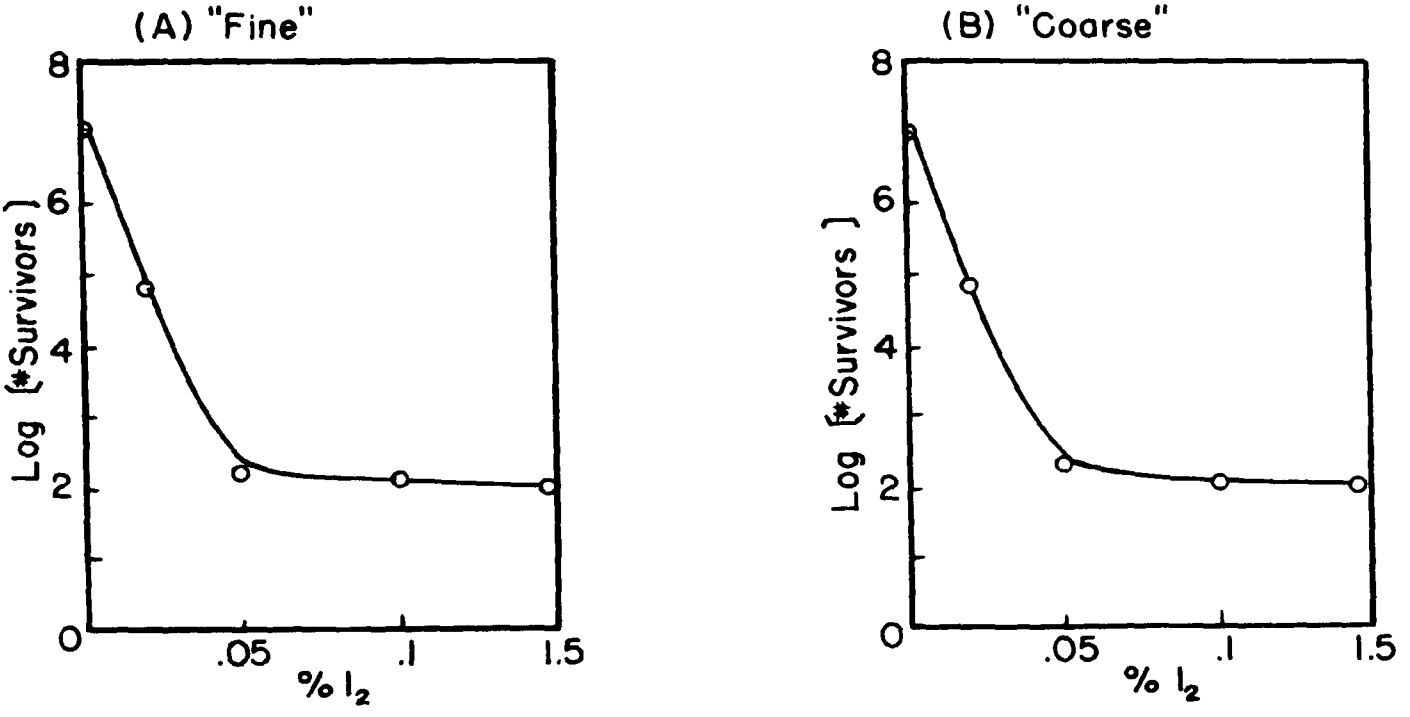


Figure 15-A,B. The reduction of the bacterial population of chicken feces in 5.0 min. at pH5.0, 25°C., by: A."Fine" I_2 suspensoid, B."Coarse" I_2 suspensoid.

each compound was expressed as the per cent available chlorine as determined by sodium thiosulfate titration (45). In Figure 16A and B, the data for sodium hypochlorite (Roman Cleanser) and Chloramine T (Wyandotte) are respectively graphed. Both are similar to each other and resemble typical "die-away" curves. The sodium hypochlorite appears to be slightly more effective in reducing the bacterial population of the chicken feces than does Chloramine T. Succinchlorimide (National Dyes) activity is presented in Figure 16C. This compound displays an activity decidedly different from that of the other two compounds in its class with respect to concentration. The slope of the bacterial reduction at low concentrations of succinchlorimide is much steeper than those of the other chlorine compounds, but rapidly changes to a far lower slope at concentrations in excess of 0.02 per cent available chlorine. Succinchlorimide appears to be less effective than the hypochlorite or the Chloramine T.

In Figure 17A and B the effect of increasing concentrations of Roccal at pH 5.0 and pH 9.0 on the disinfection of chicken feces is shown. The tenfold neutralizing dilutions were made in FDA broth containing one per cent of Tamol-N. These dilutions were incubated at 37 C and growth occurred in all tubes within

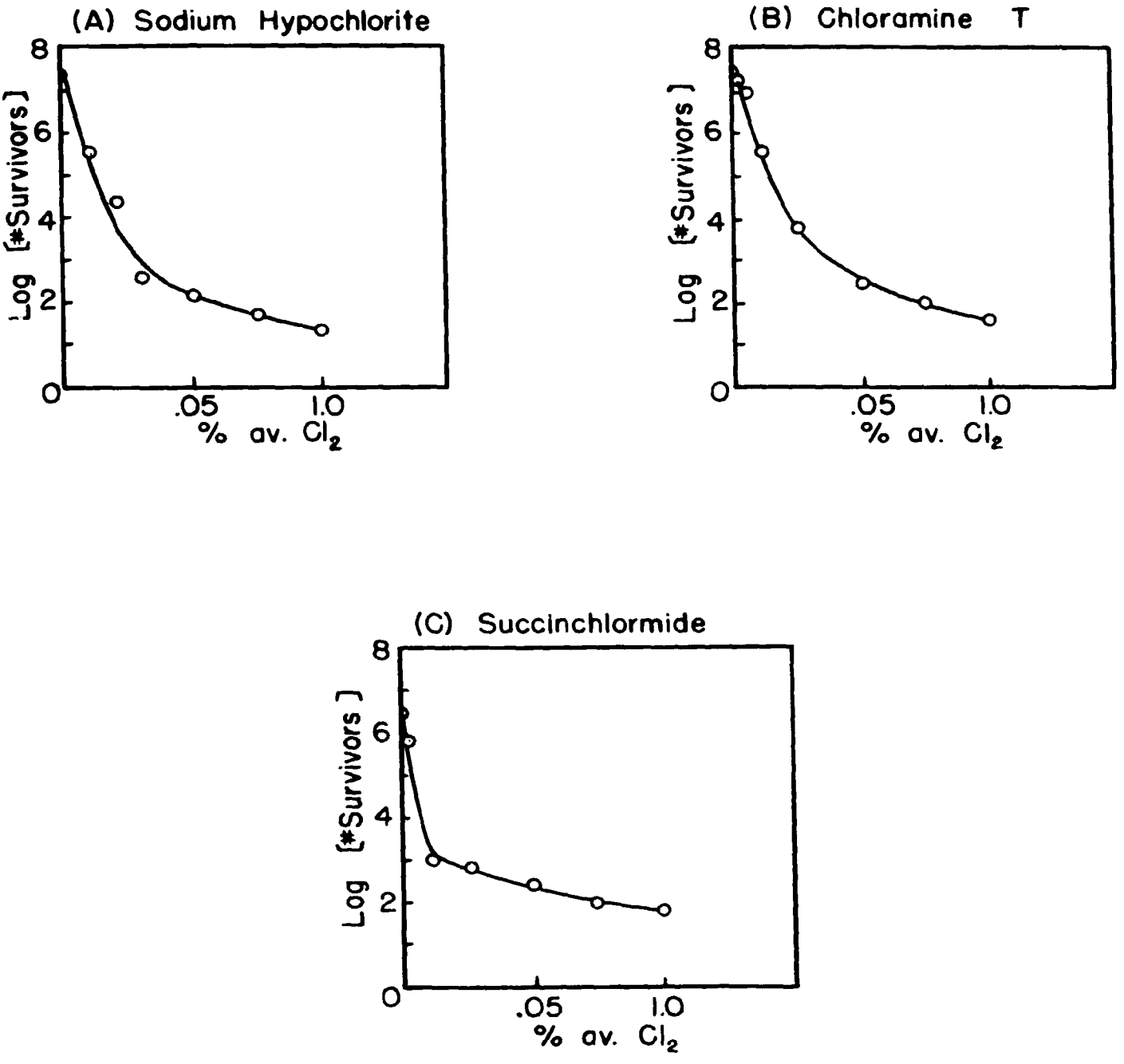


Figure 16-A,B,C. The reduction of the bacterial population of chicken feces in 5.0 min. at pH5.0, 27.5°C. by chlorine compounds.

24 hours, demonstrating that no bacteriostatic action remained following this form of neutralization. The curves obtained in this manner are very similar at both pH values; however, the curve representing disinfection at pH 9.0 is somewhat steeper at low concentrations of the compound. The maximum effectiveness in the five-minute exposure period in both cases is at about 0.02 per cent concentration, and the number of residual resistant organisms is the same in both cases.

Phenol was examined in like manner, the first dilutions were made in FDA broth to stop the action of the phenol by means of dilution and combination with the organic matter of the broth. Incubation of these broth dilutions resulted in prompt growth, demonstrating that the neutralization was effective. Figure 17C shows the sigmoid character of the survival curve obtained with increasing concentration of phenol. The extent of the horizontal branch of the curve and the steepness of the descending branch illustrate the rapid loss of germicidal activity of this compound with dilution. The concentration of phenol at which survival becomes asymptotic to concentration is at 1.0 per cent phenol.

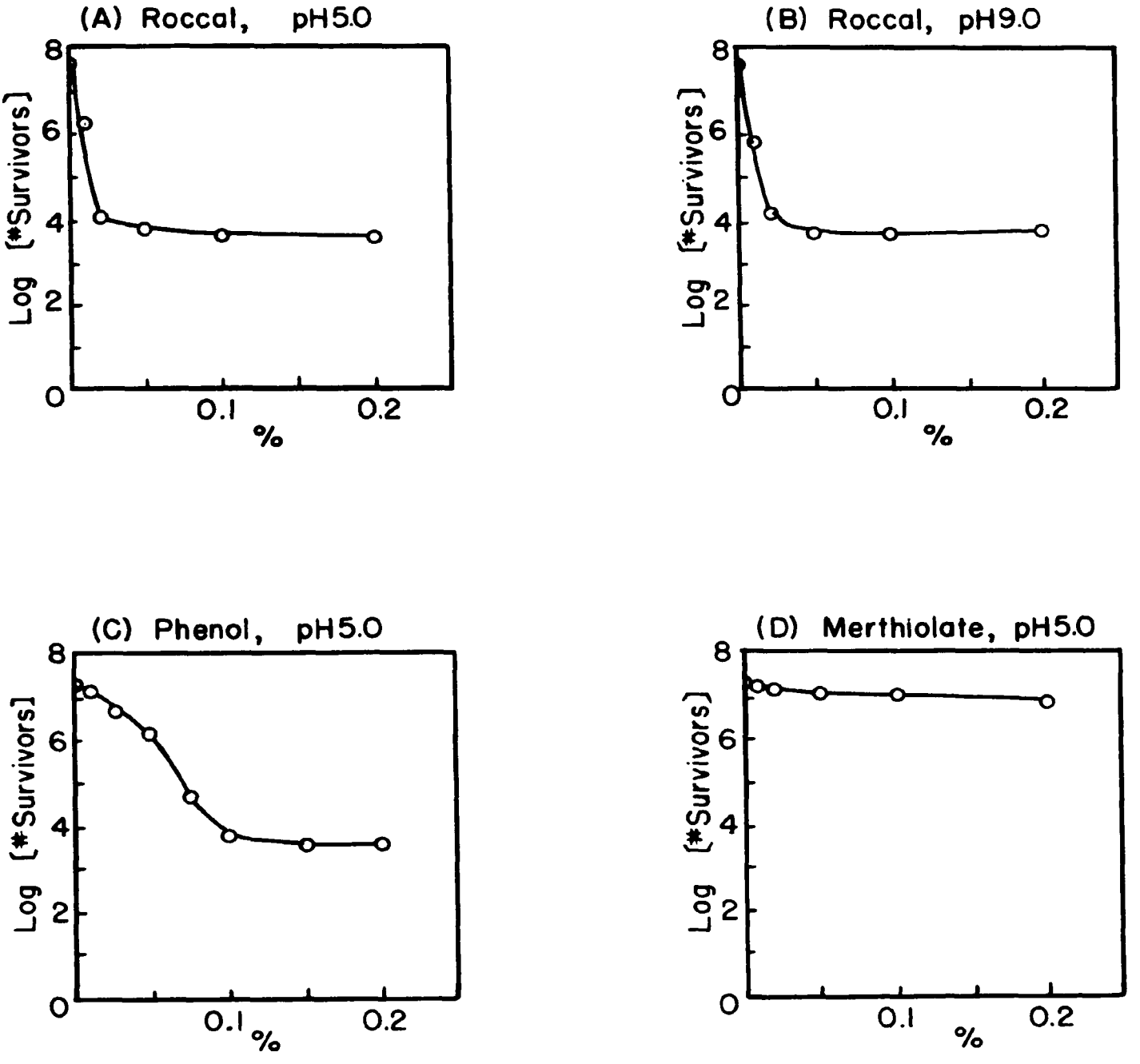


Figure 17-A,B,C,D. The reduction of the bacterial population of chicken feces in 5.0 min. at 27.5°C., by various compounds.

Merthiolate in aqueous solution at pH 5.0 was examined and the resulting survivor curve as shown in Figure 17D was obtained. Neutralization and decimal dilution were accomplished in freshly prepared Fluid Thioglycollate Medium (Difco), incubation of which demonstrated growth and hence adequate neutralization of the bacteriostatic action of merthiolate. The survival curve demonstrates that merthiolate is practically ineffective in reducing the bacterial count of chicken feces under these conditions.

The rate of disinfection of chicken feces. The five iodine preparations were all adjusted to 0.05 per cent iodine concentration at pH 5.0 in M/2 phosphate buffer. Controls were prepared of the buffer alone. After exposure periods of 1.0 and 5.0 minutes, neutralization and dilution were accomplished as previously described and samples plated for enumeration of the surviving organisms. The per cent kill, effected in these periods of exposure, was reckoned from the counts obtained by identical treatment of the fecal suspension in the phosphate buffer controls. These data are presented in Table 4. After an exposure period of 1.0 minute, the 0.05 per cent iodine in the five preparations gave an average kill of 99.82 per cent with a mean

TABLE 4

THE PER CENT KILL OF THE BACTERIAL POPULATION OF CHICKEN FECES AFTER EXPOSURE FOR THE INDICATED PERIODS TO 0.05 PER CENT IODINE AT pH 5.0 AND 27 C

0.05% I_2 as	Per Cent Kill After Exposure for	
	1.0 min	5.0 min
Suspensoid (Merck)	99.95	99.97
"Fine" suspensoid	99.37	99.84
"Coarse" suspensoid	99.98	99.99
Lugol's Solution	99.84	99.94
Iodine Tincture	99.98	99.98
Average	99.82	99.96

deviation of 0.16 per cent. This represents an accuracy slightly better than two parts per thousand. After 5.0 minutes of exposure, the preparations at this concentration caused an average kill of 99.96 per cent with a mean deviation of only 0.4 per cent, representing an accuracy of four parts per ten thousand. None of the deviations from either mean exceed the mean deviation by a factor of four, so that all values obtained can be considered as representing samples from the same specimen. It is evident that for the disinfection of chicken feces, Colloidal Iodine, Lugol's Solution and Iodine Tincture are identical in activity when measured at constant temperature, pH and concentration.

Chlorine compounds, phenol, Roccal and Merthiolate were examined in a somewhat different fashion. The experimental procedure was as given for iodine, but the intervals of exposure were extended to 30 minutes. Controls again were run in M/2 phosphate buffer and duplicate concentrations were set at pH 5.0 and pH 9.0. The effect of the phosphate buffer at pH 5.0 can be seen to be nil (Figure 18A) even after 30 minutes of exposure. In phosphate buffer at pH 9.0 there is a small but significant decrease in the numbers of viable organisms, after 30 minutes this decrease amounts to approximately 50 per cent.

The curves obtained for disinfectants in these buffers have not been corrected for this effect of the buffer alone and thus the bactericidal effect of the alkaline buffer has depressed the curves obtained at pH 9.0 somewhat.

Results with chlorine compounds are shown in Figure 18. Graph B illustrates the rate of disinfection of chicken feces by sodium hypochlorite containing 0.03 per cent available chlorine. At pH 5.0 the initial rate is greater than at pH 9.0, although after ten minutes exposure both rates have become the same. With Chloramine T (Graph C) and Succinchlorimide (Graph D), both containing 0.05 per cent available chlorine, the rate of disinfection at the acid pH is exceedingly greater than at the alkaline pH. Within the 30-minute exposure period these compounds at pH 9.0 did not effect as great a kill as they did at pH 5.0 in contradistinction to the hypochlorite. This difference would appear to be related to the dissociation of these organic compounds at these pH values to yield hypochlorite ion and subsequently the free chlorine.

In Figure 19A, the rates of disinfection produced by 1.0 per cent phenol at pH 5.0 and 9.0 are shown. There is a high rate of kill in the first minute and the rate then decreases at

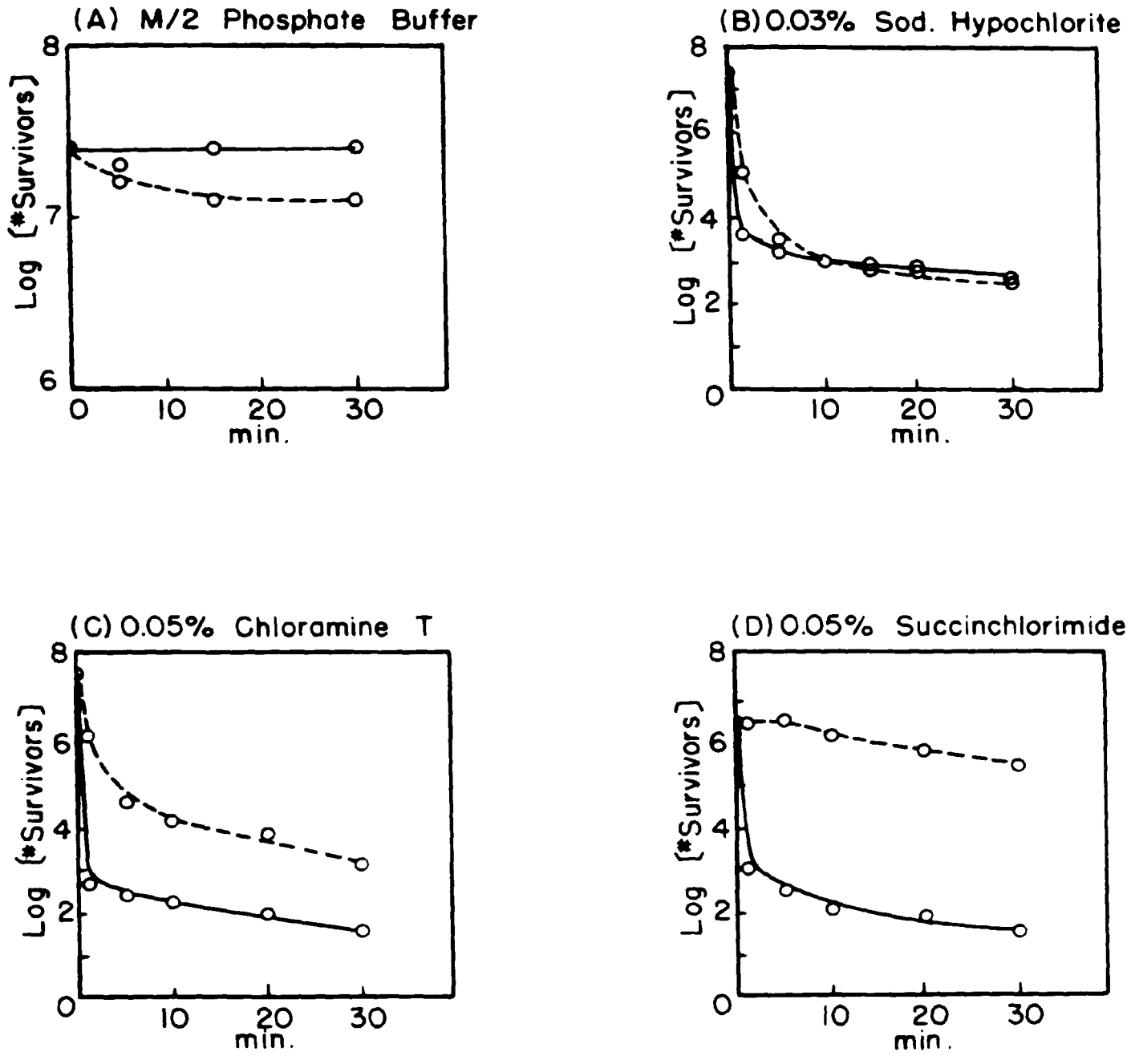


Figure 18-A,B,C,D. The rate of reduction of the bacterial population of chicken feces at pH5.0 (——) and pH9.0 (---), temperature 27.5°C. The concentration of the chlorine compounds is given as percent available Cl_2.

succeeding time intervals. Under these conditions, the hydrogen ion concentration does not appear to affect the rate of disinfection of phenol.

Roccal at 0.02 per cent concentration gives similar results to those of phenol, as shown in Figure 19B. At pH 9.0 the rate is slightly higher in the early intervals of time than is the rate at pH 5.0.

In Figure 19C the effect of 0.2 per cent merthiolate (aqueous) on the disinfection of the feces is shown. It can readily be seen that this compound has very little disinfecting power, scarcely better than alkaline phosphate buffer alone.

The changes in the bacterial flora of the feces following disinfection. Colonies were randomly picked from the plates in the preceding experiments. These fishings were planted on slants of sterile TGE agar and checked for purity by streaking on sterile plates of the same composition. The cultures were then examined microscopically as gram-stained smears and as wet mounts to observe motility. They were tested for their ability to produce catalase and to ferment Brilliant Green Lactose Bile broth in 48 hours. Twenty cultures from the control

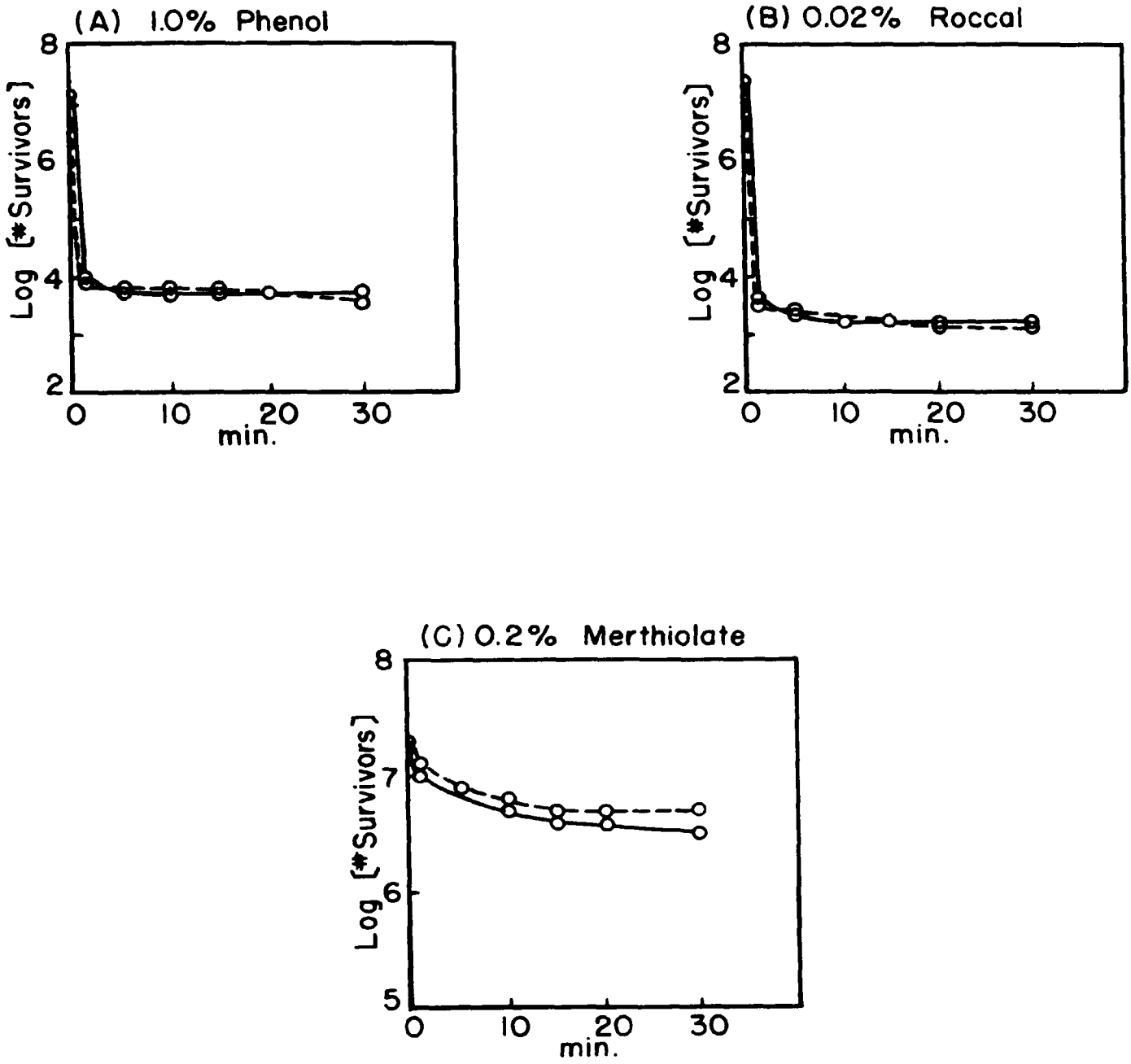


Figure 19-A,B,C. The rate of reduction of the bacterial population of chicken feces at pH5.0(——) and pH9.0(---), temperature 27.5°C.

plates were examined and ten cultures from each of the disinfectant treatments.

The significant findings from this cursory examination of the bacterial flora revealed that in the original flora the spore-forming rods comprised 20 per cent of the isolates. Following treatment with iodine compounds, chlorine compounds, phenol and Roccal the proportion of spore-forming rods increased to 50 to 90 per cent. After 30 minutes treatment with Roccal at pH 9.0, 40 per cent of the residual population were slow lactose-fermenting coliform organisms as compared to 20 per cent of the original population as coliform organisms which fermented lactose promptly.

The composition of the original bacterial flora of the chicken feces did not differ markedly from the compositions noted by Emmel (46) and by Johansson, et al. (47).

DISCUSSION

> "It is characteristic of the advance of science that less and less is found to be datum, and more and more is found to be inference." Bertrand Russell

The Measurement of Penetrability

In the experimental section the results obtained with the "penetube" technic and the disinfection of chicken feces have been presented. It is obvious that in the "penetube" technic the diffusion of a disinfectant into an agar gel column permits measurement of a linear distance traveled by that disinfectant. The endpoint measured is not necessarily the full distance that has been traveled by all molecules of the disinfectant but this endpoint does represent the point where a minimal concentration of the disinfectant has been lethal to the indicator organism in the period of exposure. This point has been taken as a reference point for the comparison of the penetration of different disinfectants or of one disinfectant under different conditions. Comparisons have been made only where the same indicator organism was used in each case. The penetration of

proteinaceous matrices by disinfectants has also been measured by means of this technic and the differential effects of the proteins on the penetration were noted above.

A few generalizations are in order. In these penetration studies it was found, perhaps fortunately for the success of the sanitarian, that the highest penetration obtained by disinfecting compounds was at the hydrogen ion concentrations at which the compounds are most germicidal. This correlation may seem to indicate that the extent of the zones of inhibition are a function of germicidal activity alone and not of penetration. That this is not the case can be seen from the studies in which iodide was added to iodine solutions with resulting increases in the zones of inhibition. Marks and Stranskov (48) showed that triiodide ions are less germicidal than molecular iodine, so that the greater zones of inhibition in the gels columns cannot be due to a greater germicidal activity of the triiodide ions. It has been demonstrated (49) that ions diffuse more rapidly than do the complementary molecules. Triiodide ions then diffused further, and although intrinsically less germicidal than iodine, caused a greater zone of inhibition. We must conclude

that the technic does actually measure penetrability and does not merely reflect differences in germicidal activity.

The disinfection of chicken feces is not as obvious a test of the penetrative power of a disinfectant. The enclosure of bacteria in organic particles is not strictly uniform and at least half of the population of the fecal suspension are free in the suspending medium. The penetration of the bacteria, whether freely suspended or enclosed in particles, must be a consideration in the disinfection process. In this respect the test bears a close resemblance to the speed of disinfection test of Anderson and Mallmann (14). The test employed in this study has the added complications of the presence of organic and particulate matter and a nonuniform population. For a disinfectant to be efficient in this test system it must have a combination of highly desirable properties, and, so, this test is a rigid one, designed to weed out "weak sisters." The conditions of this test also bear a close resemblance to many practical situations encountered in environmental sanitation and, inasmuch as it yielded fairly reproducible results in the author's hands, may find application as a rigorous test of disinfectants.

The agreement of results obtained with disinfectants as measured by the disinfection of chicken feces and by the "penetube" technic may be coincidental or may be due to the common requirement of penetration in both technics. I do not believe, however, that the fecal disinfection technic is a simple test of penetrability, although the "penetube" technic undoubtedly is such a test.

The "Penetube" Technic

The rationale underlying this technic may not at first be obvious. It must be recalled that the technic was designed to accomplish the measurement of penetrability while eliminating or avoiding the objections propounded in the Introduction.

The characteristics desired in a system for measuring the penetrative powers of disinfectants are manifold. The indication that penetration has occurred should be the visible death of bacteria. This desideratum has two implications; the first implication being that an auxanographic test provides facile measurement of results and the second that the death of the indicator organisms implies that a sufficient concentration of the disinfectant has been at a definite locus for a sufficient

length of time for disinfection to have occurred. The bacteria to be killed must be located in uniform dispersion in a definite colloidal barrier of organic or proteinacerous matter, providing a volume through which the diffusion or penetration of the disinfectant must unequivocally occur for the death of the indicator bacteria to result. The dispersed organisms must not be permitted to multiply during the exposure to the disinfectant so that a uniform concentration of cells will be maintained, all of which will be at the same physiological or morphogenetic stage of development. This last requirement, then, will prevent the reflection of differential growth rates as discussed in the Introduction, and will necessitate a subsequent treatment to permit the visible development of viable organisms. This same step of development can be combined with a neutralization step to eliminate the effects of bacteriostasis. Another desired attribute of the technic must be a capacity for wide experimental variation of components and procedures. The separation of the steps of disinfection and growth permit this possibility.

In 1921, Stiles and Adair (50) reported measurements of the coefficients of diffusion of electrolytes into gels where the gels were supported in glass tubes in a fashion similar to the

"penetube" construction. Their results are among the best determinations of diffusion coefficients. More recently, Davis et al. (51) reported a method for antibiotic testing employing linear diffusion of the antibiotic into inoculated nutrient agar in glass capillaries. According to these authors, linear diffusion assay affords many advantages over agar plate methods. There are therefore precedents for determining diffusion or penetration in a linear direction in glass tubes as has been done in the "penetube" technic.

The problem of capillary creepage of disinfectant between the walls of the glass tube and the peripheral surface of the agar columns was not a serious one. Where capillary creepage was encountered the edge of the zone of inhibition appeared as a cylinder truncated at an angle or as an inverted cone. This occurred in less than two tubes per thousand, and, inasmuch as such tubes were rejected, capillary creepage cannot be considered as a serious disadvantage.

The interpretation of the results gained using the "penetube" technic lead to the generalization that the extent or rate of penetration of a disinfectant under varying conditions of pH, temperature, presence of adjunct substances, etc., into gels of

varied composition can be measured. One substantive observation of both practical and theoretical interest is that, for any particular set of conditions investigated, there is found some minimum concentration of the disinfectant giving almost maximal extent of penetration. Stated another way, with increasing concentrations of the disinfectant a point of diminishing returns sets in which can be readily recognized on inspection of the concentration curves. These "plateau" values are presented in Table 5, along with like values obtained from the curves of the disinfection of chicken feces. The agreement of the values may only be coincidental or may indeed signify that the two dissimilar technics are correlated. This correlation, if real, would signify that the disinfection process in the experiments with chicken feces was entirely dependent upon the penetrability of the disinfectant. This view and some mathematical formulations concerning penetration and disinfection processes are discussed below.

It was believed that the amount of disinfectant diffusing into the agar columns could be related to the extent of penetration and to the nitrogen content of the columns. Iodine can be readily assayed, and thus in systems where columns containing

TABLE 5

THE CONCENTRATION OF DISINFECTANTS AT WHICH DIMINISHING EFFECTIVENESS BECOMES APPARENT IN THE PENETRATION OF GEL COLUMNS AND IN THE DISINFECTION OF CHICKEN FECES

Compound	Plateau Value in Per Cent Concentration	
	From "Penetube" Experiments	From Disinfection of Chicken Feces
Iodine (colloid)	0.05	0.05
Iodine (Lugol's)	0.05	0.05
Iodine (Tincture)	0.05	0.05
Sodium Hypochlorite	0.05	0.1
Chloramine T	0.05	0.1
Succinchlorimide	0.02	0.01
Roccal	0.02	0.02
Phenol	1.0	1.0
Merthiolate (aq.)	0.01	0.02

agar and agar plus protein additives were exposed to identical concentrations of iodine, the loss of iodine from each solution was determined. All trials gave identical losses of iodine from the solutions and still the extent of penetration varied in the manner shown in the experimental section. The nitrogen determination was the same for gelatine and the human serum albumin although lesser penetration occurred in the latter systems. It would then appear that the rate at which molecules of iodine enter the column is strictly a function of the concentration of the iodine and the cross-sectional area of the column in contact with the solution. The extent of penetration would then be a function of the concentration gradient of the unreacted iodine in the columns. The human serum albumin is then intrinsically more reactive with iodine than is gelatine and agar less reactive than either.

The Disinfection Process as Affected by Penetrability

The data and observations recorded in the experimental section may be considered in the light of the disinfection process in general in the hope that some additional light may be shed on this controversial subject.

In the early stages of the process of disinfection the organism (O) may be considered as first entering into a reversible union with molecules of the disinfectant (D) as follows:

[I] $O + D \underset{k_2}{\overset{k_1}{\rightleftharpoons}} (OD)$; where (OD) is an organism–disinfectant complex and k_1 and k_2 the equilibrium constants of the forward and reverse reactions. Succeeding this union, the fate of complex (OD) is death of the organism (O_d) at some specific reaction rate (k_3), hence:

[II] $O + D \underset{k_2}{\overset{k_1}{\rightleftharpoons}} (OD) \xrightarrow{k_3} O_d$; at a steady state, the rate of formation of (OD) must equal the rate of breakdown of the complex in accordance with the following equation:

(1) $k_1 N_f c = k_2[OD] + k_3[OD]$; where N_f is the number of free or unreacted organisms, c the concentration of the disinfectant and [OD] the number of organisms in complex formation. Equation (1) rearranges to:

(2) $\frac{N_f}{[OD]} = \frac{k_3 + k_2}{k_1 c}$; and the total number of organisms (N_t) is equal to the sum of the number of free organisms and those in complex formation (neglecting the number dead); the equation

(2) becomes:

(3) $\frac{N_t}{[OD]} = \frac{k_2 + k_3}{ck_1} + 1$; and if the maximum velocity (K) is a function of the total population and any intermediate velocity (k) a function of the number of organisms in combination with the disinfectant, then:

(4) $\frac{K}{k} = \frac{N_t}{[OD]}$, and letting $\frac{k_2 + k_3}{k_1}$ = a (another constant),

then on substituting equation (4) and this constant into equation (3), equation (5) is obtained:

(5) $k = \frac{Kc}{a + c}$.

We may now compare this theoretical treatment with the data obtained experimentally from the fecal disinfection experiences.

The death of the bacteria in the fecal samples is extremely rapid in the first minute of exposure and if we make the assumption that, in this small time interval, the rate of kill of the bacteria in the sample is logarithmic, then the death of bacteria with respect to time will be a function of the numbers of bacteria originally present, or $-dN/dt = kN$; where k is the coefficient of disinfection, N the numbers of bacteria and t the time of

exposure. Integration of this equation between zero time and one minute yields: $k = \ln N_1 - \ln N_2$; where N_1 and N_2 are the number of bacteria originally present and the number of survivors respectively. The value of k can then be found for any particular concentration of the disinfectant at constant temperature and pH. In accordance with Chick (52), the k values have been determined for a number of the compounds from the experimental data reported above. The value of e for the transformation of the logarithms to the base 10 has been omitted. On plotting these values of k against the concentration, the curve A of Figure 20 for the disinfection of chicken feces by Lugol's Solution at pH 5.0 and 27.5 C was obtained. Considering the equation of this curve to be $k = Kc/(a + bc)$, a Lineweaver-Burk plot (53) equates the value of the three constants so that the equation becomes: $k = 4.6c/(0.03 + c)$. The plot of this equation is shown in Figure 20 as curve B. That the value of constant b in this equation is unity provides a check on the agreement of the empirical equation with the theoretically derived equation (5). The experimental data and the plot of the empirical equation are in very close agreement. Data obtained with phenol (Figure 20, curve C) under the same conditions do not follow the

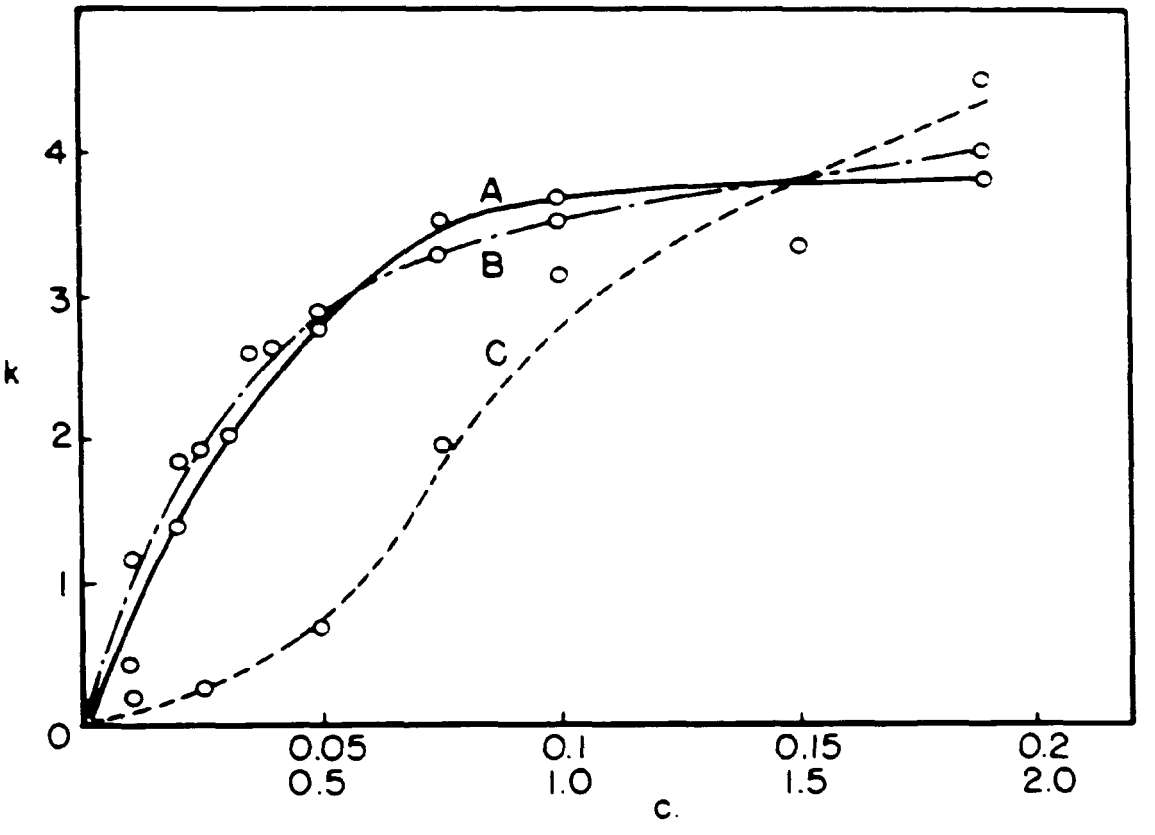


Figure 20. The rate of disinfection (k in log of kill min^{-1}) at increasing concentration (c) of Lugol's Solution (curve A), and Phenol (curve C). Curve B is plotted from $k = \frac{4.6c}{.03+c}$.

same form. This type of deviation is explicable on the basis of the irreversible reaction in formula [II] being dependent upon a multimolecular reaction between phenol and the organisms. Thus, at low concentrations of the disinfectant the rate of increase of the death velocity constant will not be as great as at intermediate concentrations. This is tantamount to saying that phenol has a high coefficient of dilution. The mathematical treatment of such situations is quite complex, but can be done in the same fashion as the treatment of deviated adsorption isotherms where "packing" of molecular layers occurs (54). This treatment will disclose the behavior of phenol to be a special case following the same general equation.

It must be noted that equation (5) is a representation of the Michaelis-Menten equation (55) for enzymatic reactions and, indeed, was derived in analogous fashion. In this derived equation, the constant K represents the maximum rate of disinfection attainable under the prevailing conditions and the constant a represents the concentration of the disinfectant at which half the maximum rate of disinfection occurs.

It can be seen that where disinfection proceeds in accordance with this kinetic picture, an organism-disinfectant

complex capable of dissociation must be formed. If the first step in disinfection is then postulated to be a collision between molecules of the disinfectant and viable organisms, this collision need not necessarily result in an immediate irreversible reaction with consequent death of the organism. If the diffusion of the disinfectants can proceed in any mean free path, then the rate at which molecules of the disinfectant will be desorbed from the surface will correspond to the equilibrium constant for the dissociation of the organism-disinfectant complex. In this study of the disinfection of chicken feces, where the penetration of the particles enclosing the organisms has been made a factor in the limitation of the disinfection process, the appearance of a relationship to this postulated disinfection mechanism has become evident. This implies that the disinfection process and the characteristic curves obtained during the course of disinfection are the result of a first order reaction rate at low concentrations of the disinfectant and a zero order reaction rate at high disinfectant concentrations. This mechanism may be further complicated by the presence of polymolecular reactions and by a nonuniform distribution of resistance of the organisms to the action of the disinfectant. It is obvious that, in

the cited experiments, the population of microbes was a miscellaneous one and that graded degrees of resistance must have and did occur. However, the percentage of resistant organisms present was only approximately 0.01 per cent of the total population and apparently this factor is of a lesser importance than might be expected.

It must be admitted that the derivation of equation (5) was not perfectly rigorous, inasmuch as the number of dead organisms was neglected. The agreement of the empirically derived equation from the experimental facts with the form of the theoretically derived equation would indicate that this neglect was not disastrous. This can be taken to mean that the interaction of the dead organisms with the remaining components of the system in any small time interval is nil.

Another interpretation can be placed upon the coincidence of form of the equations experimentally and theoretically derived for the disinfection process. The resemblance of the equations to the Langmuir Adsorption Isotherm is quite good. The non-identification of constant $\underline{a}$ with unity may merely be a consequence of the unitage employed. The observation of Knaysi (23) that yeast cells remove iodine from solution in accordance

with an adsorption isotherm might be readily taken as evidence that the disinfection mechanism proceeds in the noted order as a resultant of the adsorption mechanism. Contrariwise, although this resemblance is one necessary condition for such a mechanism, this explanation is not sufficient to explain the well-known (but poorly understood) reversal effects obtainable with mercurials and quaternary ammonium compounds. The mechanism proceeding through an organism-disinfectant complex suffices better to explain all the known facts concerning disinfection and affords a ready answer to the role of penetration by the disinfectant precedent to the ensuing lethal reactions.

The kinetics of chemical adsorption may give us additional insight into the role of penetration in disinfection. It is apparent from inspection of the curves presented in Figures 6 to 10, that there is a resemblance to adsorption isotherms. In accordance with the customary derivation of the Langmuir Adsorption Isotherm (54), analogy leads to the derivation of an equation of the same form for the rate of penetration of a disinfectant in the "penetube" technic. This treatment yields equation (6); $k_p = Kc/(b + c)$, where k_p is the length of the zone of inhibition obtained in 24 hours following exposure to the

disinfectant at concentration c at a constant temperature and pH. K then represents the maximum rate of penetration obtainable under the conditions of the test and b represents the concentration at which half the maximal rate is obtained. As in the previous case for disinfection of chicken feces, the empirical data can be fitted to this equation upon evaluation of the constants K and b. In Figure 21, the empirical data for the rate of penetration (expressed as mm per 24 hr.) of Iodine Tincture at varying concentrations into agar containing 1.0 per cent gelatine and 0.5 per cent potassium iodide are plotted (curve A). From these data, the equation $k_p = 22.7c/(0.005 + c)$ is obtained, a plot of which appears in the same figure as curve B. It is tempting, then, again to attempt to explain disinfection mechanics as being entirely dependent upon the rate of penetration of the disinfectant in accordance with equation (6). On the same grounds as before, this is neither necessary nor are there sufficient bases for so doing. It may only be a coincidence that the interconnected processes follow the same rate process. A direct causation connection between two sequential events is not sufficient in steady state mechanics for a single step to be construed as the sole rate-determining factor in the over-all

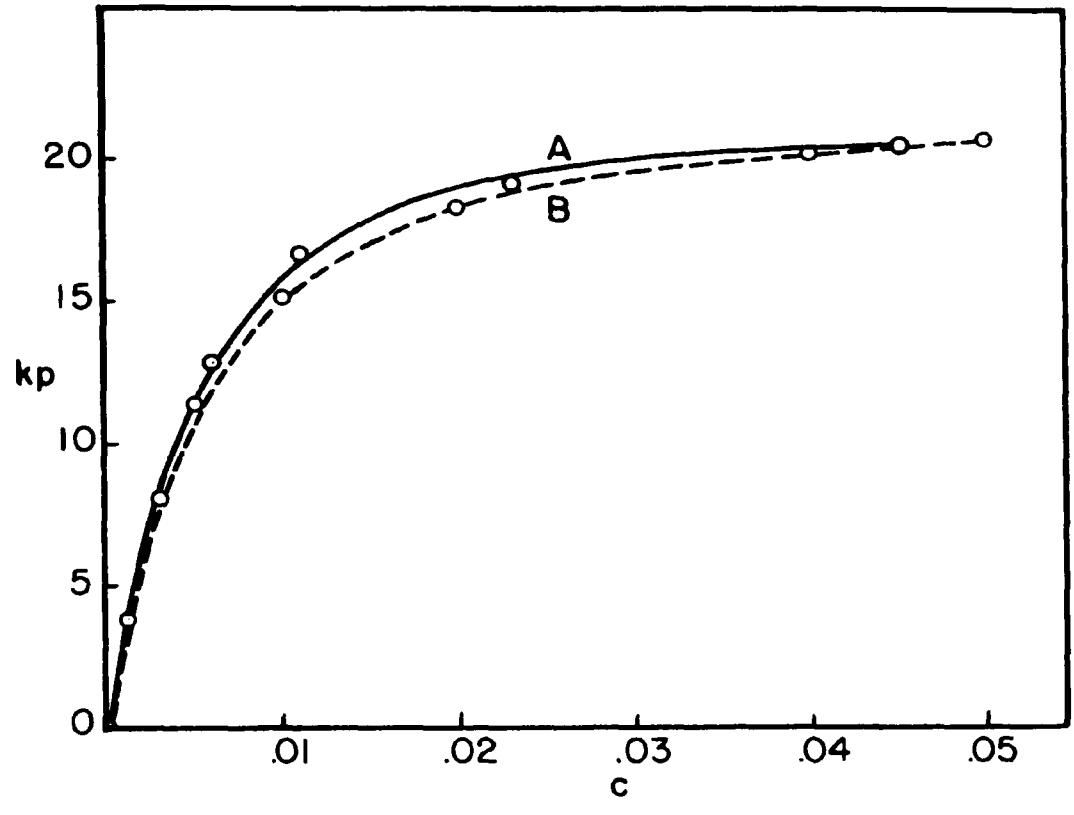


Figure 21. The rate of penetration (kp in mm.) at increasing concentration (c) of iodine in agar + 1.0% gelatine and 0.5% KI. Curve A: experimental data, Curve B: calculated from $kp = \frac{22.7c}{.005+c}$.

process. It appears to this author that the kinetics of death of microorganisms during chemical disinfection must be dictated by the adsorption of the disinfectant from the environment, the subsequent formation of a dissociable complex, penetration of the cytoplasm and a consequent irreversible reaction therein with ultimate death of the organism. The survival curves must be a function of these parametric functions and can lead to a representation of first and zero order reaction rate mechanics. The possibility of higher order reaction rates is not excluded, nor any combination with the lower order reaction rates. The nonuniform distribution of resistance in the population exposed to the disinfectant is certainly a factor influencing the events portrayed. This distribution can be likened to the distribution of activation energies among the molecules reacting in any chemical reaction and so the mechanistic approach envisioned here is capable of rationalization with the biological considerations.

The Penetrative Power of Colloidal Iodine

As has been noted in the experimental section, the penetrative power of Colloidal Iodine has been shown not to exceed

that of other iodine preparations with which it has been compared under the same conditions. These findings are in disagreement with those of Mallmann and Chandler (7) on the disinfection of avian feces and of Anderson and Mallmann (14) on the inhibition of sporulation of coccidial oocysts. These disagreements were shown to be a function of the pH of the various preparations as tested against the oocysts and fecal suspensions. When tested at equal hydrogen ion concentrations, Colloidal Iodine and Lugol's Solution were shown to be equally active in all test systems.

Colloidal Iodine does not have any intrinsically higher penetrative power than other iodine preparations. The preparation as marketed is extremely acid, and because of this acidity exhibits high penetrability and germicidal activity. Iodine, in the author's experience, has been the disinfectant possessing the best penetrative power of the various disinfectants tested. Colloidal Iodine is more efficient in this respect than are Lugol's Solution or Iodine Tincture unless these preparations are acidulated. The low toxicity and low irritant nature of Colloidal Iodine in combination with the high germicidal and

penetrative powers of iodine make Colloidal Iodine a desirable adjunct to any household first aid kit.

For environmental sanitation, the only objection to iodine in any form is its high cost as compared to chlorine. Iodine would seem to be, on other grounds, more desirable for general sanitation than chlorine. Iodine preparations are more stable than chlorine preparations at the acid pH values where both are effective. Chlorine compounds, because of this lability, are generally marketed as alkaline preparations, a condition incompatible with high germicidal activity. It would seem reasonable to this author that if crude iodine preparations could be marketed at reasonable cost iodine would be the disinfectant of choice for many jobs now preempted by chlorine. Gershenfeld and Fox (56) investigated a preparation of crude iodine dissolved in alkali with good results. Perhaps this preparation could be acidulated in the presence of some protective substance to yield a crude iodine hydrosol of low cost for use as an environmental disinfectant or sanitizer.

SUMMARY

A linear diffusion method employing agar gel stationary phases has been developed for the measurement of the penetrability of chemical disinfectants. This "penetube" technic separates the disinfection process from the growth of the indicator organisms and permits the distinction of bactericidal and bacteriostatic effects. Observations on the penetration of various disinfectants were made under different conditions by means of this new technic.

The relation of the penetrative powers of disinfectants as determined by the "penetube" technic to the disinfection of chicken feces was investigated. A hypothesis concerning the role of penetration in the disinfection process was formulated. This hypothesis concerns the formation of a dissociable organism-disinfectant complex as a necessary step in the assumption of the disinfection process as a combination of zero and first order reaction mechanisms. The possible roles of adsorption of disinfectant molecules by the organisms and of the distribution of resistance to disinfection among the component population are also discussed in relation to the hypothesis.

The high penetrability previously claimed for Colloidal Iodine was shown to be a function of its acid condition rather than an inherent property of Colloidal Iodine as such.

BIBLIOGRAPHY

1. Chick, H., and Martin, C. J. The Principles Involved in the Standardization of Disinfectants and the Influence of Organic Matter upon Germicidal Value. Jour. Hyg. 8: 654, 1908.

2. Garrod, L. P. Testing of Disinfectants in the Presence of Organic Matter. Jour. Hyg. 35: 219, 1935.

3. Salle, A. J. and Catlin, B. W. Profile Evaluations of Germicides. Jour. Am. Pharm. Assoc., Sci. Ed. 36: 129, 1947.

4. Wyss, O. Chemical Disinfectants. Ann. Rev. Microbiol. 2: 413, 1948.

5. Bruins, H. R. Coefficients of Diffusion in Liquids. Int. Crit. Tables 5: 63, 1929.

6. Vincent, M. H. Sur la Desinfection des Matieres Fecales Normales et Pathologiques; Etude de la Valeur Comparee des Divers Desinfectants Chimiques Actuels. Ann. l'Inst. Past. 9: 1, 1895.

7. Mallmann, W. L., and Chandler, W. L. On the Disinfection of Avian Fecal Material. Jour. Am. Vet. Med. Assoc. 36: 190, 1933.

8. Johns, C. K. A Method for Assessing the Sanitizing Efficiency of Quaternary Ammonium and Hypochlorite Products. Am. Jour. Pub. Health 37: 1322, 1947.

9. Seelig, M. G., and Gould, G. W. Osmosis as an Important Factor in the Action of Antiseptics. Surg. Gynec. Obst. 12: 262, 1911.

10. Nyiri, W., and Jannitti, M. About the Fate of Free Iodine upon Application to the Unbroken Animal Skin. An Experimental Study. Jour. Pharm. Exp. Ther. 44: 85, 1932.

11. Karns, G. M. The Behavior of Iodine Solutions at Liquid Solid Interfaces. I. The Wetting Power of Iodine from Various Antiseptic Solutions. Jour. Am. Pharm. Assoc. 21: 779, 1932.

12. Karns, G. M., Cretcher, L. H., and Beal, G. D. II. The Importance Thereof in the Preparation of New Iodine Antiseptics. Jour. Am. Pharm. Assoc. 21: 783, 1932.

13. Biskind, M. S. Penetration through Tissue of Iodine in Different Solvents. Proc. Soc. Exp. Biol. and Med. 30: 35, 1932.

14. Anderson, L. P., and Mallmann, W. L. The Penetrative Powers of Disinfectants. Mich. Agr. Exp. Sta., Tech. Bul. 183, 1943.

15. Etchells, J. L., and Fabian, F. W. Technic for Skin Irritation Tests: Technic for Determining Irritating Effect of Chemical Compounds. Jour. Ind. Hyg. 17: 298, 1935.

16. Beijerinck, M. W. Versl. en Meded. Kon. Akad. v. Wetensh., Afd. Natuurk., Amsterdam 3de Reeks, 6: 123, 1889. cited in: van Iterson, G. Jr., den Dooren de Jong, L. E., and Kluyver, A. J. Martinus Willem Beijerinck. Martinus Nijhoff, The Hague, 1940.

17. Kendall, A. I., and Edwards, M. R. A Method for Determining the Germicidal Value and Penetrating Power of Liquid Disinfectants. Jour. Inf. Dis. 8: 250, 1911.

18. Carnot, P., and Dumont, J. Technique d'Etude de la Penetration des Antiseptiques en Milieux Solides. Compt. Rend. Soc. Biol., 81: 1199, 1918.

19. Ruehle, G. L. A., and Brewer, C. L. United States Food and Drug Administration Methods of Testing Antiseptics and Disinfectants. USDA Circ. 198, 1931.

20. Chandler, W. L. On the Vermicidal Value of Iodine. Mich. Agr. Exp. Sta. Quar. Bul. 7: 1, 1924.

21. Knaysi, G. Disinfection I. The Development of Knowledge of Disinfection. Jour. Inf. Dis. 47: 293, 1930.

22. Knaysi, G., and Gordon, M. II. The Manner of Death of Certain Bacteria and Yeasts when Subjected to Mild Chemical and Physical Agents. Jour. Inf. Dis. 47: 303, 1930.

23. Knaysi, G. III. The Taking up of Iodine by Yeast Cells. Jour. Inf. Dis. 47: 318, 1930.

24. Knaysi, G. IV. Do Bacteria Die Logarithmically? Jour. Inf. Dis. 47: 322, 1930.

25. Knaysi, G. V. Some Properties of Frequency Curves and Their Use in Studies of Disinfection. Jour. Inf. Dis. 47: 328, 1930.

26. Chick, H. An Investigation of the Laws of Disinfection. Jour. Hyg. 8: 92, 1908.

27. Rahn, O. Injury and Death of Bacteria by Chemical Agents. Biodynamica, Normandy, Mo. 1945.

28. Fish, F. The Effect of Physical and Chemical Agents on the Oocysts of Eimeria tenella. Science 75: 292, 1931.

29. Mallmann, W. L. Practical Aspects of Disinfection. Soap and Sanit. Chem. 24 (3): 141, 1948.

30. Amann, J. v. Ultramikroskopie der Jodlosungen. Kolloid-Z. 6: 235, 1910.

31. Bordier, H., and Roy, G. Sur l'Iode Colloidal. Compt. Rend. 163: 567, 1916.

32. Chandler, W. L., and Miller, E. J. Colloidal Iodine. Jour. Phys. Chem. 31: 1091, 1927.

33. Bennet, F. C. Colloidal Iodine. Am. Jour. Pharm. 103: 51, 1931.

34. Chandler, W. L., and Ferguson, C. M. Vermicidal Iodine Preparations. Mich. Sta. Bd. Agr., Ann. Rept. for year ending June 30, 1927, 311.

35. Alderman, E. M. Vermicidal Value of Colloidal Iodine (Chandler). Thesis (M.S.) Mich. Sta. Col., 1928.

36. Chandler, W. L. Administration of Iodine Vermicide to Sheep. Mich. Sta. Bd. Agr., Ann. Rept. for year ending June 30, 1929, 159.

37. Weisner, E. S. On the Value of Colloidal Iodine in the Control of Coccidiosis in Poultry and Rabbits. Thesis (M.S.) Mich. Sta. Col., 1928.

38. Chandler, W. L. On the Control of Cecal Coccidiosis in Chickens. Mich. Agr. Exp. Sta., Tech. Bul. 127, 1932.

39. Wilson, F. H. Studies on the Treatment of Habronema Infestations of Horses. Thesis (M.S.) Mich. Sta. Col., 1939.

40. Stafseth, H. J. On the Control of Tapeworm Infestation in Chickens with Notes on the Pathology of the Intestines of the Hosts. Mich. Agr. Exp. Sta., Tech. Bul. 148, 1935.

41. Anderson, L. P. Penetrative Powers of Disinfectants. Thesis (Ph.D.) Mich. Sta. Col., 1939.

42. Gershenfeld, L., and Witlin, B. Iodine as an Antiseptic. Ann. N. Y. Acad. Sci. 53, Art. 1: 172, 1950.

43. Nyiri, W., and Dubois, L. Toxicity of Colloidal Iodine. Jour. Am. Pharm. Assoc. 20: 546, 1931.

44. Miller, E. J. Personal Communication, 1951.

45. Willard, H. H., and Furman, N. H. Elementary Quantitative Analysis. 3rd Ed. D. van Nostrand Co., Inc., New York, 1940.

46. Emmel, M. W. Bacterial Flora in Feces of the Normal Fowl. Jour. Inf. Dis. 46: 293, 1930.

47. Johansson, K. R., Sarles, W. B., and Shapiro, S. K. The Intestinal Microflora of Hens as Influenced by Various Carbohydrates in a Biotin-Deficient Ration. Jour. Bact. 56: 619, 1948.

48. Marks, H. C., and Strandskov, F. B. Halogens and Their Mode of Action. Ann. N. Y. Acad. Sci. 53, Art. 1: 163, 1950.

49. Williams, J. W., and Cady, L. C. Molecular Diffusion in Solution. Chem. Rev. 14: 171, 1934.

50. Stiles, W., and Adair, G. M. The Penetration of Electrolytes into Gels. III. The Influence of the Concentration of the Gel on the Coefficient of Diffusion of Sodium Chloride. Biochem. Jour. 15: 620, 1921.

51. Davis, W. W., Parke, T. V., Daily, W. A., Rushton, L. M., and McGuire, J. M. Some New Procedures and Instruments Useful for Microbiological Antibiotic Testing by Diffusion Methods. V. A Test Procedure Employing Linear Diffusion into Inoculated Agar-Filled Glass Capillaries. Jour. Am. Pharm. Assoc., Sci. Ed. 39: 331, 1950.

52. Chick, H. The Theory of Disinfection. A System of Bacteriology 1: 179, 1930.

53. Lineweaver, H., and Burk, D. The Determination of Enzyme Dissociation Constants. Jour. Am. Chem. Soc. 56, 658, 1934.

54. Brunauer, S. The Adsorption of Gases and Vapors. I. Physical Adsorption. Princeton University Press, Princeton, N. J., 1943.

55. Michaelis, L., and Menten, M. L. Die Kinetik der Invertinwirkung. Biochem. Z. 49: 333, 1913.

56. Gershenfeld, L., and Fox, D. The Bactericidal Efficiency of Crude Iodine in (0.1 N) Alkali. Am. Jour. Pharm. 120: 279, 1948.