CHARACTERIZATION OF AN AGAR COMPONENT WHICH INFLUENCES ANTIBIOTIC ACTIVITY

A Thesis

Presented to

the Faculty of the Department of Biology

University of Houston

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

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by

Frank Joseph Hanus

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ABSTRACT

Bacto agar reversed the effect of basic antibiotics while acidic and amphoteric antibiotics were not markedly effected. The reversal of activity was shown to be due to the presence of a diffusable, non-dialyzable sulfated polyanionic carbohydrate. This substance was shown to bind cationic dyes and form insoluble complexes with some basic antibiotics. Antibiotic activity was not markedly affected by the pore size of the agar or by the presence of diffusable, physiologically active substances in the agar.

Much of the antagonism of Bacto agar was removed by precipitation of the agar with ethanol or by extraction of polymerized agar with ammonium hydroxide. It is possible that the ammonium hydroxide extraction may be commerically feasible and will yield an agar product suitable for the evaluation of antibiotic activity.

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INTRODUCTION

Agar is a complex polysaccharide which is readily extractable from various species of algae belonging to the genus Gelidium. It is composed of two carbohydrates, agarose and agaropectin (Araki, 1959). Agarose is made up of a number of alternating units of β-D-galactopyranose and 3,6-anhydro- α-L-galactopyranose. Agaropectin forms a less rigid gel than does agarose, and appears to contain the same hexose residues esterified with sulfuric acid and pyruvic acid.

A common characteristic of agar is its ability to form stable gels over a wide range of temperatures and concentrations. This feature has made it suitable as a supporting medium for the growth of microorganisms, immunological reactions and electrophoretic studies.

Recent evidence has indicated that agar is not an inert polymer of galactose but that it contains substances which interfere with electrophoretic studies (Brishammar, et al., 1961; Hjerten, 1961; Wunderly, 1960), immunological reactions (Arima and Kurokawa, 1963), and with various microbiological assays in which agar is used. For example agar has been shown to affect the growth of N. gonorrhea, N. meningitidis (Frantz, 1942; Gould et al., 1944; Ley and Mueller, 1946; Casman, 1947), Hemophilus vaginalis (Dukes and Gardner, 1961) and pleuropneumonia like organisms (Yaphe, 1959). Factors have been isolated from agar which alter the growth of type 2 Dengue virus (Schulze and

Schlesinger, 1963), EMC virus (Takemoto and Liebhaber, 1961), mengo encephalomyelitis virus (Colter et al., 1964; Campbell and Colter, 1965). Metallic cations and amino acids (Hayashi and Nonaka, 1962), thiamin (Day, 1942), biotin (Robbins, 1939, 1941), niacin and p-aminobenzoic acid (Ryan, 1950) have been detected in small quantities in agar.

Numerous reports indicate that agar may alter the activities of many antimicrobial agents. Agar has been shown to interfere with phenols (Sands et al., 1963; Sands and Bennett, 1964; Gray and Taylor, 1952; Waksman and Woodruff, 1942; Cook, 1954), acridine (Barker, 1948, 1949), crystal violet (Rose and Miller, 1939), 5-diazouracil (Greenberg, 1960), fatty amines (Hanus and Bennett, 1964), quaternary ammonium compounds (Sherwood, 1942; Hoogerheide, 1945; Quisno et al., 1946), and oleates and stearates (Alexander and Soltys, 1946). The activities of polymyxin (Sands, 1965; Bechtle and Scherr, 1958), kanamycin, neomycin (Sands, 1965), bacitracin (Bechtle and Scherr, 1958), nystatin (Hazen and Brown, 1951; Eisenberg, 1956), streptomycin (Sands, 1965; Iyer and Iyer, 1960), dihydrostreptomycin and furadantin (Truant, 1958) are affected by the presence of agar in the medium. Spaulding and Anderson (1951) noted that organisms that appear resistant to drugs when tested by the disc method are often destroyed by antibiotics in the patient's tissue. Different commercial agars may also produce variable results in evaluations of antibiotics (Sands, 1965; Bechtle and Scherr, 1958; Greenberg, 1960; Parag, 1961; Branch et al., 1960).

This investigation was undertaken with three goals in mind. The first was to determine what factors in agar were responsible for the reduction of antibiotic activity. The second was to determine the mechanism by which these factors act. The third was to devise an efficient and economical method of purification of agar which would render it suitable for the evaluation of antibiotics.

MATERIALS AND METHODS

Organism. A culture of Staphylococcus aureus from the University of Houston stock culture collection was used throughout this investigation. The organisms were maintained on nutrient agar at 4 C and transferred periodically. The inoculum for the various experiments was prepared by growing the organisms for 24 hr at 37 C on nutrient agar and washing them from the agar with sterile isotonic saline. A Klett-Summerson photoelectric colorimeter with a blue filter was used to obtain uniform solutions with a turbidity of 100 Klett units and a cell density of 2.4×10^8 Cells/ml.

Antibiotics. The compounds used in this study were polymyxin, neomycin, kanamycin sulfate, streptomycin sulfate, aureomycin HCl, Terramycin HCl, declomycin HCl, vancomycin, novobiocin sodium, and penicillin G sodium. Concentrated aqueous solutions of the antibiotics were filter sterilized through an ultrafine Morton fritted glass filter and aseptically diluted to the desired concentration in sterile deionized distilled water.

Tube dilution tests. In the initial experiments to determine if agar had any effect on the activity of antibiotics, double strength nutrient broth was prepared and diluted to single strength with 3% Bacto agar or deionized distilled water. Final agar concentration was 1.5%.

Five ml of each medium was pipetted into a series of

screw cap tubes and autoclaved. The tubes were then placed in a water bath at 45 C. The tubes were removed individually from the water bath and 0.1 ml of the antibiotic solution was aseptically added to each tube. The tube was then shaken on the Vortex Jr. Mixer. One-tenth ml of a 10 fold dilution of the standardized cell suspension was then added and the tube was again shaken. When the agar had solidified the tubes were incubated at 37 C for 48 hr. At the end of this period the tubes were examined under 10X magnification for growth.

The approximate inhibitory concentration of antibiotic was first determined by a screening test where concentrations of 0.01, 0.1, 1.0, 10, 100, and 1000 ppm were employed. Once the inhibitory range was determined it was divided into 10 increments. For example if the inhibitory range was between 1 and 10 ppm, the increments were 1 ppm. All data presented are averages of four determinations with the minimum inhibitory concentrations given in parts per million (ppm) of inhibitor (w/v).

Preparation of extracted agars. Washed agar cubes were prepared from Bacto agar using a technique similar to that reported by Schulze and Schlesinger (1963). A 3% aqueous solution of agar was autoclaved for 20 min at 121 C. It was then poured into a shallow pan and allowed to gel. The gel was cut into small cubes and placed in an equal volume of deionized distilled water. The water was changed

every 24 hr. The wash water was stored at 4 C for subsequent investigations.

Ethanol precipitated agar was prepared by the addition of warm ethanol to melted washed agar. The ethanol was slowly added with constant stirring until a stable flocculation appeared. The precipitate was filtered using a Buchner funnel, washed twice with ethanol and incubated overnight in fresh ethanol. The precipitate was again filtered, washed five times with ether and air dried.

Granular NH_4OH extracted Bacto agar was prepared by suspending 50 grams of Bacto agar in 500 ml of a 0.1 N NH_4OH solution. The suspension was incubated at 37 C, the supernatant was decanted at the end of 8 hr, and fresh NH_4OH solution was added. The suspension was incubated at 37 C overnight. The NH_4OH solution was decanted and the granular agar was resuspended in 500 ml of deionized distilled water. The water was changed at the end of 3 hr and at 12 hr intervals for 3 days. The granular agar was removed from the water by filtering through Whatman #1 filter paper in a Buchner funnel and dried on a watch glass at 45 C overnight.

Polymerized NH_4OH extracted agar was prepared from 2% agar cubes. The cubes were placed in an equal volume of 0.1 N NH_4OH and incubated at 37 C. The NH_4OH solution was decanted after 3 hr and fresh NH_4OH was added. The NH_4OH was changed every 12 hr for 3 days and was then decanted. The cubes were washed and stored in deionized

distilled water.

The ether extraction of agar was carried out using a Soxhlet extraction apparatus. Five grams of agar were placed in the Soxhlet cup and extracted with 100 ml of diethyl ether continuously for 6 hr. The agar was then air dried and the residual ether was evaporated in an oven at 40 C. The residue was weighed and resuspended in 10 ml of acetone.

Preparation of agar plates. Antibiotic activity was determined by placing Difco antibiotic sensitivity discs on agar plates prepared with the various agars. The agar media were prepared by melting 3% solutions of the agars with flowing steam. A 2% solution was prepared in the case of the NH_4OH extracted agar cubes. Concentrated nutrient broth (10% w/v) and deionized distilled water were added to the melted agars to adjust all the media to concentrations of 1.5% agar and 0.8% nutrient broth. The media were then sterilized in the autoclave at 121 C for 20 min. One ml of the standardized inoculum was added at 45 C and thoroughly mixed. Ten ml of medium were pipetted into each petri dish and allowed to solidify. The sensitivity discs were placed on the medium and allowed to diffuse for 2 hr at 4 C. The plates were then incubated for 24 hr at 37 C and the diameter of the zone of inhibition was measured under 10X magnification.

Once it was established that some factor in agar could drastically reduce the size of the zone of inhibition of

basic antibiotics while not markedly affecting acidic or amphoteric antibiotics, a series of experiments were designed to illucidate the nature of the factor. These experiments included dye diffusion experiments in Bacto and purified agars, experiments to determine the effect of agar concentration on zone size produced by antibiotics and dyes, and experiments in which Bacto agar was overlayed with a purified agar to determine diffusability and dialyzability of the agar antagonist.

Agar plates for the comparison of the diffusion of dyes were prepared from 1.5% aqueous solutions of the various agars melted under flowing steam in the autoclave. Ten ml of each agar solution were pipetted into petri dishes and allowed to solidify. Thirteen and seven-tenth mm sensitivity discs were placed on the agar medium and 0.05 ml of a 0.25% solution of the various dyes were pipetted to the center of the disc. The plates were then incubated at 37 C for 24 hr. Zones of diffusion were measured as the diameter of the zone of detectable color under 10X magnification. Cationic dyes employed were methylene blue and crystal violet. Anionic dyes used were eosin B and erythrosin.

Agar plates employed to determine the effect of variation of agar concentration and pore size on antibiotic activity and dye diffusion were prepared in the same manner as in the above experiments, however the agar concentration was varied from 0.75% to 4%. Nutrient broth concentration was the same in all antibiotic plates. In these experiments

only Bacto agar and ethanol precipitated agar were employed.

Experiments to determine diffusability and dialyzability of the factor reversing antibiotic activity were carried out using agar plates made up of 2 layers of 1.5% agar. Five sets of plates were prepared. The composition of each set is shown in Table 1. Agar plates for methylene blue diffusion experiments were prepared in the same manner using 1.5% agar solutions containing no nutrient broth.

All layered plates were kept at 4 C for 24 hr to allow diffusion of antagonistic factors. At the end of this time the antibiotic plates were allowed to reach room temperature, and 0.1 ml of standardized inoculum was spread over the surface. Antibiotic sensitivity discs were placed on the surface of the media, allowed to diffuse for 2 hr at 4 C and incubated at 37 C for 24 hr. Methylene blue discs were placed on dye diffusion plates and allowed to diffuse for 24 hr at 37 C.

Purification of the antagonistic factor. Further information regarding the nature of the antagonistic factor in agar was obtained by additional study of the water extract from Bacto agar cubes. The extract from the first and second washings were pooled and further purified. One volume of the water extract was treated 3 times with 4 volumes of diethyl ether in a separatory funnel. The ether fraction was discarded. Two 50 ml samples of the aqueous extract were placed in dialysis bags and dialyzed at room temperature with constant stirring against 2 liters of deionized distilled water. The dialysates were changed

TABLE 1. Preparation of layered agar plates

Set	Bottom Layer	Top Layer
1	Bacto agar Nutrient broth Distilled water	Bacto agar Nutrient broth Distilled water
2*	Bacto agar Nutrient broth Distilled water	Ethanol ppt. agar Nutrient broth Distilled water
3	Ethanol ppt. agar Nutrient broth Aqueous extract	Ethanol ppt. agar Nutrient broth Distilled water
4	Ethanol ppt. agar Nutrient broth Distilled water	Ethanol ppt. agar Nutrient broth Distilled water

* A sheet of sterile dialysis tubing was sandwiched between the layers of Bacto and ethanol ppt. agar in set 5.

at 3, 6, 12, and 24 hr. One of these samples was then treated twice with 20 grams of washed activated charcoal. Two other 50 ml samples of extract were similarly dialyzed against 3 M NaCl and 0.1 N NH_4OH. These 2 samples were then dialyzed against deionized distilled water to remove the NaCl and NH_4OH. Five ml of 1 N NH_4OH was added to a 45 ml sample of extract. This sample was allowed to sit for 4 hr at room temperature, neutralized with 2 N HCl and then dialyzed against water. Each sample was then incorporated in ethanol precipitated agar plates to determine if any of the activity of the sample had been removed by treatment.

Five ml of acetone residue solution from the Soxhlet extraction of Bacto agar was incorporated into 100 ml of ethanol precipitated agar medium and autoclaved to remove the acetone. Agar plates were then prepared to test for antagonistic activity.

Reaction of extract in liquid media. The antibiotic activity of polymyxin, neomycin, streptomycin, novobiocin and penicillin was determined in nutrient broth prepared with deionized distilled water and nutrient broth prepared with pooled water dialyzed extract. A procedure was employed similar to that described for determining the minimum inhibitory concentrations for antibiotics in the tube dilution tests.

Specially designed incubation bottles were used to determine if the extract must come in contact with the organism in order to reverse antibiotic activity. Extract

from the pooled first and second aqueous extractions was concentrated by evaporation. One hundred fifty ml of extract were placed in a dialysis bag and suspended in front of a heated air blower. The volume was reduced to one-tenth the original volume and then dialyzed against water. The water was changed at 3, 6, and 12 hr. The concentrated extract was stored at 4 C and dialyzed before use.

The 250 ml incubation bottles consisted of a reagent type pyrex bottle and a glass stopper with a ground glass seal. A concentric tube was located in the ground glass stopper extending downward into the bottle. The top of the tube could be closed off by a screw cap. The bottle was assembled with a dialysis bag securely fastened to the lower section of the tube. Two hundred ml of nutrient broth were placed in two of the dialysis bottles and sterilized by autoclaving. After the bottles had cooled, a stock solution of antibiotic was added to the broth so that twice the minimum inhibitory concentration was present in the bottle. In one of these bottles, 20 ml of sterile water was introduced through the screw cap top into the dialysis bag. In the other bottle 20 ml of autoclaved concentrated water extract was introduced into the dialysis bag. The bottles were allowed to equilibrate overnight at 4 C. Each bottle was inoculated with 0.4 ml of standardized inoculum and incubated at 37 C for 24 hr.

Spectrophotometric analyses of extract reactions. One-tenth ml of 0.25% methylene blue solution was intro-

duced into two 250 ml Erlenmeyer flasks, each containing 50 ml of water. A dialysis bag containing 10 ml of water was placed in one flask and a dialysis bag containing 10 ml of unconcentrated water dialyzed aqueous extract was introduced into the other. Both flasks were placed in a shaking incubator at 37 C and allowed to equilibrate for 24 hr. The concentration of methylene blue remaining outside the dialysis bags was compared spectrophotometrically using a Beckman DK-2A recording spectrophotometer. A similar experiment with polymyxin was employed to show the ability of the extract to concentrate polymyxin.

Alteration of the spectrum of methylene blue. One-tenth ml of 0.25% methylene blue solution was added to 10 ml of water and to 10 ml of dialyzed extract. The spectra were compared on a Beckman DK-2A recording spectrophotometer using a water reference.

Reaction of extract with antibiotics. One thousand ppm solutions of polymyxin, neomycin, kanamycin, streptomycin, novobiocin, vancomycin, chloramphenicol, declomycin, and penicillin were prepared in 10 ml of extract to show any visible reaction of the antibiotic with the extract. These solutions were allowed to react at room temperature for 3 hr and then observed for the presence of flocculation or the presence of turbidity.

Reaction of extract with metallic cations. One-tenth molar solutions of $BaCl_2$, $FeCl_3$, $ZnCl_2$, $MnCl_2$, $MgCl_2$, LiCl, KCl, and NaCl were prepared in extract to show any

visible reaction between multivalent and monovalent cations and the extract. These solutions were treated in the same manner as the antibiotics.

Chemical properties of the extract. The aqueous extract and the NH_4OH extract from the agar cubes were analyzed to determine some of the general chemical properties of these materials. Dry weights were obtained for each of 8 consecutive extractions. Five ml of extract were placed on each of 2 tared watch glasses and dried at 60 C for 24 hr. Weight of the material removed by each extraction was then calculated. Several samples of dried residue were pooled and ashed in a tared crucible.

Carbohydrate content of the dried residue was determined by an enthrone test (Umbreit et al., 1957) and by the method of Dubois et al. (1956). Sulfate was measured by titration of the HCl hydrolyzed (1 N HCl in the autoclave at 121 C for 2 hr) residue and of the ashed residue by the method of Snell and Snell (1949). Free and amino nitrogen were determined by Nesslerization and comparison with a NH_4Cl standard.

One dimensional paper chromatograms were run at room temperature on Whatman #1 paper for 8 hr. Butanol-acetic acid-water (4:1:5) solvent system was used for one set of chromatograms and isopropanol-pyridine-acetic acid-water (8:8:1:4) was used for the other. Galactose and glucose standards were run on each chromatogram with acid hydrolyzed extract, NH_4OH treated extract and raw extract. Chromato-

grams were sprayed with diphenylamine and aneline reagents (Bailey and Bourne, 1960) and with ammonical silver nitrate (Henry, 1964) and dried in a New Brunswick chromatographic oven at 90 C for 5 min.

Statistical considerations. All results are recorded as the average of two experiments, with the exception of experiments in which zones of inhibition or dye diffusion was measured. In these cases four zones were measured.

To establish the variation inherent in the experimental conditions, duplicate experiments were run on 8 consecutive days. Zones of inhibition were measured on NH_4OH extracted polymerized agar and Bacto agar for neomycin and penicillin. The mean, the sample standard deviation, and the relative standard deviation were calculated by the method of Snedecor (1956).

RESULTS

Effect of agar on the minimum inhibitory concentration of antibiotics. In control tubes containing no inhibitor heavy growth of S. aureus occurred at the surface of the agar and slightly below the surface. In sub-inhibitory concentrations of inhibitor, the organism consistently produced individual colonies on the surface and slightly below the surface. Growth in liquid medium produced a generally uniform turbidity with a slight yellow sediment.

When the inhibitory activities of ten antibiotics were compared in nutrient broth and nutrient broth solidified with Bacto agar, it was noted that the minimum inhibitory concentrations of some antibiotics were greatly increased in the agar medium (Table 2). These data show that the basic antibiotics, polymyxin, neomycin, kanamycin and streptomycin were much more strongly effected by the presence of agar than were the amphoteric or acidic antibiotics.

Extraction of antagonistic factor. It is possible to remove much of the antagonistic effect of agar by extraction with water or NH_4OH or by precipitation with ethanol (Table 3, Fig. 1, 2). Ethanol precipitation and extraction of polymerized agar cubes with NH_4OH were the most effective methods used. Extraction of granular agar with 0.1 N NH_4OH and a water extraction of agar cubes were not as effective, but yielded an agar that was as free of antagonistic factors as Difco Nobel agar. Extraction with hot ether did not greatly reduce the interference.

TABLE 2. Effect of agar on the inhibitory activities of antibiotics against *S. aureus*

Antibiotic	Minimum inhibitory concentration (ppm)* Nutrient Broth	Bacto Agar
Polymyxin	4.0	220.0
Neomycin	0.1	3.0
Kanamycin Sulfate	0.5	3.0
Streptomycin Sulfate	2.25	6.25
Aureomycin HCl	0.15	0.27
Terramycin HCl	0.46	0.68
Declomycin HCl	0.07	0.13
Vancomycin	0.50	0.70
Novobiocin Sodium	0.90	1.00
Penicillin G Sodium	0.10	0.10

* Average of 4 determinations

TABLE 3. Effectiveness of various extractions of agar

	Diameter of zone of inhibition (mm)						
	Bacto	Ether Extracted	NH_4OH Extracted Granular	Bacto Washed	Noble Agar	Ethanol Ppt.	NH_4OH Extracted Polymerized
Polymyxin	10	13	16	17	15	22	22
Neomycin	18	21	21	25	21	32	30
Kanamycin	25	24	32	37	34	47	44
Streptomycin	16	17	20	25	21	27	27
Tetracycline	37	38	38	40	38	42	39
Erythromycin	30	30	30	33	31	34	34
Chloramphenicol	25	25	25	26	25	26	26
Novobiocin	30	29	31	32	31	32	32
Penicillin	45	45	47	49	48	50	48

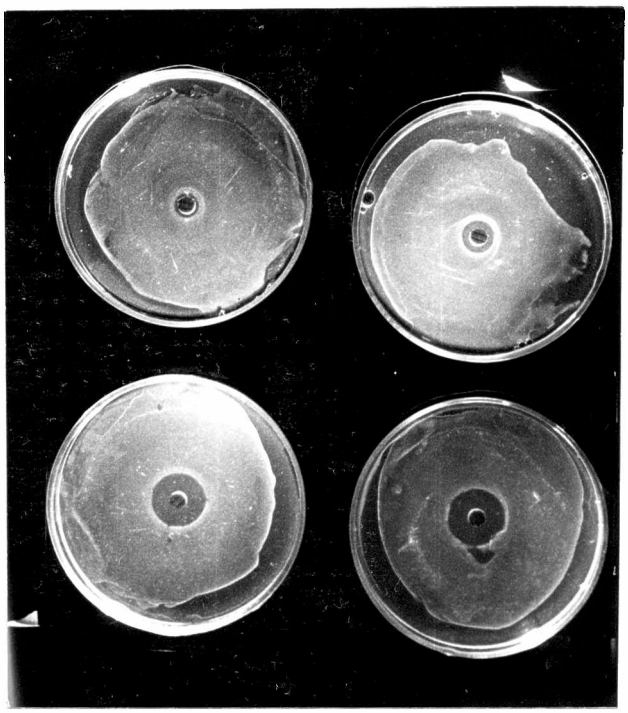


FIG. 1. Effect of extracted agars on the inhibitory zone of polymyxin.

Bacto agar	Washed agar
ETOH ppt. agar	NH_4OH extracted agar cubes

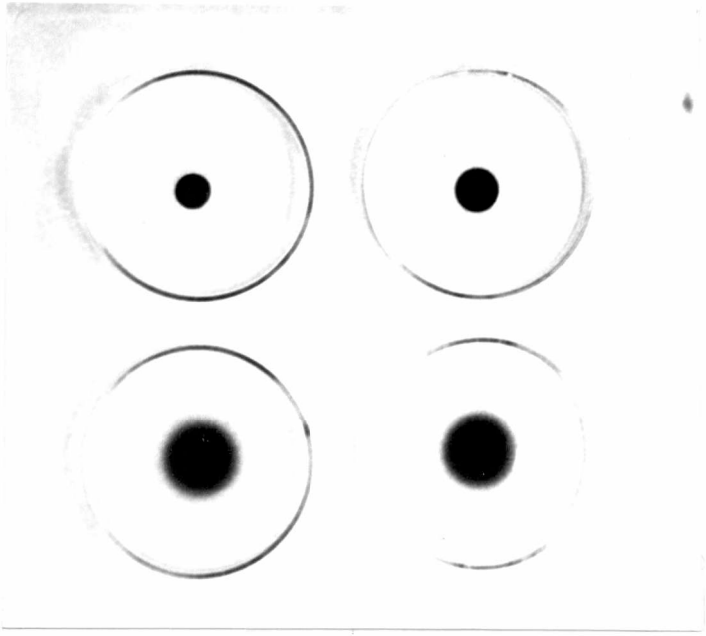


FIG. 2. Effect of extracted agars on the zone size of methylene blue diffusion.

Bacto agar	Washed agar
ETOH ppt. agar	NH_4OH extracted agar cubes

Effect of Bacto agar and ethanol precipitated agar on the diffusion of cationic and anionic dyes. Figure 3 shows a comparison of the zone of diffusion of cationic and anionic dyes in Bacto agar and in the less antagonistic ethanol precipitated agar. Bacto agar drastically reduced the diameter of the zone of diffusion of the cationic dyes, methylene blue and crystal violet. The zones of diffusion of erythrosin and eosin B were the same in Bacto and ethanol precipitated agars.

Variation in pore size of agar. The size of the zones of inhibition of antibiotics were compared in media of different Bacto agar concentrations in Fig. 4. The zones of the basic antibiotics, neomycin and polymyxin, are greatly reduced in size by increasing the agar concentration. The acidic antibiotics, novobiocin and penicillin, are only slightly affected by the increased agar concentrations.

When similar experiments were carried out on ethanol precipitated agar, none of the antibiotics tested were greatly affected by variations in the agar concentrations (Fig. 5).

Figure 6 is a comparison of the zones of diffusion of erythrosin and methylene blue in gels of different Bacto agar concentrations. Methylene blue is strongly affected by increasing the agar concentration from 0.75% to 4.5%, while there is little difference in the size of the zone for erythrosin. When erythrosin and methylene blue were compared on ethanol precipitated agar (Fig. 7) neither

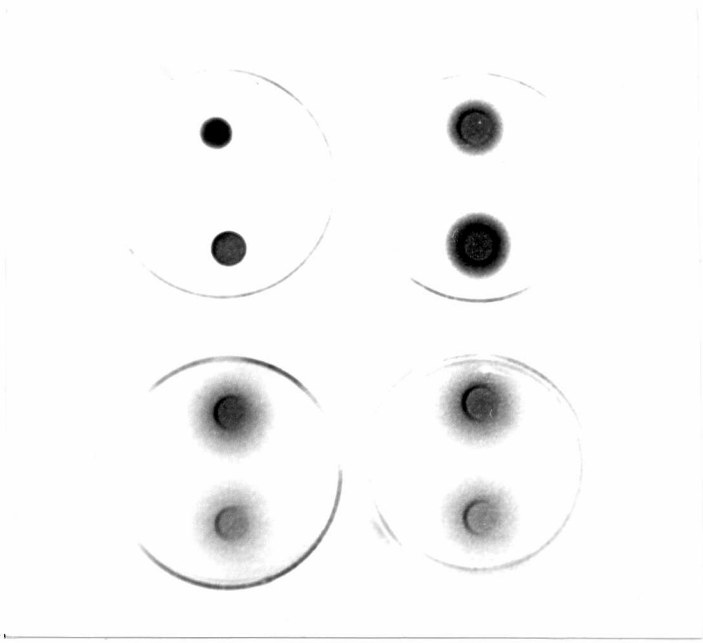


FIG. 3. Diffusion of dyes in Bacto and ethanol precipitated agar.

Left: plates prepared with Bacto agar

Right: plates prepared with ethanol ppt. agar

Dyes listed from top to bottom: crystal violet, methylene blue, erythrosin, eosin B

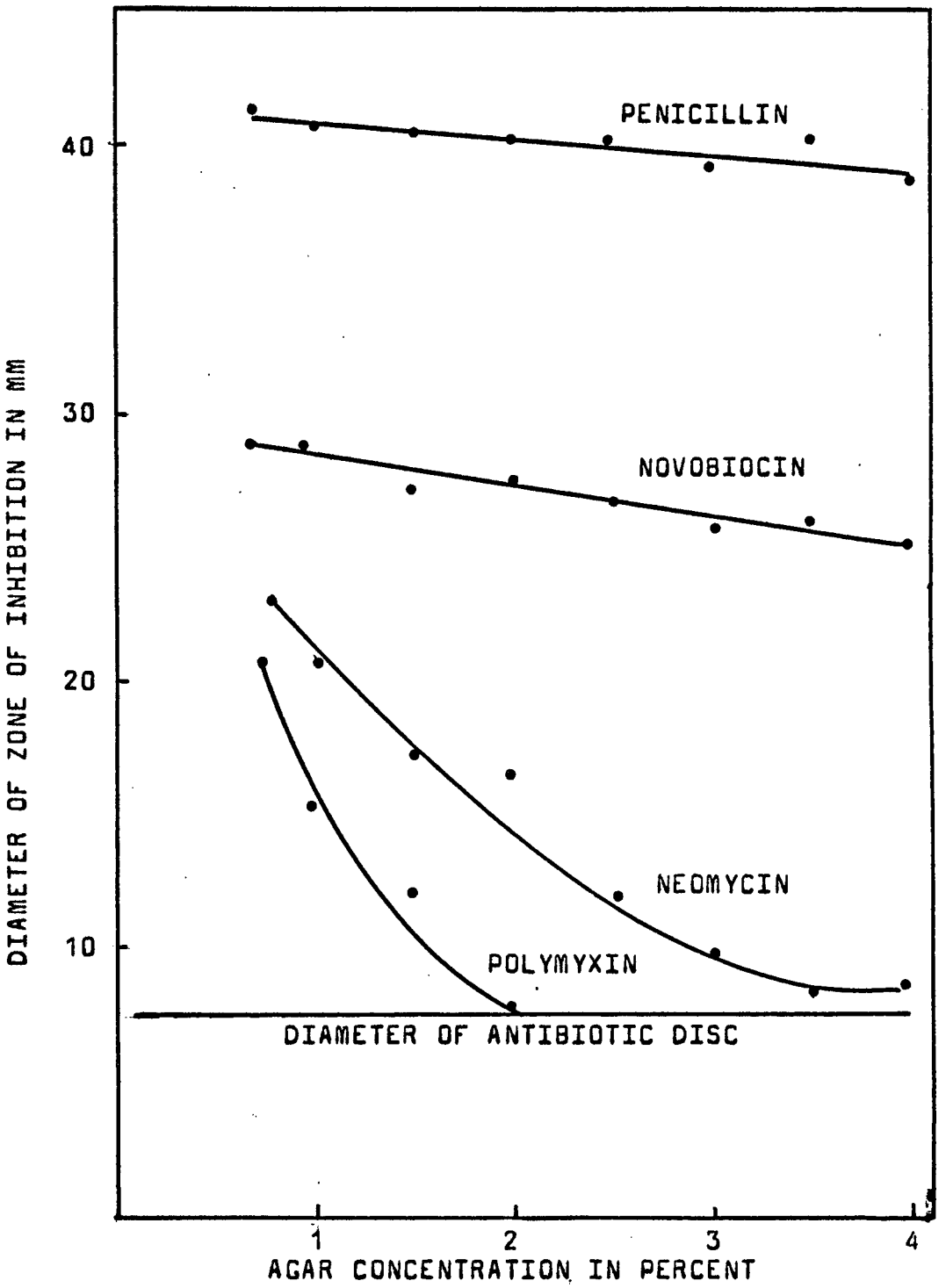


FIG. 4. Effect of Bacto agar concentration on the diameter of the zone of inhibition of four antibiotics

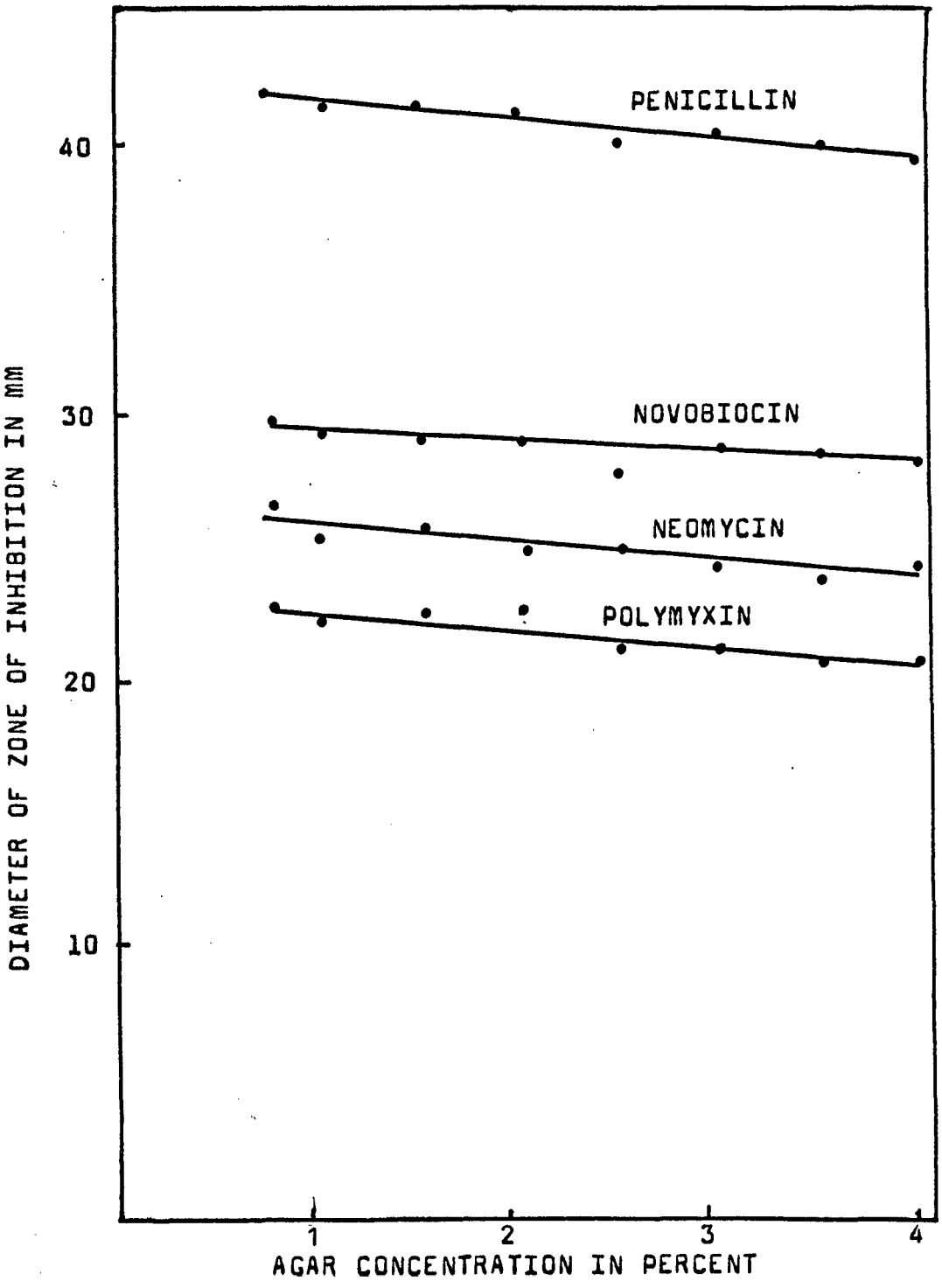


FIG. 5. Effect of ethanol precipitated agar concentration on the diameter of the zone of inhibition of four antibiotics.

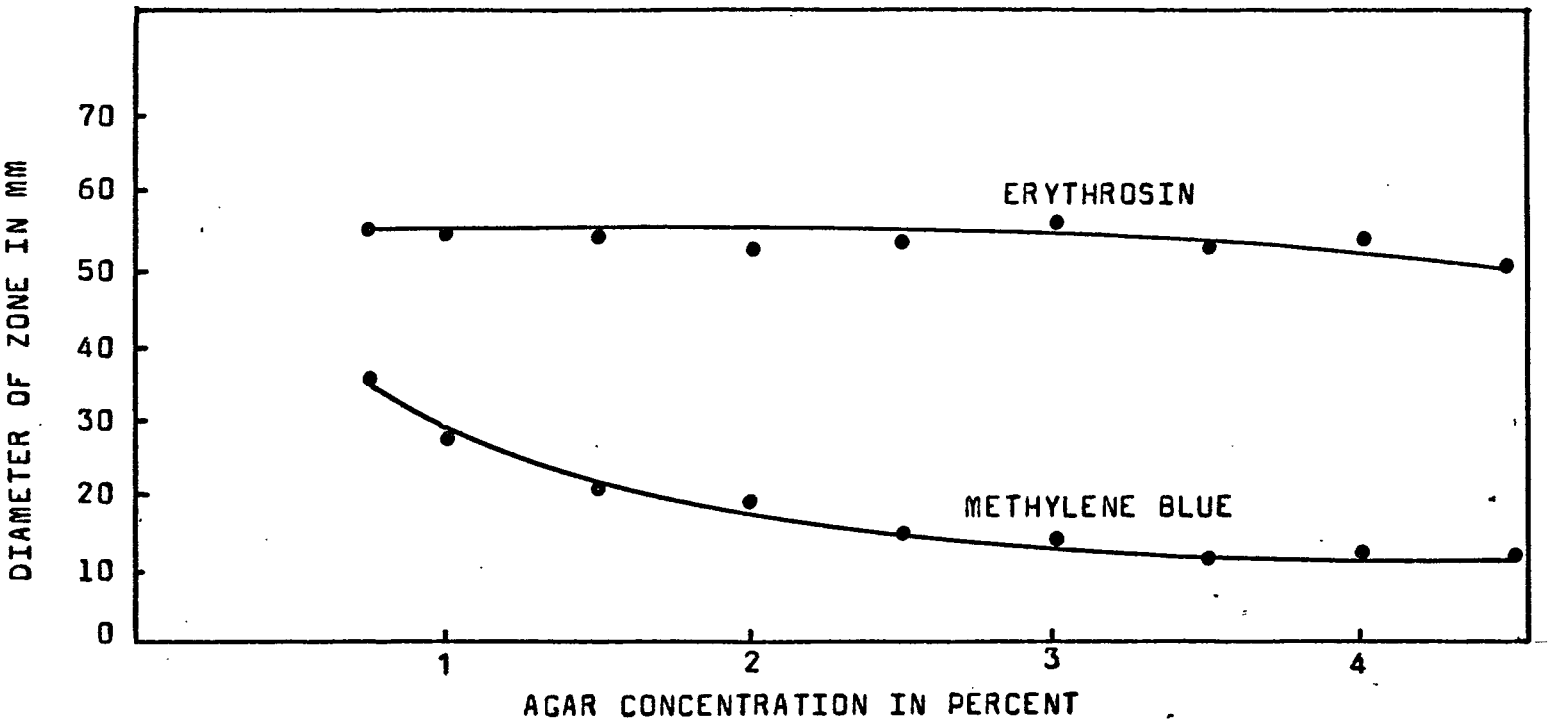


FIG. 6. Effect of Bacto agar concentration on diffusion of anionic and cationic dyes.

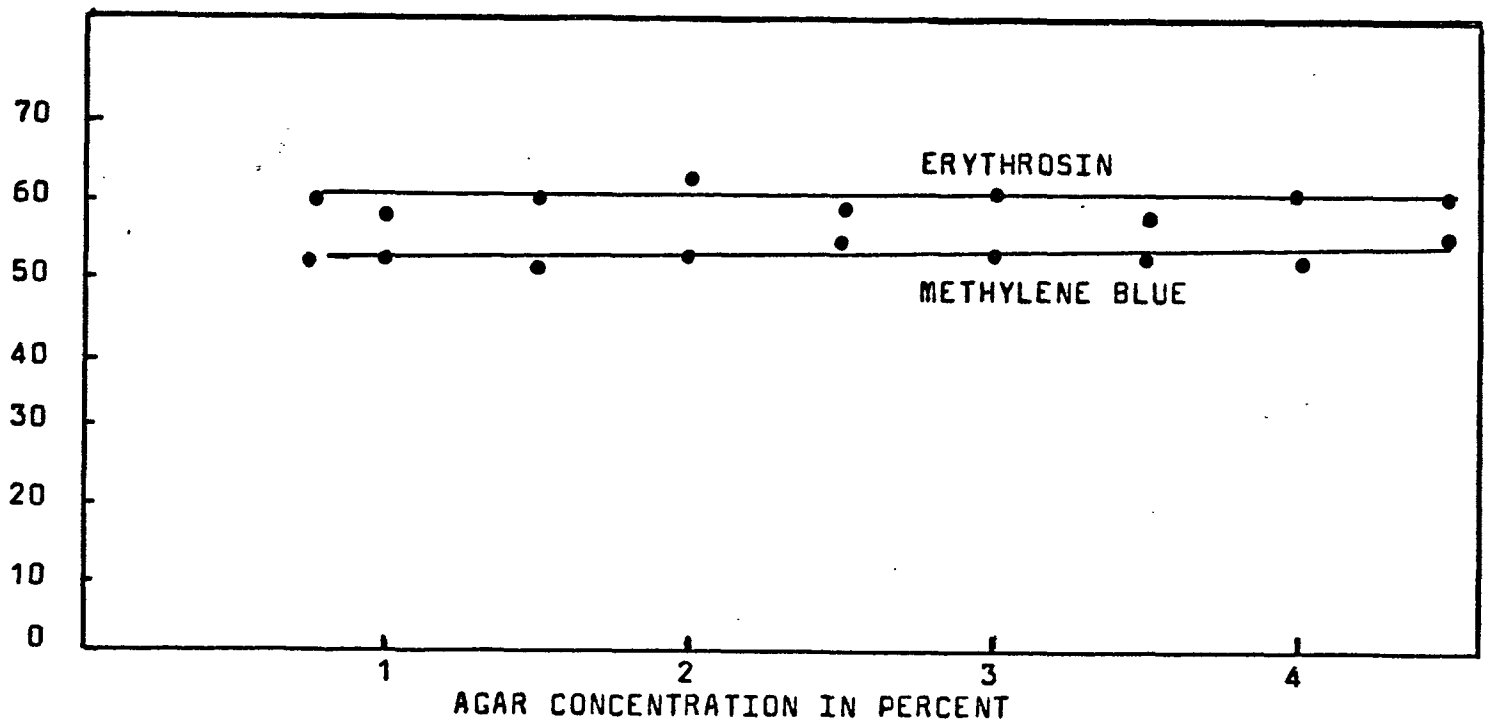


FIG. 7. Effect of ethanol precipitated agar on diffusion of anionic and cationic dyes.

dye was affected by variation in the agar concentration.

The results thus far show that cationic antibiotics and cationic dyes are strongly affected by Bacto agar. Amphoteric or anionic molecules are not greatly affected. Much of this effect may be removed by extraction of polymerized agar with NH_4OH or by precipitation with ethanol. Variations in the pore size of the agar, produced by changing the Bacto agar concentration, only slightly affected the zone size of acidic antibiotics and anionic dyes. The zone size of basic antibiotics and cationic dyes varied greatly with changes in Bacto agar concentration, but when ethanol precipitated agar was used, little change was seen.

Diffusability and dialyzability of the antagonistic factor. Table 4 shows the effect of layering ethanol precipitated agar over Bacto agar. It is evident that after 24 hr the factor in agar that reverses antibiotic activity and inhibits diffusion of basic dyes diffuses readily into the ethanol precipitated agar layer (Fig. 8, 9). It was shown by the interposition of dialysis tubing between the agar layers that the agar antagonistic factor was non-dialyzable in nature.

The non-dialyzability of the agar antagonistic factor was confirmed by experiments carried out in a liquid medium. The water extract of agar, when incorporated into nutrient broth, reversed the activity of those antibiotics affected by agar (Table 5). The antagonist, therefore, had not been removed by dialysis of the water extract.

Experiments carried out in the specially designed

TABLE 4. Diffusion of the antagonistic factor in layered plates

	Diameter of zone (mm)				
	ETOH ppt. / ETOH ppt.	ETOH ppt. / Bacto	ETOH ppt. dialysis Bacto	ETOH ppt. / ETOH ppt. + Ext.	Bacto / Bacto
Polymyxin	18	9	17	9	8
Neomycin	31	19	29	19	18
Kanamycin	32	19	28	20	18
Novobiocin	32	31	29	31	30
Methylene blue	35	20	31	17	20

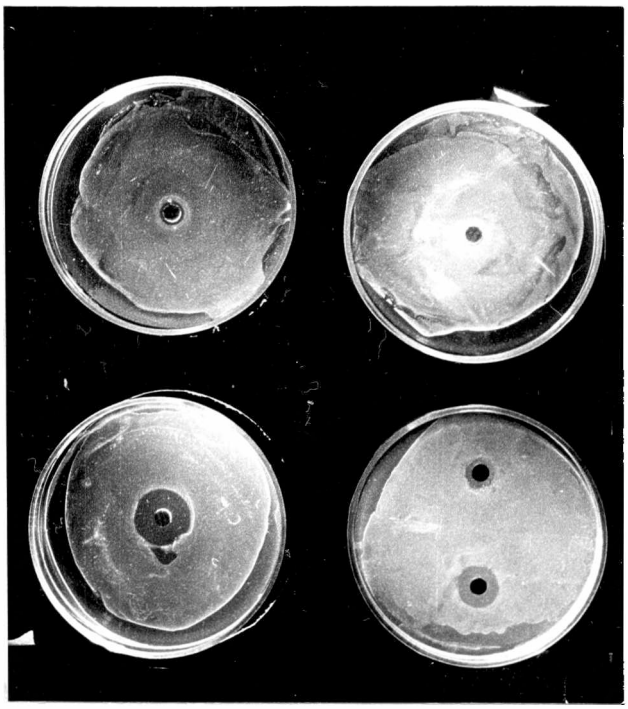


FIG. 8. Zones of inhibition of polymyxin on layered agar plates.

Bacto over Bacto	ETOH ppt. over ETOH ppt. + extract
Ethanol ppt. over ethanol ppt.	Ethanol ppt. over Bacto Dialysis tubing between layers on lower zone

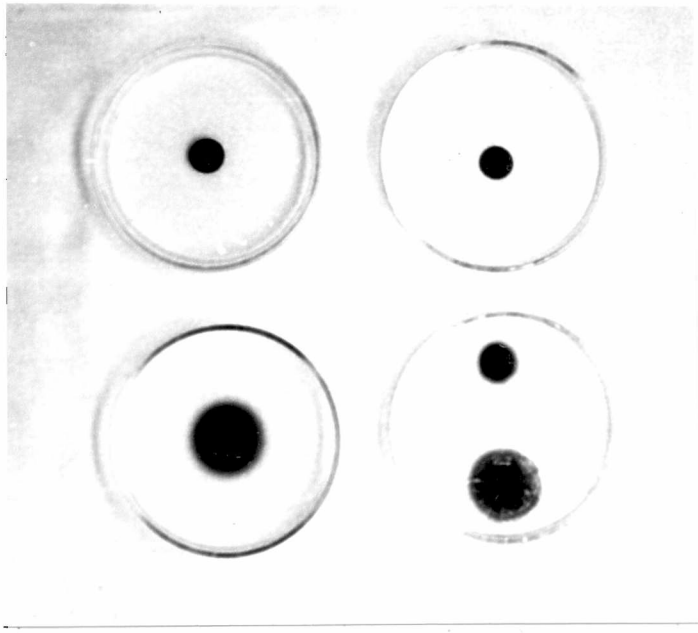


FIG. 9. Zones of diffusion of methylene blue on layered agar plates.

Bacto over Bacto	ETOH ppt. over ETOH ppt. + extract
Ethanol ppt. over ethanol ppt.	Ethanol ppt. over Bacto Dialysis tubing between layers on lower zone

TABLE 5. Activity of extract in nutrient broth

Antibiotic	Minimum inhibitory concentration (ppm)*	
	Broth Prepared in Water	Broth Prepared in Extract
Polymyxin	3.0	250
Neomycin	0.09	3.2
Streptomycin Sulfate	1.8	6.0
Novobiocin Sodium	0.9	1.0
Penicillin G. Sodium	0.1	0.1

* Average of 4 determinations

incubation bottles demonstrated that the dialyzed extract retained its ability to reverse the action of polymyxin and neomycin even when separated from the organism by dialysis tubing (Fig. 10) thus indicating that the extract acts directly upon the antibiotic rather than upon the organism.

Purification of antagonistic factor. Results of the expetiments designed to purify the factor are presented in Table 6. Dialysis against water or NaCl, or extraction with ether did not remove the antagonistic factor from the water extract. Treatment with charcoal produced a slight reduction in activity. NH_4OH irreversibly deactivated the extract. No activity was found in the ether-soluble material present in agar.

Spectrophotometric analysis of extract reactions. Methylene blue reacted readily with the non-dialyzable portion of the aqueous agar extract. When a solution of methylene blue was allowed to dialyze to equilibrium with an extract sample, the extract produced over a 70 fold increase in the concentration of the methylene blue in the dialysis bag. A similar experiment demonstrated that the extract caused a 10 fold increase in the concentration of polymyxin within the dialysis bag. No effect was shown with anionic dyes or acidic antibiotics.

Alteration of the spectrum of methylene blue. When methylene blue was reacted with the extract there was a substantial decrease in the height of the α and β absorp-

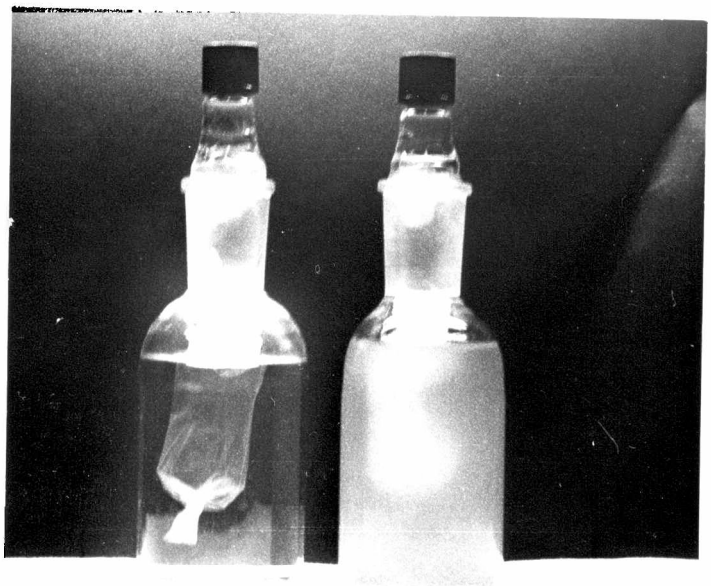


FIG. 10. Inactivation of polymyxin by non-dialyzable factor in aqueous agar extract.
Left: Dialysis tubing contains water
Right: Dialysis tubing contains extract

TABLE 6. Activity of extract after purification

	Diameter of zone of inhibition (mm)				
	Polymyxin	Neomycin	Kanamycin	Streptomycin	Methylene Blue
Bacto Agar	9	16	22	15	20
Ethanol ppt. agar prepared with:					
Ether extracted extract	9	15	23	16	21
Water dialyzed extract	10	16	21	15	20
NaCl dialyzed extract	9	15	20	15	21
Charcoal treated extract	11	17	25	17	24
NH_4OH dialyzed extract	19	30	47	22	31
NH_4OH treated extract	19	29	40	21	33
Ether soluble material	20	32	44	26	35
Water	21	33	46	25	35

tion peaks of the compound with a corresponding increase in the μ band. This is shown in figure 11.

Reaction of extract with antibiotics. Figure 12 shows the visible reaction of the aqueous extract with some of the antibiotics affected by agar. There was visible turbidity produced with kanamycin, streptomycin and neomycin. The extract caused a stable flocculation and precipitation of polymyxin.

Figure 12 also shows that no visible reaction occurred when novobiocin, vancomycin, chloramphenicol, declomycin or penicillin were dissolved in the extract.

Figure 13 shows that some multivalent metallic cations produced turbidity in the extract solutions while the monovalent cations did not. These results indicate that multivalent cations such as polymyxin, neomycin, barium, and ferric ions, can combine with active factors in the extract to produce a stable turbidity, and in some cases, flocculation and sedimentation of the complexes formed.

Chemical properties of the extract. Data are presented in figure 14 for the total dry weight and carbohydrate content of material extracted from Bacto agar cubes for each of 8 aqueous extractions. Table 7 gives a breakdown of the general chemical groups found in this extract and in the NH_4OH extraction of agar cubes. Both extractions yielded a large amount of a sulfated carbohydrate material with approximately 3 hexose units per sulfate group.

The acid hydrolyzed extract contained large quantities of material similar to galactose in rf value and color

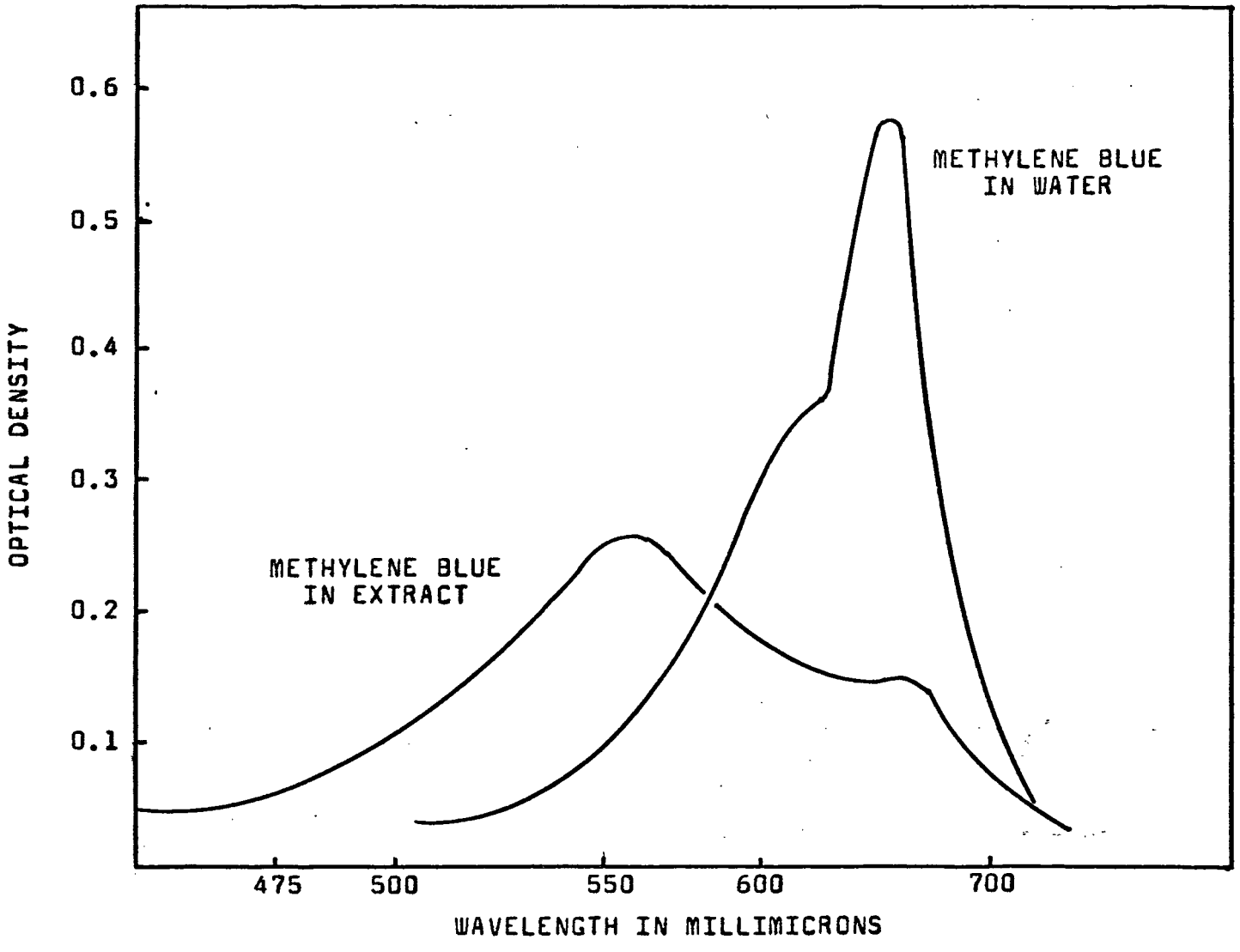


FIG. 11. Effect of agar extract on the absorption spectrum of methylene blue.

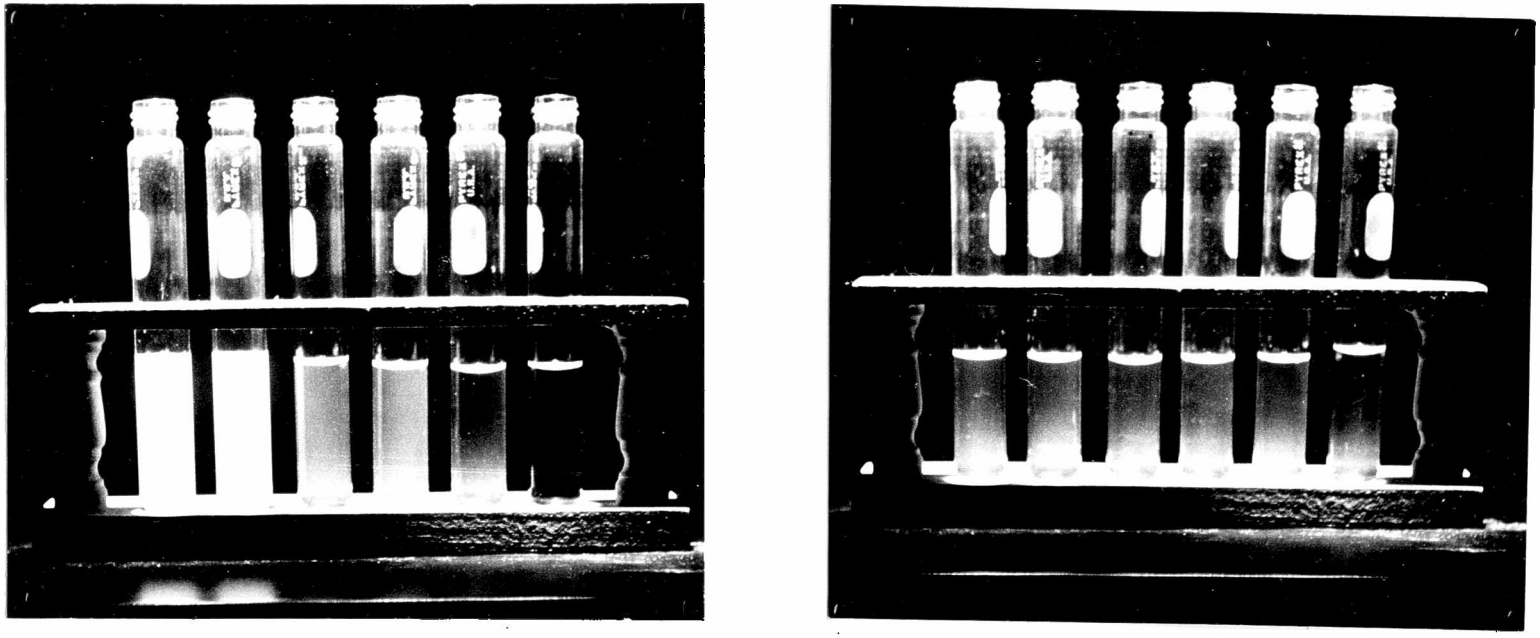
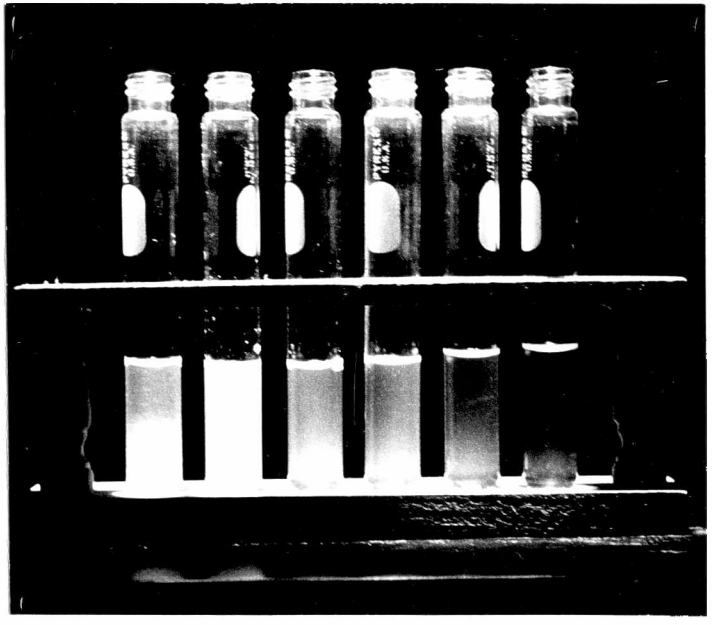


FIG. 12. Precipitation of antibiotics by agar extract.

Left to right: polymyxin, neomycin, kanamycin, streptomycin, extract, water.

Left to right: novobiocin, vancomycin, chloramphenicol, penicillin, declomycin.



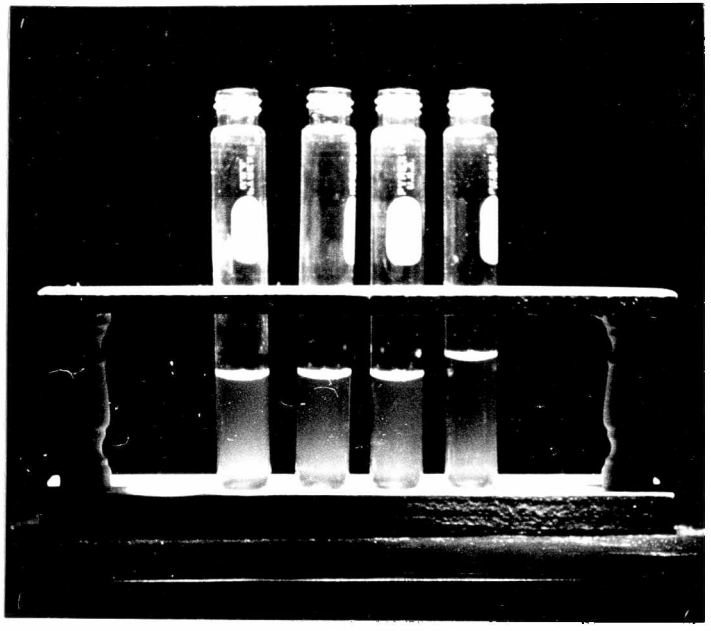


FIG. 13. Precipitation of metallic cations by agar extract.

Left to right: $BaCl_2$, $FeCl_3$, $ZnCl_2$, $MnCl_2$, $MgCl_2$, extract.

Left to right: LiCl, KCl, NaCl, extract.

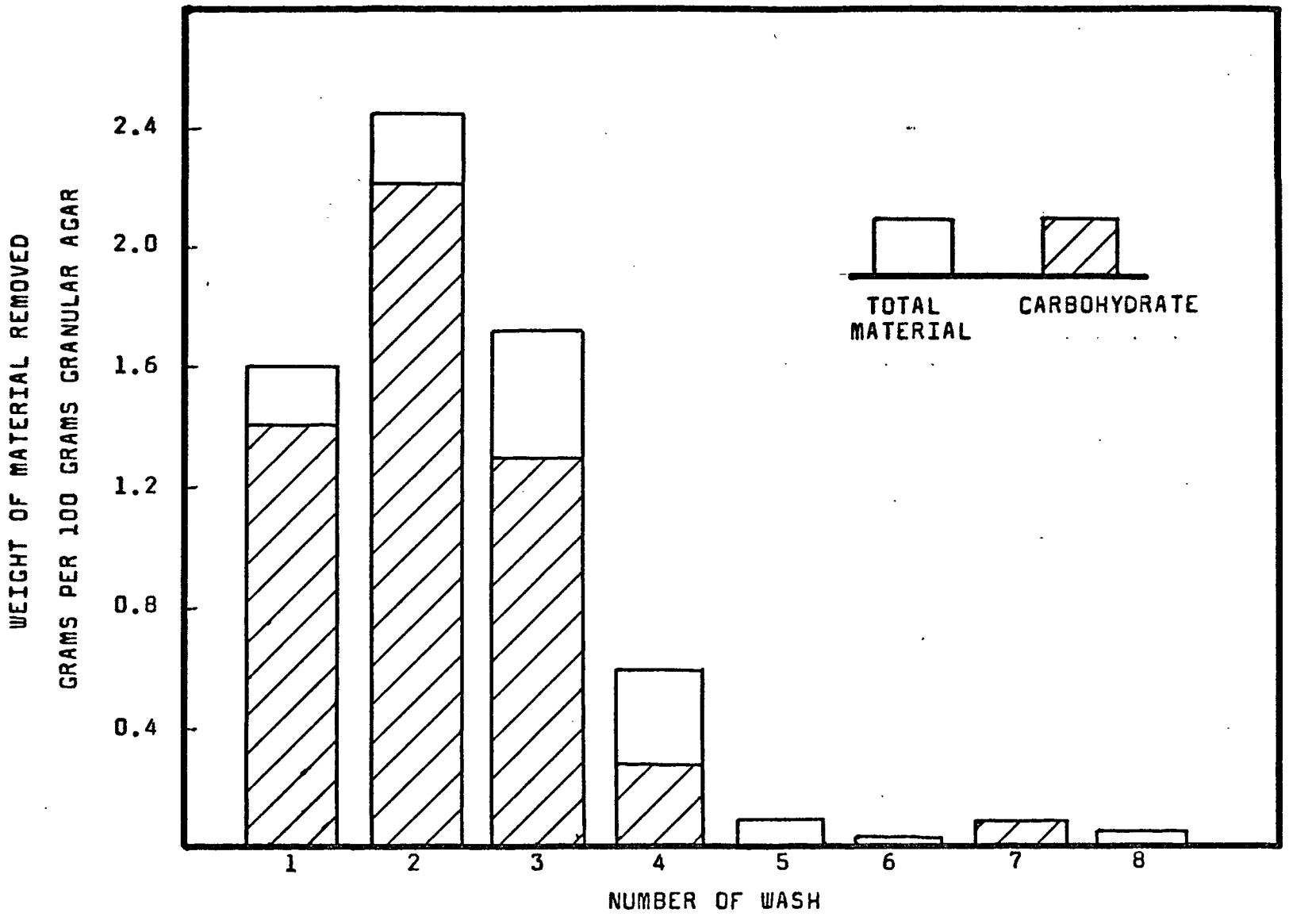


FIG. 14. Dry weight of material removed by each of eight successive water extractions.

TABLE 7. Chemical Properties of material extracted from polymerized agar

	grams/100 grams agar	
	Water Ext.	NH_4OH Ext.
Total amount of water extractable material	6.35	7.97
Carbohydrate		
Anthrone	5.29	6.37
Dubois et al. method	5.12	6.30
Nitrogen		
Total	0.31	-
Amino	0.08	-
Sulfate		
Hydrolyzed extract	0.89	1.01
Ashed extract	0.92	1.12
Ash other than sulfate	0.15	0.17

reaction. The NH_4OH treated extract and the untreated extract contained small quantities of this component. A component was detected in the butanol-acetic acid-water chromatogram of the acid hydrolyzed extract which was similar to gluconic acid lactone in rf value and color reaction. This component was not detected in other chromatograms. The untreated extract and the NH_4OH treated extract contained a great deal of carbohydrate material with an rf value of less than 0.01. This probably corresponds to the undigested polysaccharide.

Statistical considerations. Results of 8 experiments with two antibiotics, performed on consecutive days, are presented in Table 8. The differences observed between Bacto agar and NH_4OH extracted agar are greater than that which would be expected from experimental error.

TABLE 8. Variation in zone size in eight consecutive experiments

Experiment Number	Diameter of zone of inhibition (mm)			
	Neomycin		Penicillin	
	Bacto Agar	NH_4OH Extracted Cubes	Bacto Agar	NH_4OH Extracted Cubes
1	10	30	48	45
2	11	26	48	48
3	11	26	46	46
4	10	33	48	42
5	9	31	48	47
6	10	30	49	48
7	9	30	50	45
8	11	29	47	47
Mean	10.1	29.4	48	46
Sample Standard Deviation	0.84	2.41	1.19	2.00
Relative Standard Deviation	8.3%	8.2%	2.5%	4.3%

DISCUSSION

Agar is ideal for use in microbiological investigations because of its low cost and ease of preparation. Since few organisms are capable of digesting it, it is more suitable for use than starch or gelatin gels. Its interference with activities of antimicrobial agents; however, makes results somewhat questionable when investigations are carried out using agar in the test medium.

There are three ways in which agar might interfere with the testing of antimicrobial agents. Fleming (1943), Hoogerhaide (1945), and Bechtle and Scherr (1958) felt that antagonism might result from limitation of free diffusion and convection of the antimicrobial agents. It is also possible that some of the physiologically active substances reported in agar may render the organisms less sensitive to antibiotics. Many antibiotics are inactivated by the presence of metallic cations (Donovick, et al., 1948; Zolli et al., 1961; Weinberg, 1960; Price, 1957; Newton, 1953). It is also possible that antibiotics are bound to substances in the agar gel which reduce their activity. Quisno et al. (1946) found that granular agar could adsorb large quantities of a quaternary ammonium compound.

The results presented here indicate that the antagonist is not a small dyalyzable particle such as thiamin, biotin, or metallic cations. The results also show that the pore size of the agar gel has little to do with anti-

biotic activity. Agar's reversal of antibiotics is related to the presence of a sulfated polysaccharide which is present in the agar gel and which may adsorb basic antibiotics.

Since purification of agars has been shown to reduce their interference for some antimicrobial agents (Greenberg, 1960; Sands and Bennett, 1964; Sands, 1965) attempts were made to purify Bacto agar to remove the deleterious factors. Table 3 shows the results of these various extractions. Ethanol precipitation and extraction of polymerized agar with NH_4OH removed the largest amount of antagonistic material. Extraction with water substantially improved the quality of the agar.

In an effort to determine if positively charged molecules were affected by agar in the same manner in which basic antibiotics were affected, cationic and anionic dyes were diffused in Bacto agar plates and ethanol precipitated agar plates. Figure 3 shows that the positively charged methylene blue and crystal violet do not diffuse freely in Bacto agar. The increased diffusion of cationic dyes in ethanol precipitated agar cannot result from an increase in pore size of the agar due to precipitation of the agar because erythrosin and eosin B diffuse to the same extent in both agars.

Figure 4 shows that the zone produced by antibiotics does not vary greatly as a result of variations in the concentration of the agar. The zones produced by basic antibiotics are drastically reduced by increasing agar

concentrations. When similar experiments were performed in ethanol precipitated agar, none of the antibiotics were greatly affected by variations in agar concentrations. This indicates that variations in pore size of the agar do not markedly affect the size of the zone of inhibition of antibiotics tested. It appears then, that limitation of diffusion has little to do with antagonism of antibiotics. The increased interference shown in Bacto agar with increased concentration probably results from increased concentration of antagonistic substances. Figures 6 and 7 show the results of similar experiments performed with methylene blue and erythrosin. It is obvious that the same hypothesis holds true here. The large variations of zone size of methylene blue in only Bacto agar probably result from the variations in the concentration of factors in Bacto agar which bind this positively charged dye. 1

Table 4 and figures 8 and 9 show the effect of layering ethanol precipitated agar over Bacto agar. This experiment was designed to determine if the antagonistic factors present in Bacto agar are free to diffuse into the upper layer of ethanol precipitated agar, and if interposition of dialysis tubing between these layers would stop the movement of this factor to the ethanol precipitated layer. The results show the factor to be diffusable and non-dialyzable. If the factor which reverses the activity of antibiotics were a small particle such as a growth factor or metallic ion, one would expect the factor to be

both diffusable and dialyzable. This experiment shows that this is not the case. The experiment also shows that the aqueous extract obtained by washing agar contains a diffusable antagonistic factor.

The aqueous extract of agar, when incorporated into nutrient broth, reversed the activity of the same antibiotics antagonized by agar (Table 5). Novobiocin and penicillin were not antagonized.

The dialyzed extract reduced the activity of neomycin and polymyxin even when separated from the organism by a dialysis tubing. This again shows the factor to be non-dialyzable since the dialyzed extract retained its activity, and it shows the factor must react with the antibiotic and not the organism in order to reverse the activity of antibiotics. This indicates that growth factors and metallic cations are not the substances in agar that reverse antibiotic activity. Ryan (1950) showed that Bacto agar contained negligible amounts of vitamin impurities. It is unlikely that the small amount of vitamins and impurities in Bacto agar could influence the activity of antibiotics when a complex substance such as nutrient broth is used as a growth medium.

Results of experiments designed to purify the extract are presented in Table 6. This again shows the factor to be present in large quantities in the water extract. These results again indicate the factor is non-dialyzable against water or a high concentration of NaCl. The extract was

shown to be deactivated by NH_4OH. This may have been due to hydrolysis of the active factor in the extract or to a combination of the extract with the NH_4^+ resulting in a complex which was nonreactive with basic antibiotics.

No antagonistic activity was present in the ether extracted material. This may be correlated with the results in Table 3 which show no reduction in antagonistic activity of the ether extracted Bacto agar. The reduction in diffusion of methylene blue in all cases paralleled the reversal of antibiotic activity.

When methylene blue was dialyzed to equilibrium with the extract, the extract produced over a 70 fold increase in the concentration of the dye within the bag. The extract was also shown to concentrate polymyxin. Since there is not a 1:1 relationship between methylene blue and reactive sites on sulfated polysaccharides (Walton and Ricketts, 1954), it was not possible to determine how many such sites were available in the water extract to react with methylene blue molecules.

Agar and other sulfated polysaccharides such as heparin and chondroitin sulfate are capable of causing a shift in the visible spectrum of methylene blue (Kelly, 1955; Walton and Ricketts, 1954; Levine and Shubert, 1953; Michaelis and Granick, 1945). There is a direct relationship between metachromatic activity and sulfate content (Walton and Ricketts, 1954). Figure 11 shows the shift in the spectrum of methylene blue induced by reaction with the extract.

This spectral shift is almost identical to those observed for other polyanions when reacted with basic metachromatic dyes such as crystal violet and methylene blue (Shubert and Levine, 1955; Walton and Ricketts, 1954; Levine and Shubert, 1953; Michaelis and Granick, 1945). These results indicate high concentrations of polyanionic material in the dialyzed extract.

If large quantities of polyanionic material such as a sulfated polysaccharide were present in the extract, one would expect formation of complexes with polycationic substances such as polymyxin and neomycin. Figure 12 shows that the basic antibiotics do form a complex with the extract. Polymyxin actually reacts to such an extent that the complex flocculates and settles out of suspension. Acidic and amphoteric antibiotics produced no visible change in the extract. Polyvalent metallic cations were also capable of producing turbidity in the extract, while monovalent cations produced no visible change.

The results of the reactions with methylene blue, cationic antibiotics and polyvalent metallic cations indicates the presence of a polyanionic molecule in the extract. Other investigators (Takemori and Nomura, 1960; Takemoto and Liebhaber, 1961; Agol and Chumakova, 1962; Schulze and Schlesinger, 1963; Colter et al., 1964) have isolated a water soluble sulfated polysaccharide with a molecular weight of approximately 50,000 (Campbell and Colter, 1965). The data presented in Table 7 indicate the

presence of a sulfated polysaccharide with approximately three hexose units per sulfate. This represents the factor in agar responsible for the reversal of antibiotic activity.

These experiments have shown Bacto agar to interfere with the activity of basic antibiotics. The action of agar on antibiotics is closely paralleled by its action on cationic dyes. Spectral shifts induced in methylene blue and complex formations with basic antibiotics and multivalent metallic cations indicate the substances responsible are polyanionic molecules. This is borne out by a chemical determination. Limitation of diffusion and reversal by dialysable growth factors and metallic ions have been eliminated as possible causes of antibiotic reversal.

SUMMARY

Bacto agar reversed the effect of basic antibiotics while acidic and amphoteric antibiotics were not markedly affected. The reversal of activity was shown to be due to the presence of a diffusable, non-dialyzable sulfated polyanionic carbohydrate. This substance was shown to bind cationic dyes and form insoluble complexes with some basic antibiotics. Antibiotic activity was not markedly affected by the pore size of the agar or by the presence of diffusable, physiologically active substances in the agar.

Much of the antagonism of Bacto agar was removed by precipitation of the agar with ethanol or by extraction of polymerized agar with ammonium hydroxide. It is possible that the ammonium hydroxide extraction may be commercially feasible and will yield an agar product suitable for the evaluation of antibiotic activity.

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