THE EFFECT OF CERTAIN ANTIBIOTICS, CRYSTAL VIOLET AND SODIUM AZIDE ON THE "IN-VITRO" CELLULOSE DIGESTION BY RUMINANT MICROORGANISMS

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THE EFFECT OF CERTAIN ANTIBIOTICS, CRYSTAL VIOLET AND SODIUM AZIDE ON THE "IN-VITRO" CELLULOSE DIGESTION BY RUMINANT MICROORGANISMS.

By

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Introduction

Ruminating animals, such as the bovine, the sheep, and the goat, consume and digest large quantities of cellulose-containing roughage feeds. Cellulose is a major source of carbohydrate energy. Repeated investigations have failed to show the presence of cellulase in the digestive juices of the ruminants (8). The microbial population of the rumen is mainly responsible for the breakdown of cellulose into soluble products available to the animal. Henneberg (cited by Huffman 13) and Baker and Harriss (1) studied microscopically the partially decomposed contents of the rumen and observed cellulolytic bacteria within the eroded cavities of the plant material. Gall et al. (12) and Hungate (14) isolated in pure culture many types of these cellulolytic microorganisms in numbers high enough to designate them as major physiological types in the rumen.

The final products of ruminant cellulose digestion are mainly butyric acid, propionic acid, and acetic acid (7), and gases. The intermediary stages in the conversion of cellulose to the fatty acids most likely are cellobiose, glucose, lactic acid, and pyruvic acid (7, 23, 33). The fatty acids are absorbed through the wall of the rumen by the blood, from which they are probably either oxidized directly for energy or synthesized into storage fats or carbohydrates (10).

All the constituents of a complex microbial association inhabiting a natural milieu are influenced, directly or indirectly, by other members of the population (32). Undoubtedly, synergistic, symbiotic, and antagonistic relationships exist within the population. However, on a given substrate, the ruminal population seems to remain fairly stable (12). One net resultant of the ruminal microflora is an active cellulolytic fraction. This fraction and other microorganisms are concerned with proteolysis, starch hydrolysis (30), urea hydrolysis, and the fermentation of hexoses to fatty acids. The associative population also possesses the ability to synthesize amino acids (18), vitamins (16), bacterial protein (20), and bacterial carbohydrates (1), for the use of other members of the microbial population and the ruminant itself.

The present study was undertaken to investigate the effect of penicillin, streptomycin, neomycin, chloromycetin, crystal violet, and sodium azide upon the "in vitro" cellulose digestion by the mixed ruminant microflora. This "in vitro" work was initiated to gain information concerning the cellulolytic activity of streptococci isolated from the rumen. It was known that streptococci grow in the presence of sodium azide but are inhibited by crystal violet. The mixed ruminal microflora were seeded into a

medium containing cellulose (filter paper) and various bacteriostatic agents. Cellulose digestion occurred in the presence of crystal violet but was almost completely inhibited by sodium azide, suggesting that the streptococci were not involved in cellulose digestion. These observations indicated the possibility of utilizing bacteriostatic or bactericidal agents in the study of ruminal population. Several antibiotics with diverse bacterial spectra were obtained for this study.

Any "in vitro" method that deals with a mixed bacterial population must simulate as closely as possible the environmental conditions encountered "in vivo". No change should occur in the proportional numbers and specific activity of the microflora under the artificial conditions to assure the same metabolic efficiency that occurs naturally. Marston (23) considered the following as the main factors that determine the normal rumen population:

1. low oxygen partial pressure
2. high carbon dioxide pressure
3. a continuous supply of phosphate and inorganic nitrogen from the saliva
4. temperature of 40±1°C.
5. the massive inoculum of associative organisms in the residual contents of the rumen
6. the buffering capacity of the saliva

Another factor is the continuous removal of the products of

cellulose digestion through the wall of the rumen.

Woodman and Evans (33) studied the production of fatty acids "in vitro". The inoculum was the ninth subculture from the rumen contents of the sheep which had been incubated at 37°C. in the presence of filter paper cellulose. The fermentation was carried on in a flask at 37°C., and the action was allowed to proceed until the cellulose disappeared. The method of these workers failed to simulate the conditions of the rumen satisfactorily. Strict anaerobiosis was not maintained, the pH was not regulated, the fatty acids were not removed, and the temperature of incubation was lower than that existing in the rumen.

Marston (23) devised a method that more closely approximated the conditions of the rumen by regulating the pH, maintaining the temperature at 40°C ± 1, and maintaining anaerobiosis by passing nitrogen continuously through the system. Louw et al. (19) further improved the "in vitro" technique by introducing a method for removing the fatty acid products of digestion. The fermentation was carried on in a semi-permeable sac that was suspended in a large volume of an aqueous growth medium. The fatty acids dialyzed out of the field of fermentation. Louw's results showed that the rate of cellulose digestion was more rapid in the semi-permeable bag than in an ordinary glass container.

Experimental

Crystal violet and sodium azide study

The technique utilized in the streptococci study was simpler than that later devised for the antibiotic work. The fermentation vessels were round-bottom, 500-ml flasks fitted with two-holed rubber stoppers for the nitrogen gas inlet and outlet. Seven grams each of #12 Whatman filter paper (ground to pass through the medium sieve of a Wiley mill) were weighed into eleven flasks. Two ml each of 1M $MgSO_4$, two ml of 2M $(NH_4)_2SO_4$, two ml 0.5M $CaCl_2$, five ml of 1M KH_2PO_4, 0.5 mg $FeSO_4$, 0.25 mg $CuSO_4$, 0.5 mg $ZnSO_4$, and 0.3 mg $CoCl_2$ was added to each flask. Two ml of 4 per cent azide was added to three flasks, ten ml of 1:10,000 dilution of crystal violet was added to three flasks, and three flasks were maintained as positive controls. Enough distilled water was added to each flask to make the final volume 250 ml, after 200 ml of the rumen ingesta was added. The other two flasks were negative controls for ascertaining the cellulose content of the ruminal ingesta. The flasks with the rubber stoppers, rubber tubing, collection flask, and other equipment were sterilized prior to use by autoclaving at 15 pounds pressure for 15 minutes.

The rumen samples were collected from a Holstein cow on an ordinary hay and grain ration. The sample was filtered through four layers of cheese cloth into a sterile two-liter flask. The liquid was

pressed from the solid ingesta manually. The sample was taken at 12:30 PM to assure an active cellulolytic microflora and immediately transported to the laboratory. Two hundred ml aliquots were added to each flask and then mixed in a mechanical shaker while nitrogen gas was passed serially through the system. The nitrogen passed through a 200 ppm Roccal solution and sterile water, respectively, before entering the first flask. The flasks were then placed in a thermostatically-controlled water bath set at 40° C and nitrogen gas was again bubbled vigorously through the flasks for five minutes. The gas rate was then decreased. The pH was checked periodically and maintained at pH 6.2-6.8 with 1N Na_2CO_3. Every 24, 36, and 48 hour period of incubation one series consisting of a control, an azide flask, and a crystal violet flask was removed. To stop the fermentation in the incubated flasks and the negative controls, 10 ml of a 1:10 solution of Roccal was added to the samples and refrigerated at 4°C until analyzed.

Antibiotic study

The antibiotics used in the study were chosen mainly for their diversity of bacterial spectrum and, to a lesser extent, because of their use as growth stimulants for young, growing animals by Stern & McGinnis, (28). The antibiotics employed were as follows: Penicillin-G

(Crystalline Sodium Salt), Lot No. 06298-F, Parke, Davis, and Company; Streptomycin Merck (Calcium Chloride Complex) Lot No. 2147, Merck & Company; Neomycin Sulfate, Research No. 9283-4, The Upjohn Company; and Chloromycetin (Synthetic), Lot No. 134007, Parke, Davis, and Company.

To simulate more closely the conditions of the normal rumen, the method devised by Louw et al. (19) was utilized in the second series of experiments. A semipermeable container was suspended in a large, brown-glass bottle containing a complex salt solution similar to the one Burrough's et al. (5) found to be most stimulating to cellulose digestion by ruminant microorganisms. This solution contained per liter 20 ml 1N NaH_2PO_4, 20 ml 1M $NaHCO_3$, 10 ml 1M $MgSO_4$, 10 ml 2M $(NH_4)_2SO_4$, 10 ml 0.5M $CaCl_2$, 10 ml 1M KCl, .002 gms $FeSO_4$, .002 gm $CoCl_2$, .002 gm $ZnSO_4$, and .001 gm $CuSO_4$. The final pH after autoclaving was 7.0 ±1. The semi-permeable material was a "Visking" cellulose sausage casing, 1 3/4" in diameter, closed by #10 rubber stoppers at both ends (Figure #1). The top rubber stopper contained two small holes and a third larger hole. The cellulose allowed the by-products of cellulose digestion to dialyze out of the field of fermentation and nutrients to dialyze in. An air driven stirrer was set in the liquid outside the "Visking" sac. This would augment dialysis by removing the dialysate as rapidly as it passed through the dialyzing membrane.

Anaerobic conditions were maintained by continuously bubbling oxygen-free CO_2 through one of the two holes in the top #10 stopper of the "Visking" sac. Oxygen traces were removed from the CO_2 by bubbling the gas through a chromous acid solution as employed by Hungate (14). The gas was then washed in distilled water, dried over anhydrous $CaCl_2$, sterilized by passing through an 18-inch column of sterile cotton, and then led into the fermentation system. Each flask had its individual gas supply. The gas volume was regulated by means of screw clamps and the tank pressure. The rumen ingesta and the cellulose were mixed by increasing the flow rate of the gas. The gas outlet consisted of a Kjeldahl bulb which provided a trap for liquids and foam that were pushed up the outlet during a period of high gas flow rate. When the flow rate was decreased, the trapped liquids flowed back into the fermentation vat.

The third and larger hole in the top #10 rubber stopper was used as an orifice through which was added the rumen ingesta, the antibiotics, and to remove samples periodically for antibiotic assay and pH determinations. A #00 rubber stopper was inserted into the larger hole during the normal course of fermentation.

The #12 rubber stopper which fitted into the outer brown glass jar also contained three holes. The largest was cut to fit tightly the top #10 rubber stopper from which the semi-permeable bag was hung. Another held the glass tubing

through which the mercury-sealed, air-driven stirrer passed. A large-bore hypodermic needle made the third hole. The needle allowed the equalization of air pressure when the entire apparatus was autoclaved.

Four grams of filter paper cellulose were weighed into the "Visking" sac with the bottom #10 rubber stopper in place. The bag was then suspended in the brown-glass bottle from the #12 rubber stopper. The top #10 stopper was fitted into the "Visking" sac and the #12 stopper, holding the former tightly in place. The gas inlets and outlets, the hypodermic needle, and the stirrer shaft were plugged with cotton. The entire fermentation apparatus was sterilized by autoclaving for 15 minutes at 15 pounds pressure. The complex salt solution was sterilized in two-liter batches in three-liter flasks by autoclaving for 30 minutes at 15 pounds pressure.

The rumen sample was collected in a one liter Ehrlenmeyer flask fitted with a three-hole rubber stopper which held a large funnel, a gas inlet, and a gas outlet. The entire apparatus was wrapped in paper and sterilized by autoclaving. A large, heavy balloon was filled with oxygen-free carbon dioxide and was attached by rubber tubing to the gas inlet of the collection flask. The gas flow was controlled with screw clamps. The samples were collected from a Holstein cow with a large rumen fistula. The animal was on a normal hay-and-grain diet. During the collection

period, rubber gloves were worn. The fistula cap was carefully removed and the rubber gloves were "washed" with ruminal ingesta to remove some of the contaminating microorganisms. A pocket was dug into the rumen contents to facilitate the removal of the more fluid portions at the lower level of the rumen. At this time the screw clamp between the collection flask and the carbon dioxide bag was opened to wash the air from the collection flask with carbon dioxide (CO_2). The sample was removed from the pocket with a sterile porcelain cup. The ingesta was filtered through six layers of cheese cloth and the semi-solid portion was squeezed manually to remove the ruminal liquor in close contact with the partially disintegrated hay. The liter flask was completely filled with the ruminal fluid. Throughout the collecting operation, carbon dioxide was bubbled through the flask to maintain as near to anaerobic conditions as possible.

At the laboratory, 200 ml aliquots of the ruminal fluid were pipetted into the four "Visking" cellulose casing sacs which contained the cellulose substrate. Two hundred ml were also pipetted into a negative control. Five ml of the ingesta were also added to a test tube containing 10 ml of 7 per cent formalin for bacterial counts. The fermentation vessels were placed in a water bath with the temperature thermostatically maintained at $40^{\circ}C \pm 1$. Carbon dioxide was bubbled through each flask to maintain anaerobiosis and to

mix the rumen fluid with the finely ground cellulose. (See photos 1 and 2).

The antibiotic selected was first added to the three-liter flasks containing the complex salt solution. The final antibiotic concentration in the nutrient solution equaled the concentration that would exist inside the "Visking" sacs. The salt solution was then added to the jar outside the sac until the aqueous levels outside and inside the bag were the same. The antibiotic was then added to the ruminal ingesta. Mixing by the vigorously bubbling carbon dioxide was continued for five minutes. A sample was removed to assay the effective antibiotic level and to check the pH of the solution. The rate of gas flow was then decreased to impart a very slow movement to the ingesta. The pH and the antibiotic were checked periodically. The pH was maintained at pH 6.2-6.8 by using 1N Na_2CO_3 to neutralize the fatty acids. After the fermentation system was complete, the air-driven stirrers were started to keep the liquid outside the "Visking" sausage casing moving to aid dialysis. Fermentation was terminated by adding 10 ml of a 1:10 solution of Roccal to the rumen ingesta and storing the ingesta under refrigeration.

Antibiotic assay

The four antibiotics--penicillin, streptomycin, neomycin, and chloromycetin--were assayed by a single, expedient method which varied only in the pH of the medium.

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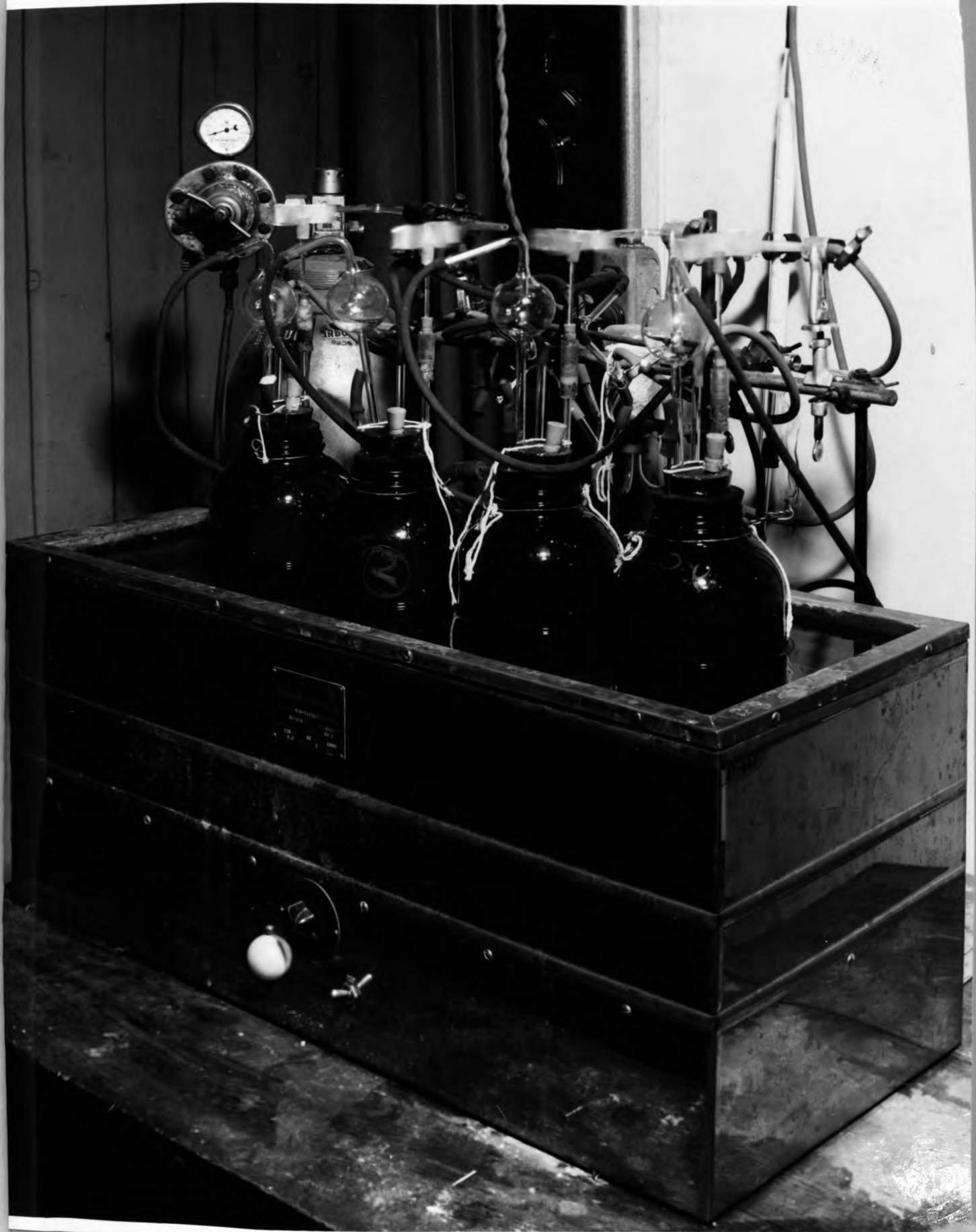

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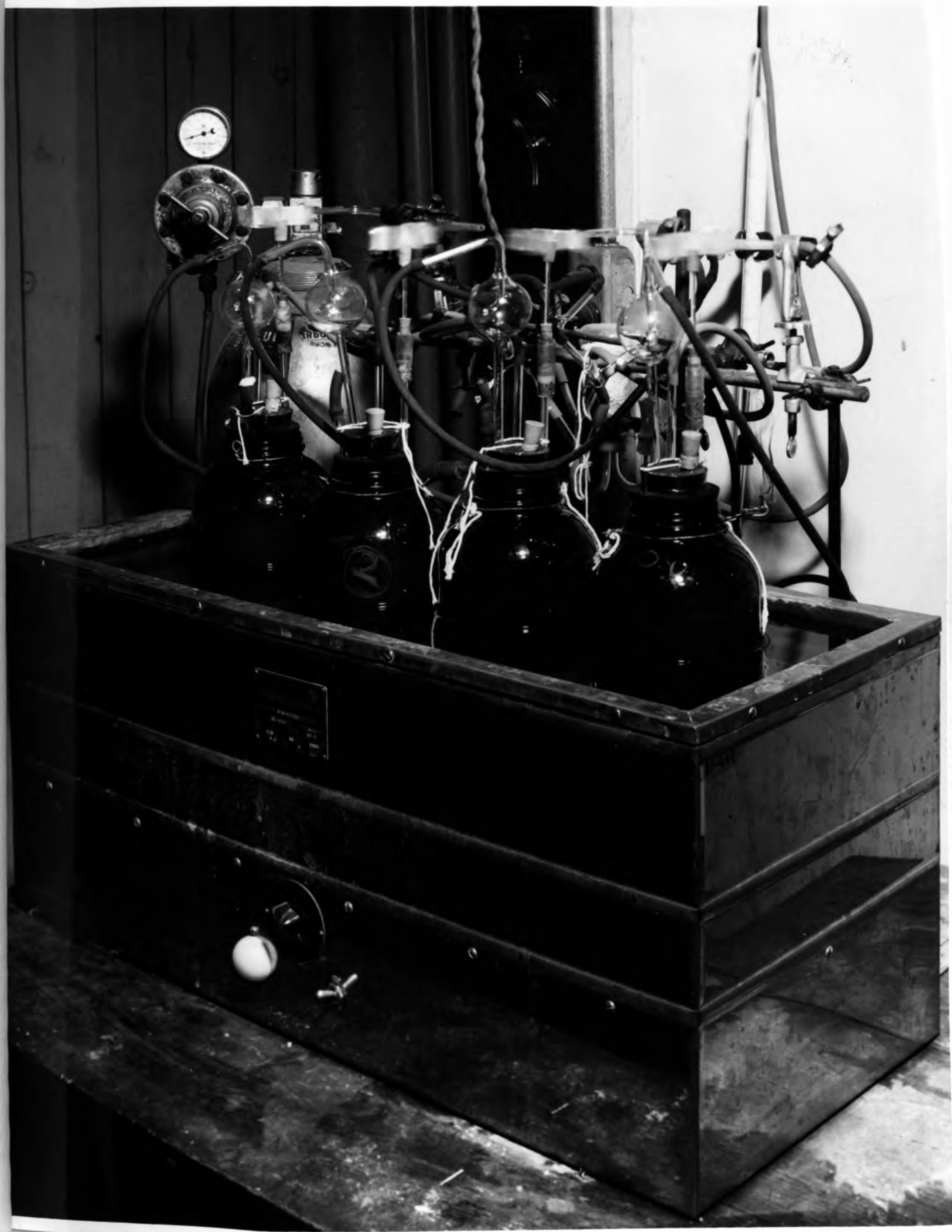
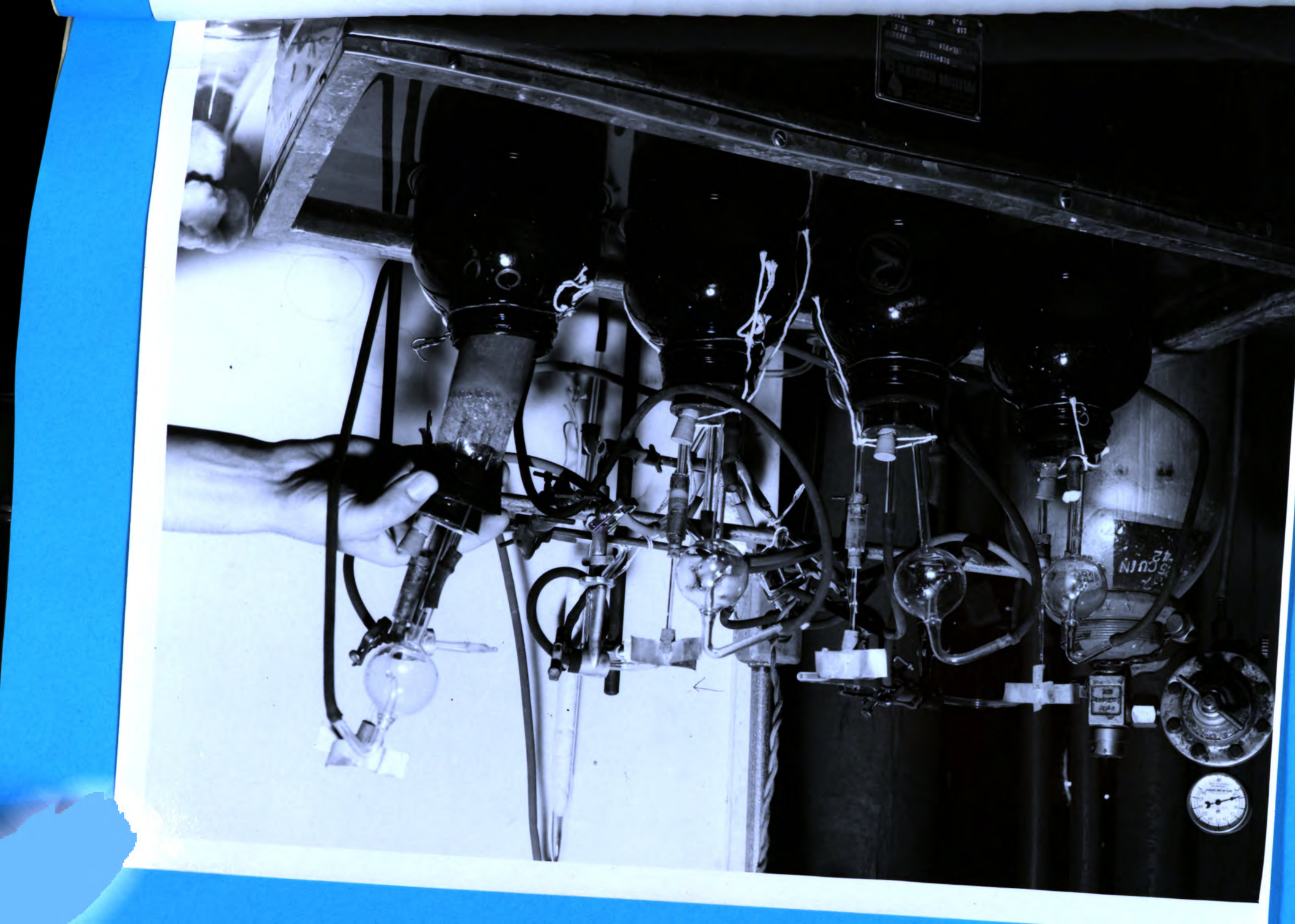




A modification of the paper disc plate method of Loo, Shell, Thornberry, et al. (17) proved most valuable for this work. Paper discs were used as antibiotic reservoirs instead of the usual porcelain cylinders. The filter paper disc was filled by carefully lowering the disc with forceps to the antibiotic solution until contact was made. The liquid saturated the disc by capillarity. The disc was then placed on an agar plate seeded with an organism sensitive to the antibiotic. The antibiotic diffused from the disc to form a zone of inhibition when the plate was incubated. The diameter of the zone of inhibition is proportional to the concentration of the antibiotic.

The test organism employed was a spore suspension of Bacillus subtilis A. T. T. C. 3R8788. The viable plate count on the spore suspension was 27,000,000 per milliliter. Enough of the test organism was added to the seed agar to give a final concentration of 250,000 spores per ml.

Twenty milliliters of the base agar was added to each Petri dish. After the base hardened, four ml of seed agar was pipetted onto the base agar and allowed to solidify. The plate was inverted and refrigerated at 4°C until used. The assay agars employed were standard assay media obtained from Difco Laboratories, Inc. Bacto-mycin assay agar was used for neomycin, streptomycin, and chloromycetin. The pH was depressed to pH 7.0 for chloromycetin. Pen-assay agar and Pen-assay seed agar were used for penicillin.

Standard curves of each antibiotic were obtained by dissolving a weighed quantity of the dry antibiotic in a known volume of phosphate buffer with pH 7.9 for neomycin and streptomycin, pH 6.0 for penicillin, and pH 7.0 for chloromycetin. F. D. A. "working standards" were used for streptomycin and penicillin. The stock solutions were diluted to give concentrations equal to the highest concentration employed in the fermentation vessel. The highest concentration was serially diluted to form five gradient concentrations. Each series of five concentrations was absorbed on paper discs which was placed on one seeded agar plate. Standard curves were based on six replicates. The zones of inhibition resulting after incubation at 37°C for six hours were measured and the standard curves were prepared by plotting the concentration of the antibiotic against the zone of inhibition.

The fermentation ingesta was sampled periodically for the antibiotic assay. Five tenths ml of the ingesta was removed after thoroughly mixing the slurry for five minutes. Each plate contained a filter paper disc wetted with samples from each of the four fermentation vessels and one disc with an antibiotic standard to check against variations in the individual plates, time of incubation, etc. Each antibiotic assay was done in triplicate.

Cellulose determination

The method of Crampton and Maynard (6) was employed to determine the residual cellulose in the "visking" sac after the period of fermentation. The ingesta inside the bag was quantitatively added to evaporating dishes and dried at 75°C for 48 hours. The dry weight was obtained and a five tenths gram aliquot was weighed for the cellulose determination. The negative control which had not been incubated contained the four grams of undigested cellulose plus the 200 ml of the ingesta. Analysis of this control gave the amount of the cellulose added with the 200 ml of ingesta. This figure was added to the four grams of cellulose in the incubated fractions to give the total original cellulose. Subtracting the residual cellulose from the total cellulose gave the cellulose digested. From this, the percent of cellulose digested was calculated (corrected for the blank control).

Method of bacterial counts

The microscopic method used for bacterial counts was developed in this laboratory by Bortree et al. (4). The stain was prepared by saturating 10 ml of ethanol with crystal violet (gentian violet). One ml of the ethanol solution was added to 49 ml of distilled water, mixed thoroughly, and filtered.

The samples for bacterial counts were taken at the termination of the fermentation period. The negative control

was taken before incubation ensued. Five ml of the ruminal ingesta were pipetted into 10 ml of 7 per cent formalin and thoroughly shaken. Three ml of this solution were pipetted into 22 ml of distilled water. After mixing, one ml of this solution and one ml of the dye were transferred into eight ml of distilled water in a test tube and shaken well. This resulted in a final dilution of 1:250. The test tube was gently heated over a low flame until the solution bumped gently (about 60-65° C). Before cooling, a blood pipette was filled with the bacterial suspension and a drop was placed on a clean Petroff-Hauser counting chamber. The cover slip was carefully placed over the "well" to avoid the inclusion of air bubbles in the chamber. The bacteria in one hundred small squares were counted for each sample. The number of bacteria was computed by the following formula: Number of bacteria per ml =

$$\frac{\text{Bacterial count} \times \text{Dilution} \times 20 \times 20 \times 1000 \times 50}{100 \text{ (number of small squares counted)}}$$

20 x 20 = mu each side of square

50 = depth of well with coverslip on Petroff-Hauser chamber

1000 = conversion factor of mu to mm

Data

Crystal violet and sodium azide study

Streptococci were isolated from the rumen on a medium containing 1 per cent tryptone, 0.5 per cent yeast extract, 0.5 per cent glucose, and 1.5 per cent agar. Sodium azide in a final concentration of 1:2500 was incorporated in the medium to depress organisms other than the desired streptococci (22,27). The sample of the rumen ingesta was diluted serially in the melted agar to a dilution of 10^{-12}. The tubes were incubated at 37°C for 48 hours. Discrete colonies appeared in the tubes up to a dilution of 10^{-7}, designated 10,000,000 organisms per milliliter. Four colonies were picked and pure cultures were isolated by streaking on the tryptone-yeast extract-glucose agar. The four streptococci, when in pure culture, were classified as a variant of Streptococcus bovis, differing from the type species by producing only acid in litmus milk (Table I). The type species produces acid, curdles the milk in 3-5 days, and reduces the litmus.

The cellulolytic activity of the total ruminal population in the presence of 1:2500 sodium azide and 1:200,000 crystal violet decreased cellulose digestion for the first twenty-four hour period, and increased cellulose digestion during the next twenty-four hour period (Table II).

TABLE 1. Characteristics of Streptococci Isolated from the Rumen of a Cow on a Hay-Grain Ration

	Streptococci Number				
	7	11	16	17	*S. bovis**
1. Colonies	Small, punctiform, smooth, raised				
2. Morphology	Diplococci, long and short chains				
3. Gram reaction	+	+	+	+	+
4. Temperature relation[1]					
10°C	-	-	-	-	-
37°C	+	+	+	+	+
45°C	+	+	+	+	+
5. Growth in NaCl broth[1]					
2% NaCl	+	+	+	+	+
4% NaCl	-	-	-	-	-
6% NaCl	-	-	-	-	-
6. Growth in 0.1% methylene Blue[1]	-	-	-	-	-
7. S F medium at 45°C[1]	+	+	+	+	
8. Catalase Production	-	-	-	-	-
9. Growth in 20% Bile[1]	+	+	+	+	+
10. Growth in 30% Bile[1]	+	+	+	+	+
11. Thermal resistance[1]					
60°C for 20 min.	+	+	+	+	+
60°C for 30 min.	+	+	+	-	+
12. Action on blood agar	no greening; no hemolysis				
13. Hydrolysis of starch	+	+	+	+	+
14. Liquefacation of gelatin	-	-	-	-	-
15. Final pH in glucose broth	4.1	4.2	4.3	4.2	4.0-4.5

TABLE I (Continued)

	Streptococci Number				
	7	11	16	17	S. bovis*
16. Litmus milk	acid only; no curdling; no reduction				acid, curdled in 3-5 days, followed by reduction of litmus
17. Esculin	+	+	+	+	+
18. Sodium hippurate	-	-	-	-	-
19. Fermentation reaction[2]					
a. glycerol	-	-	-	-	-
b. inulin	+	+	+	+	±
c. maltose	+	+	+	+	+
d. mannitol	-	-	-	-	±
e. raffinose	+	+	+	+	+
f. sorbitol	-	-	-	-	±
g. trehalose	+	+	+	+	±
h. sucrose	+	+	+	+	+
i. galactose	+	+	+	+	+
j. lactose	+	+	+	+	+
k. cellobiose	+	+	+	+	
l. arabinose	+	+	+	+	±

[1] (+) growth, (-) no growth

[2] (+) acid, (-) no acid

*According to Bergey's Manual of Determinative Bacteriology.

TABLE II. The Influence of Sodium Azide and Crystal Violet on the "In Vitro" Digestion of Cellulose

Period of Incubation	No Inhibition			1:2.500 Sodium Azide			1:200,000 Crystal Violet		
	total cell.	cell. digested	% dig.	total cell.	cell. digested	% dig.	total cell.	cell. digested	% dig.
24 hours	6.3	1.8	28%	6.3	-0.3	0	6.3	1.2	19%
36 hours	6.3	2.8	44%	6.3	012	3%	6.3	2.7	43%
48 hours	6.3	4.2	66%	6.3	0.4	6%	6.3	4.4	70%

Antibiotic study

1. Rate of disappearance of the antibiotics from rumen liquor

The antibiotics in the aqueous phase of the rumen ingesta were measured periodically to obtain some indication of the rate of disappearance of the antibiotic during the course of fermentation. Five tenths ml samples were removed after the ingesta had been mixed thoroughly. The zone of inhibition of each assay was evaluated in terms of an antibiotic unit (penicillin) or micrograms per ml (streptomycin, neomycin, chloromycetin) by comparing with the standard curve. (Diagrams 2, 3, 4). The antibiotic persistency curves were obtained by plotting the effective concentration against incubation time (Diagrams 5, 6, 7, 8).

Within the first 40 minutes, penicillin in the aqueous phase dropped rapidly to one-fourth of the original value where it remained for the remainder of the fermentation.

Streptomycin persisted at levels equal to and greater than the original concentrations. The fermentation vessel containing 50 u gm/ml of streptomycin indicated a value of 70 u gm/ml on the first assay. After twenty-seven hours, the assayed value fell to the original 50 u gm/ml. Possibly a synergistic action with some constituent of the rumen was responsible for the increased streptomycin activity.

Neomycin was not significantly removed from the aqueous phase of the fermenting rumen liquor by action of the

rumen microflora or constituents of the ingesta. A slow gradual disappearance was evident, but 25 hours was necessary for the concentration to decrease 50 per cent.

Conversely, chloromycetin rapidly disappeared from the rumen liquor. After 30 minutes, the chloromycetin ingesta, with the original value of 50 u gm/ml decreased 30 per cent, while the ingesta with an original concentration value of 25 u gm/ml decreased 56 per cent. The six-hour assay indicated that no chloromycetin was detectable in the aqueous phase of the rumen liquor.

2. Percent digestion of cellulose

The percent of cellulose digested by the ruminant microflora was calculated to show the effect of the antibiotics on the cellulolytic fraction of the associative population (Table III). The lower concentrations of penicillin stimulated cellulose digestion but the 15 units of penicillin per ml significantly decreased cellulose digestion. Streptomycin did not significantly affect the cellulolytic activity of the microflora with the concentration of 12.5 u gms/ml. The high concentrations, 25 u gms/ml and 50 u gms/ml decreased cellulose digestion.

Neomycin, up to 25 u gms/ml did not decrease cellulose digestion over the positive control but the stimulatory effect of neomycin on cellulose digestion decreased with an increase in concentration of neomycin. Chloromycetin, in the concentrations employed, decreased cellulose digestion by the ruminal microorganisms.

3. Effect of antibiotics on bacterial population

Bacterial counts were made to obtain an indication of the effect of the antibiotics on bacterial proliferation (Table III). All three concentrations of penicillin depressed the multiplication of the microbial population approximately to the same degree. The bacterial counts in the presence of streptomycin decreased with an increase in the concentration of the antibiotic. Neomycin and chloromycetin showed trends similar to the effect of streptomycin.

TABLE III. Influence of Certain Antibiotics on Cellulose Digestion by Rumen Organisms[1]

	Total original cellulose (gms)	Cellulose digested (gms)	Percent digestion (%)	Deviation from positive control (%)	Bacterial counts (organism/ml (1×10^9)
Penicillin					
15.0 u/ml	5.25	1.28	24.4	-13.4	8.2
7.5	5.36	2.41	45.0	+ 7.2	7.5
5.0	5.10	2.25	44.1	+ 6.3	7.4
0.0	5.26	1.99	37.8	--	10.3
neg. control	5.29	0	0	--	6.4
Streptomycin					
50 u/ml	3.89	1.04	26.7	- 4.0	5.5
25	3.84	0.92	24.0	- 6.7	6.8
12.5	3.84	1.23	32.0	+ 1.3	9.5
0.0	3.84	1.18	30.7	--	13.6
neg. control	4.39	0	0	--	6.4

(Continued)

TABLE III. (Continued)

	Total original cellulose (gms)	Cellulose digested (gms)	Percent digestion (%)	Deviation from positive control (%)	Bacterial counts (organism/ml (1 x 10^9)
Neomycin					
25.0 ug/ml	4.43	1.30	29.3	+ 3.2	5.3
12.5	4.33	1.32	30.5	+ 4.4	7.0
6.25	4.18	1.42	34.0	+ 7.9	9.5
0.0	4.63	1.21	26.1	--	9.0
neg. control	4.33	0	0	--	6.5
Chloromycetin					
50.0 ug/ml	4.32	0.66	15.3	-17.5	6.2
25.0	4.32	1.01	23.4	- 9.4	8.8
0.0	4.22	1.35	32.0	--	10.0
0.0	4.32	1.49	33.6) 32.8	--	10.3
neg. control	4.42	0	0	--	6.4

[1] The table is corrected for negative control to equal 0% digestion.

SCHEMATIC DRAWING of the APPARATUS

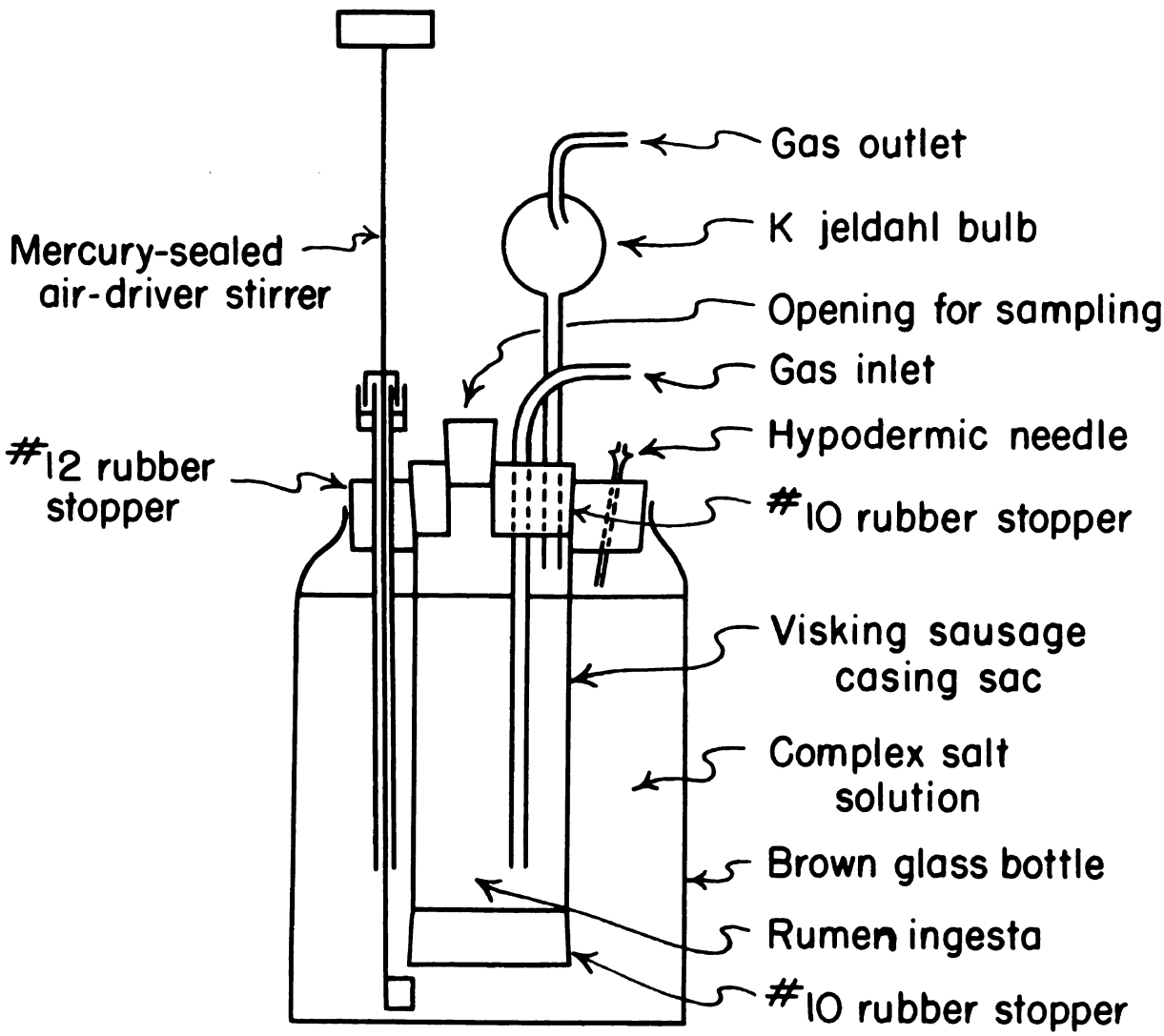


Figure 1

FIGURE II

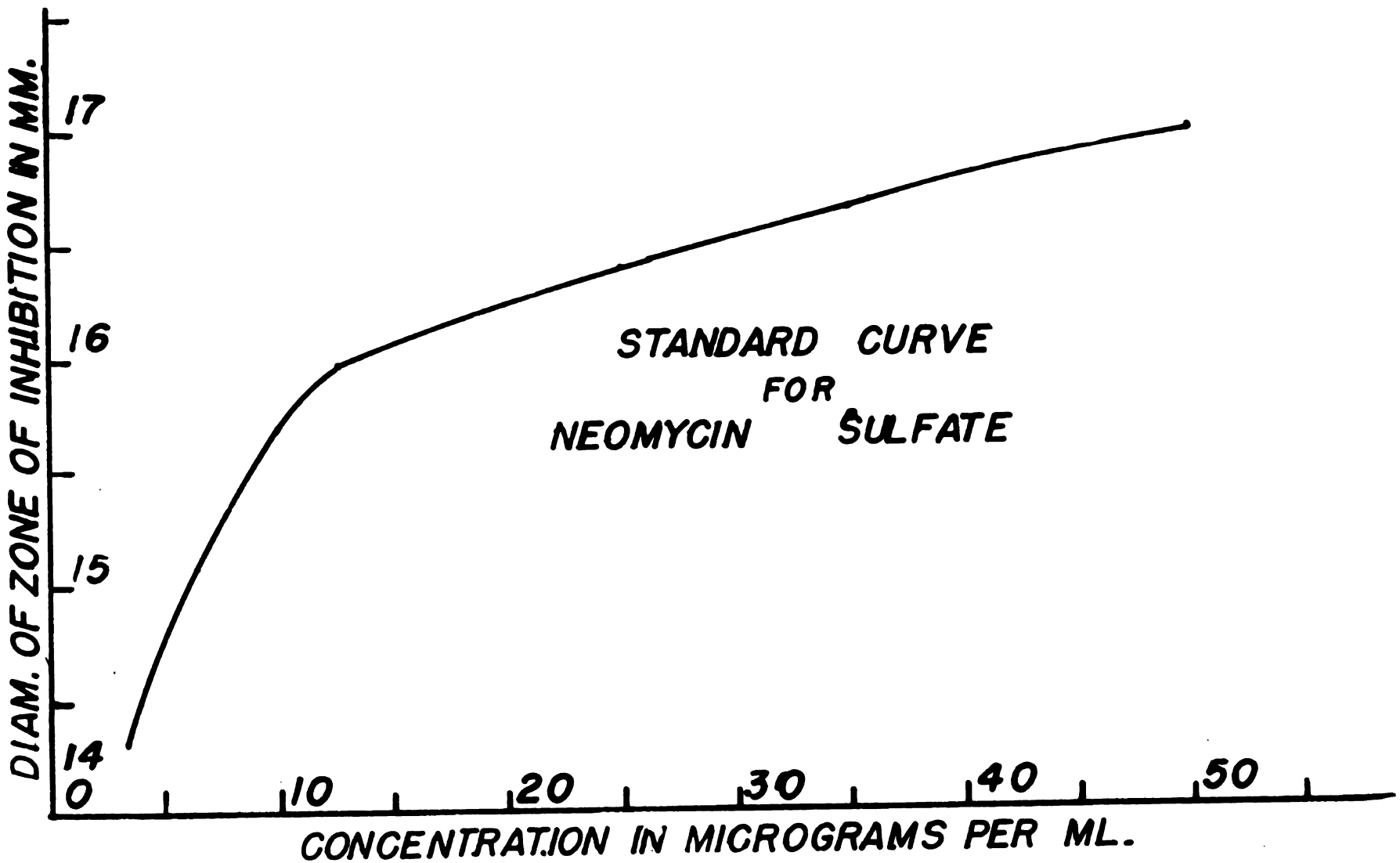

DIAM. OF ZONE OF INHIBITION IN MM.
17
16
15
14
0
10
20
30
40
50
CONCENTRATION IN MICROGRAMS PER ML.
STANDARD CURVE
FOR
NEOMYCIN SULFATE

FIGURE III

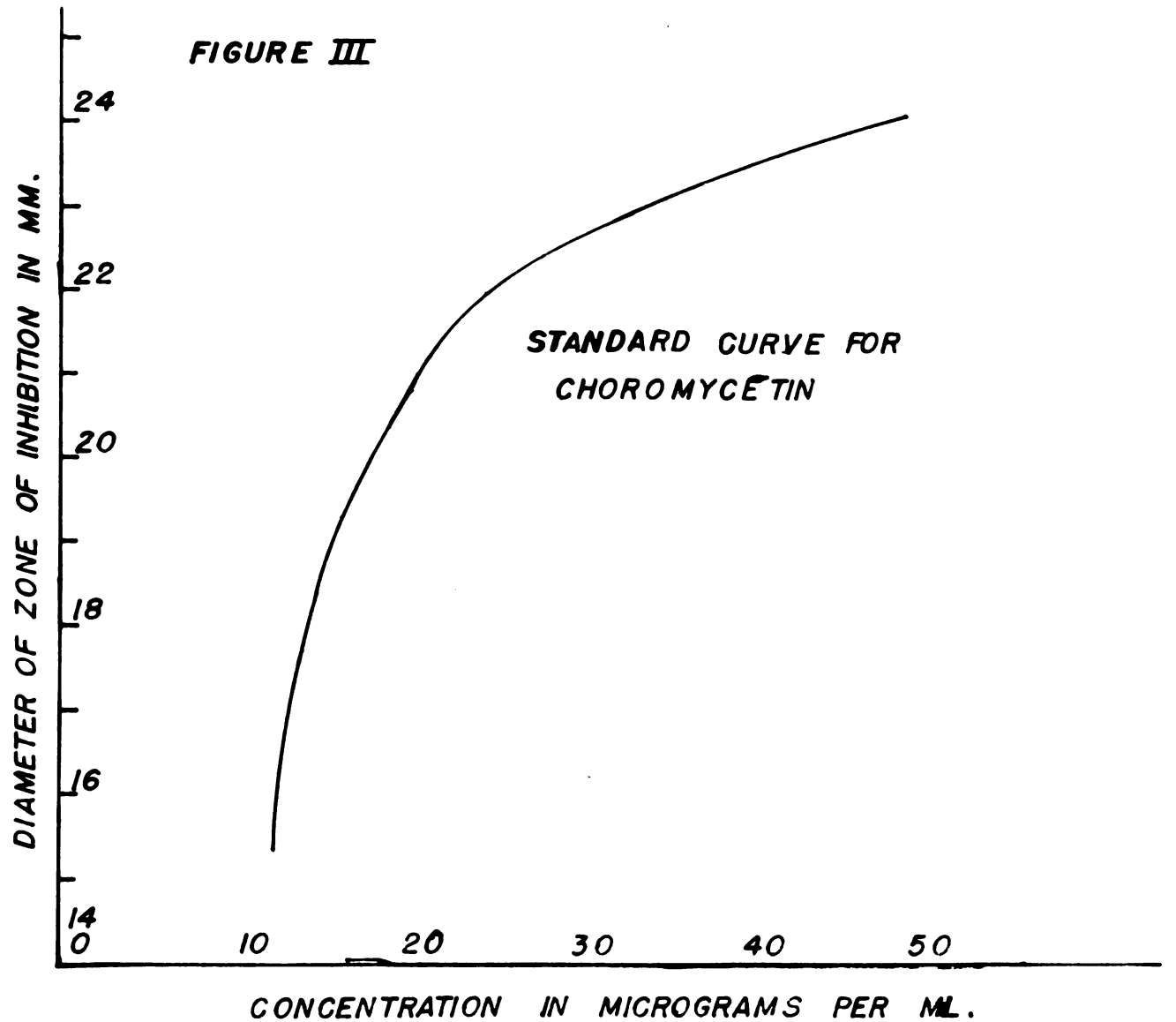
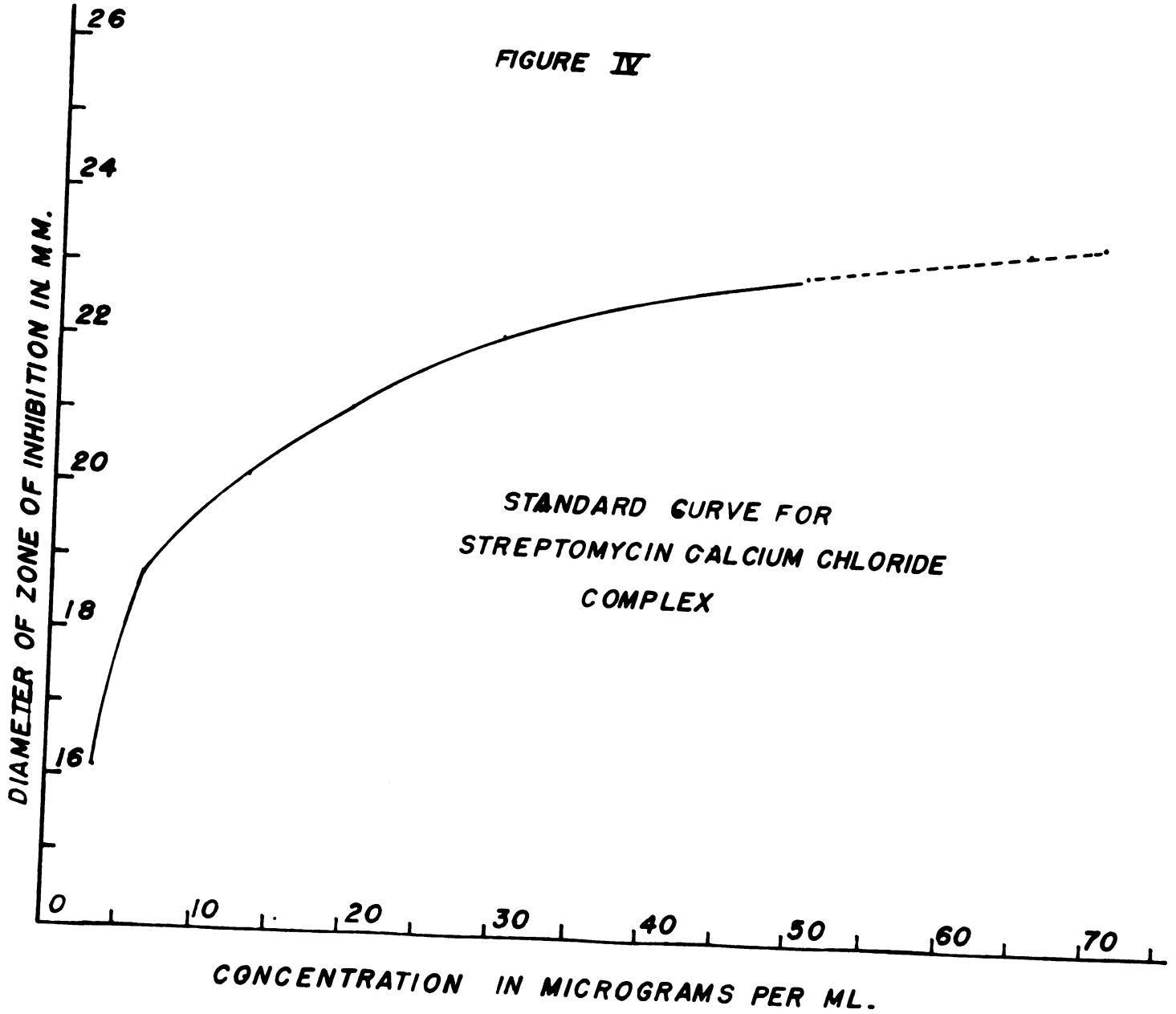
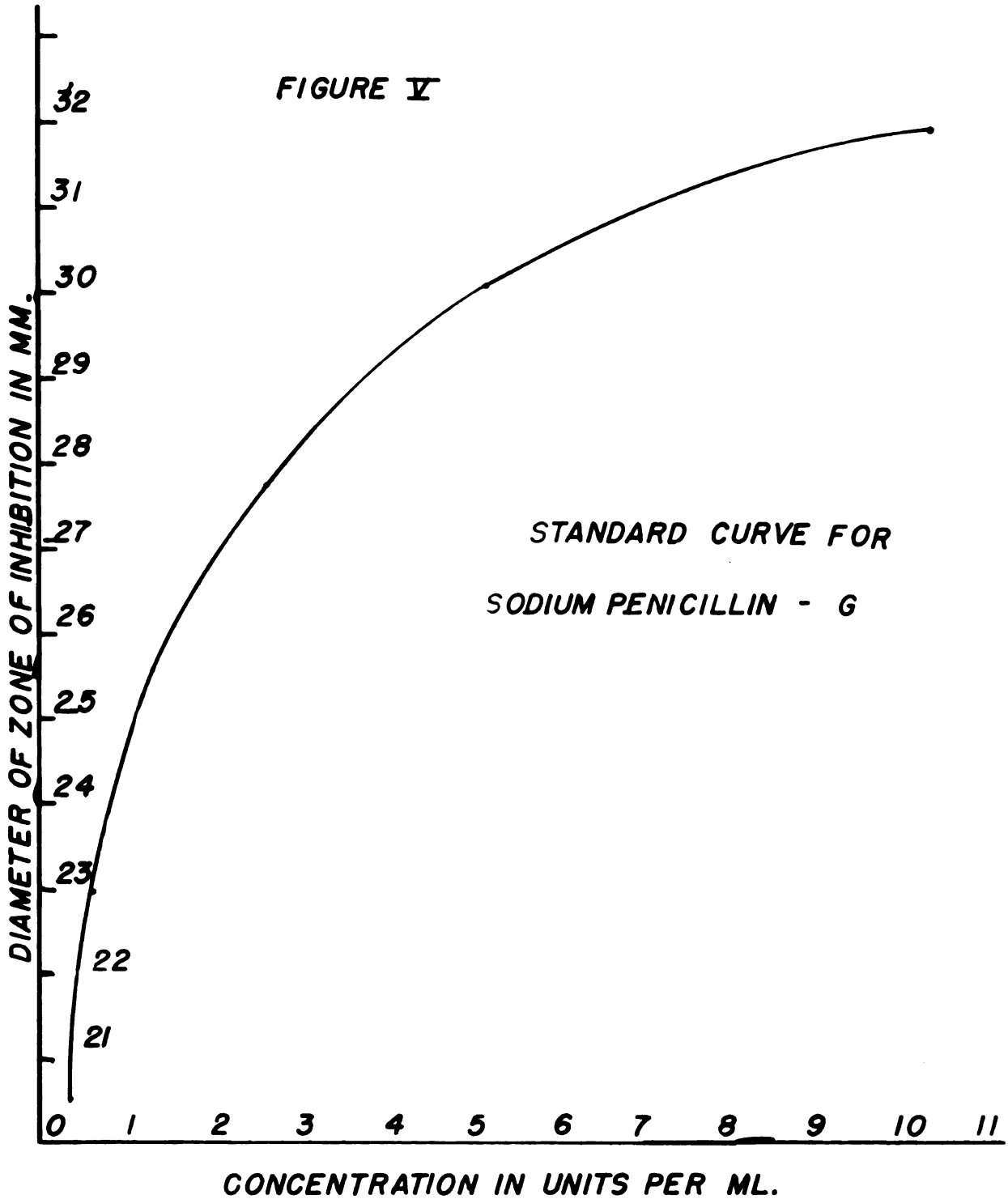

DIAMETER OF ZONE OF INHIBITION IN MM.
24
22
20
18
16
14
0
10
20
30
40
50
CONCENTRATION IN MICROGRAMS PER ML.
STANDARD CURVE FOR
CHOROMYCETIN

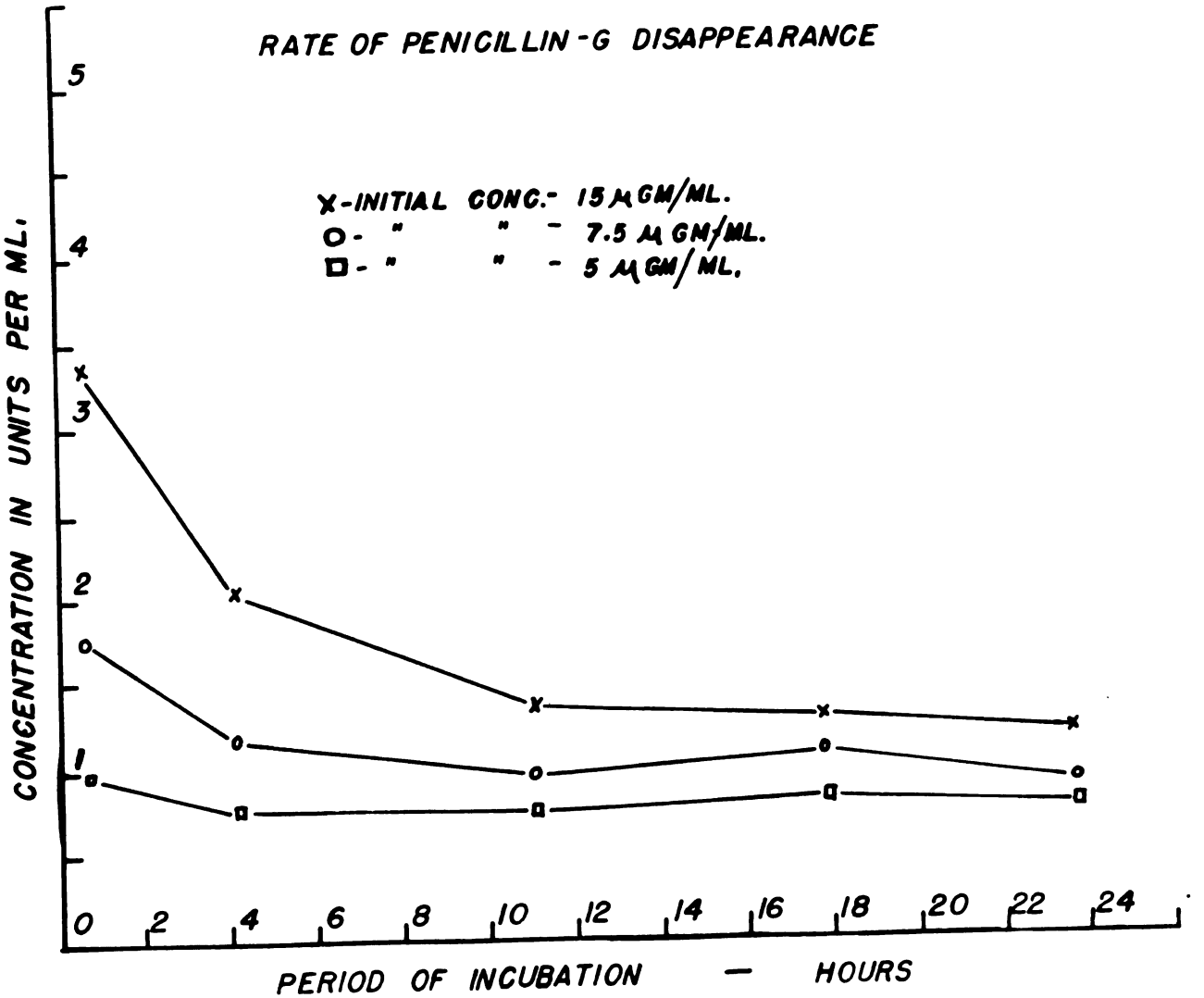
FIGURE IV

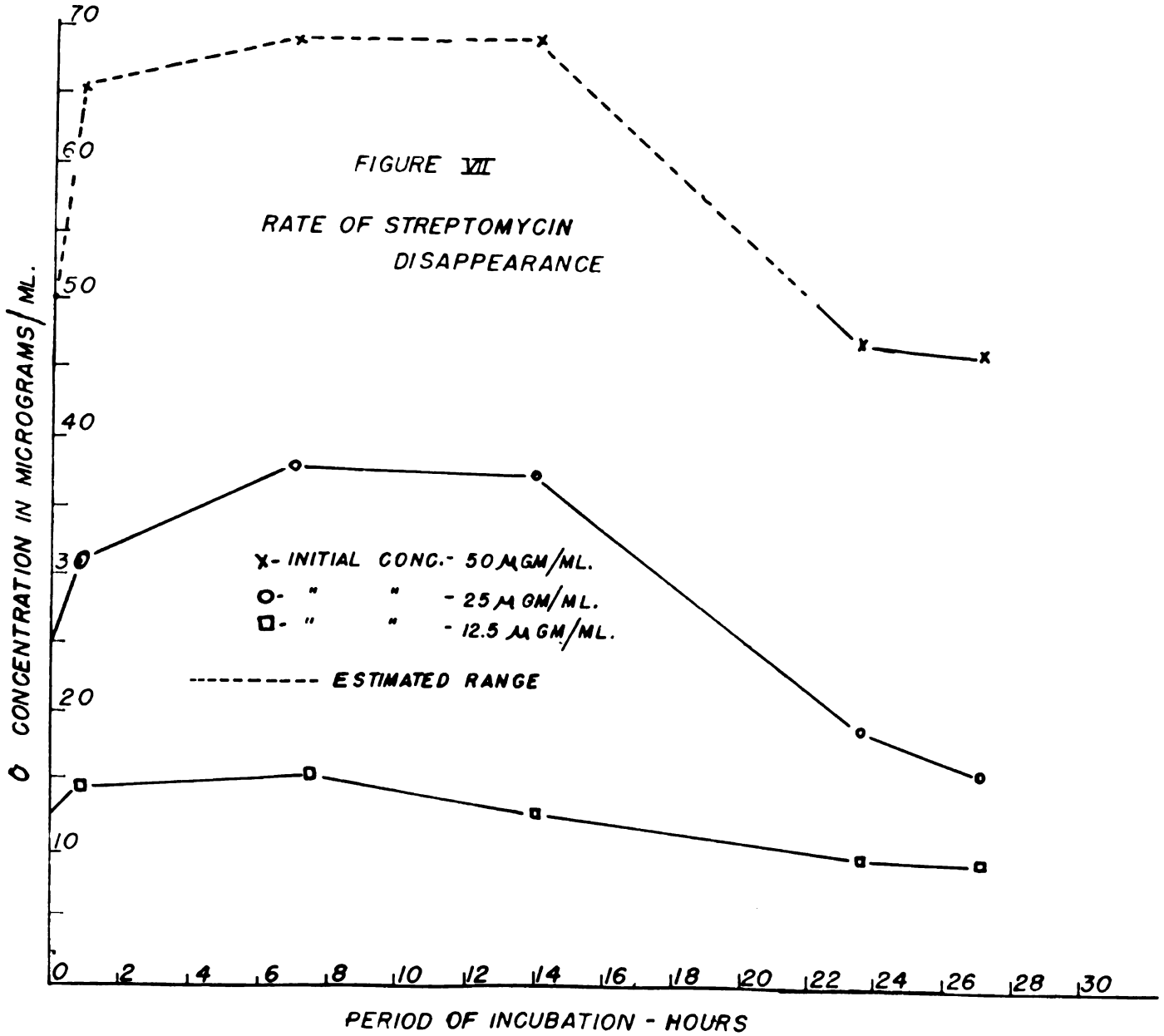

26
24
22
20
18
16
0
10
20
30
40
50
60
70
DIAMETER OF ZONE OF INHIBITION IN MM.
CONCENTRATION IN MICROGRAMS PER ML.
STANDARD CURVE FOR
STREPTOMYCIN CALCIUM CHLORIDE
COMPLEX

DIAMETER OF ZONE OF INHIBITION IN MM
32
31
30
29
28
27
26
25
24
23
0

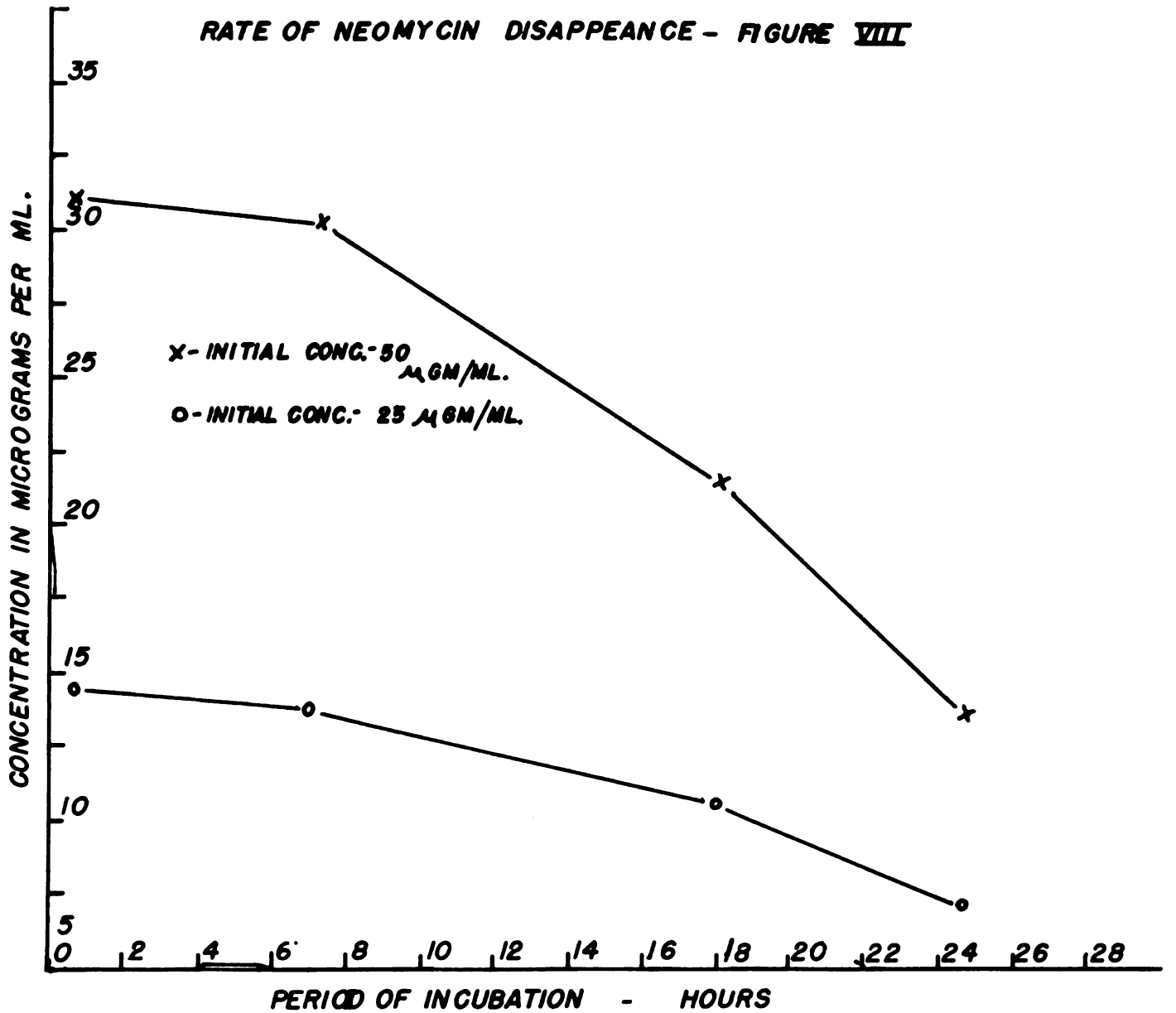
FIGURE V


DIAMETER OF ZONE OF INHIBITION IN MM.
32
31
30
29
28
27
26
25
24
23
22
21
0
1
2
3
4
5
6
7
8
9
10
11
CONCENTRATION IN UNITS PER ML.
STANDARD CURVE FOR
SODIUM PENICILLIN - G


RATE OF PENICILLIN-G DISAPPEARANCE
X-INITIAL CONC.- 15 MGM/ML.
O- " " - 7.5 MGM/ML.
□- " " - 5 MGM/ML.
CONCENTRATION IN UNITS PER ML.
1
2
3
4
5
0
2
4
6
8
10
12
14
16
18
20
22
24
PERIOD OF INCUBATION — HOURS


70
60
50
40
30
20
10
0
FIGURE VII
RATE OF STREPTOMYCIN
DISAPPEARANCE
CONCENTRATION IN MICROGRAMS/ML.
X- INITIAL CONC.- 50 MGM/ML.
O- " " - 25 MGM/ML.
□- " " - 12.5 MGM/ML.
---------- ESTIMATED RANGE
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
PERIOD OF INCUBATION - HOURS

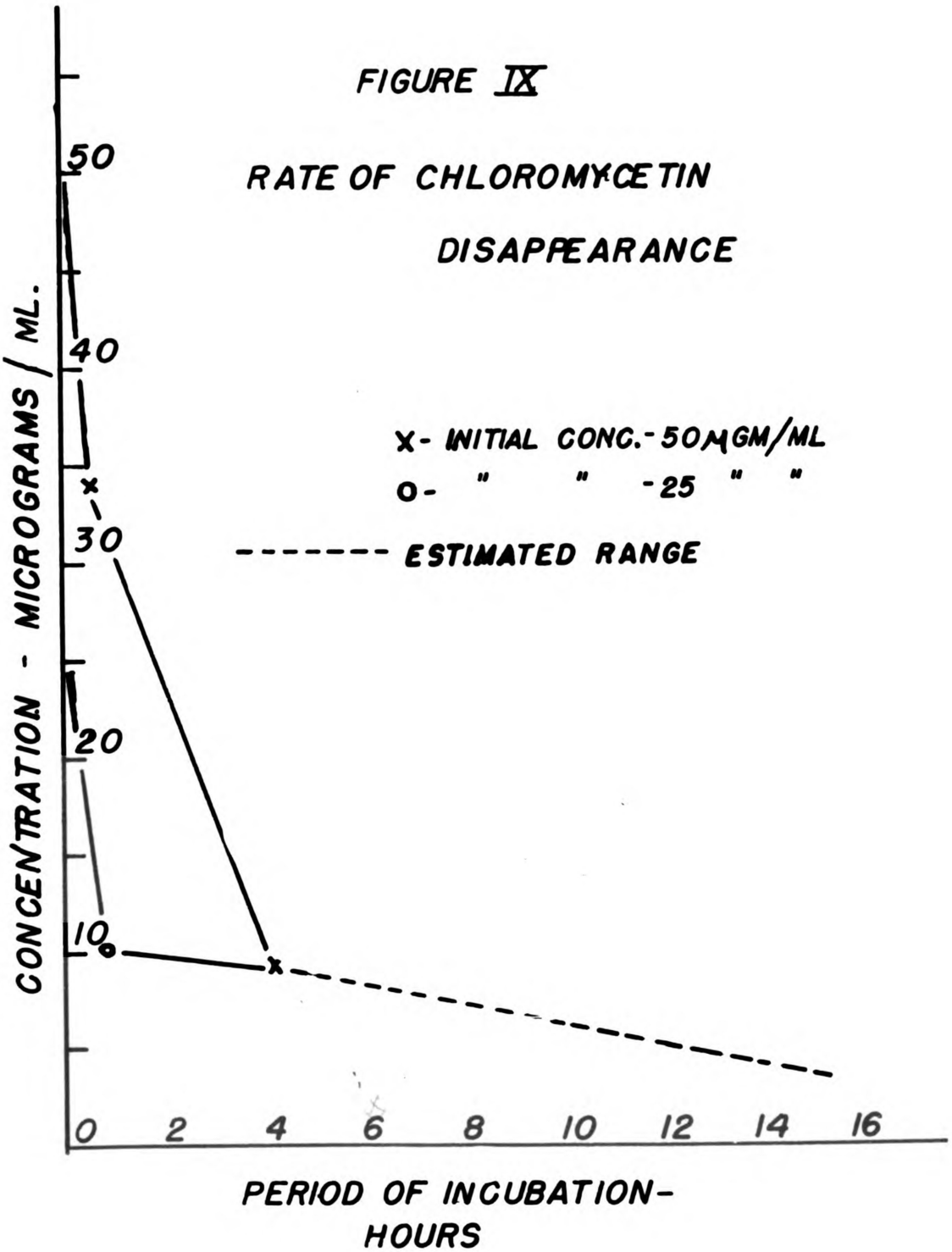
RATE OF NEOMYCIN DISAPPEANCE - FIGURE VIII


RATE OF NEOMYCIN DISAPPEANCE - FIGURE VIII
CONCENTRATION IN MICROGRAMS PER ML.
35
30
25
20
15
10
5
X - INITIAL CONC. - 50 μGM/ML.
O - INITIAL CONC. - 25 μGM/ML.
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
PERIOD OF INCUBATION - HOURS


CONCENTRATION - MICROGRAMS / ML.

FIGURE IX

RATE OF CHLOROMYCETIN DISAPPEARANCE



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DISCUSSION AND CONCLUSION

The purpose of these series of experiments was to determine the effect of certain antibiotics, crystal violet, and sodium azide on "in vitro" cellulose digestion by the total ruminant microflora. For many years, antibiotics and other bacteriostatic agents have been used in isolation work to inhibit selectively undesirable types of organisms (9, 21, 29). Another important use of some of these bacteriostatic agents is to aid in identifying certain bacteria which are able to grow in the presence or absence of these deleterious agents (24,26). The above-mentioned techniques suggested the possibility of defining the cellulolytic fraction of the rumen microflora in terms of resistance or sensitivity to the antibiotics, sodium azide, and crystal violet.

The initial work with the crystal violet and sodium azide indicated that the cellulolytic microorganisms were sodium azide- sensitive and crystal violet- resistant with the concentrations employed. The activity of the substances on the ruminal microflora was pronounced. However, the limitations of the antibiotic study permit only cautious characterizations of the ruminal microflora in regard to sensitivity and resistance.

Bliss and Todd (2) compared eight antibiotics in regard to the minimal inhibitory concentrations for gram

positive cocci and gram negative bacilli. These investigators found that four units/ml of penicillin G were necessary to inhibit the most resistant microorganism studied and the average range of sensitivity was about .06-.08 units/ml. Chloromycetin inhibited gram positive cocci in concentrations from 1.2 to 10 ugms/ml and inhibited gram negative bacilli in concentrations from 1.25 to 25 ugms/ml with the exception of the genus Pseudmonas which required 100 ugms/ml. With streptomycin, a microorganism is considered sensitive if it is inhibited by concentrations of 10 ugms/ml or less (25). In view of the above observations, the cellulolytic fraction of the ruminal microflora can be considered to be resistant to neomycin, resistant to penicillin G, moderately sensitive to streptomycin, and sensitive to chloromycetin.

When studying the activity of antibiotics against Clostridia, Bliss and Worth (3) observed that most strains were inhibited by penicillin G in concentrations of 1 ugm/ml or less; by neomycin in concentrations of 10-1,000 ugms/ml; by chloromycetin from 1-10 ugms/ml; by streptomycin from 100-1,000 ugms/ml. The present findings indicate that the fastidious, anaerobic cellulolytic bacteria from the rumen are more resistant to penicillin G that the obligate anaerobic Clostridia; more sensitive to streptomycin; and about equally sensitive to chloromycetin. The large range of inhibition of neomycin on the Clostridia prevents any comparison with

the effect on the cellulose digestors.

The most important staining procedure utilized in bacteriology is the Gram stain. Correlation with the fundamental properties of the bacterial cell has caused the division of bacteria into two large, general groups: gram positive species and gram negative species (15). Penicillin G is active primarily against gram positive organisms but also affects certain gram negative bacteria not including E. coli, Hemophilus, and Brucella (32). Streptomycin is active principally against gram negative and acid fast organisms; neomycin, mainly against streptomycin-resistant gram negative and acid fast bacteria; chloromycetin, against both gram positive and gram negative bacteria and rickettsiae (25). Gall et al. (12) isolated 17 cultures of ruminal bacteria from the paunch of a bull and 23 cultures from the rumen of sheep in dilutions which indicated that these organisms were present in the rumen in numbers of 10 billion and 100 billion per ml. Because of close agreement with the slide counts made on the ruminal ingesta, she indicated these isolants as some of the predominating organisms in the rumen. Twenty-two of these cultures showed breakdown of cellulose "in vitro". Most of the isolated bacteria were gram positive, non-motile, anaerobic rods. A few were gram negative cocci. The effect of neomycin and chloromycetin on the cellulose digestors correlates with the gram reaction; no correlation with penicillin G and moderate correlation with

streptomycin. It must be realized, however, that the gram stain varies with the technique employed, the media used for growing the organism, and with other environmental conditions, (Knaysi, 15). Also, it must be kept in mind that the defining of antibiotic resistance or sensitivity on the basis of the gram reaction of the bacterial cell is only a relative concept.

The antibiotic assays were performed to estimate the effective concentrations of the antibiotics in the rumen ingesta. The paper disc assay method was sufficiently sensitive to detect the concentrations present throughout the fermentation for penicillin, streptomycin, and neomycin. Chloromycetin could not be detected after six hours of incubation in the ruminal ingesta. Therefore, it can only be assumed that following the first assay the concentration of chloromycetin was less than 9 ugms/ml.

Cohen (cited by Waksman 31) found that streptomycin is adsorbed on cellulose and other substances. In this regard, Waksman suggested that the activity of the streptomycin was affected only insofar as the concentration of the free streptomycin in the medium was reduced. However, since the antibiotic is adsorbed on the cellulose substrate of the cellulolytic bacteria, the streptomycin is still available for bacteriostatic or bactericidal activity. This "adsorption effect" probably applies to the other antibiotics. Unfortunately, the adsorbed antibiotic cannot be estimated by the

assay method employed. The effective concentration of the antibiotic is the sum of the concentration in the aqueous phase (as shown by the assays) and the quantity of antibiotic adsorbed on the cellulose.

Recent studies with growing rats have shown that certain antibiotics increase the growth rates of these animals (28). Other workers are incorporating antibiotics in the rations of ruminants to observe any changes in the growth of young animals and any changes in the physiological processes of mature animals. Any effect realized would undoubtedly be the result of a change in the microbial population inhabiting the intestinal tract. The present "in vitro" study was also employed to indicate possible stimulatory antibiotics. The data suggested that the lower concentrations of penicillin, streptomycin, and neomycin stimulated cellulose digestion. The concentration of chloromycetin observed showed no stimulatory activity. It is well known that subinhibitory concentrations of antibiotics stimulate the growth of sensitive bacteria (25). In view of this fact, no postulates can be made regarding the stimulatory activity of penicillin, streptomycin, chloromycetin, and neomycin on cellulose digestion by the ruminal microorganisms in the procedures used.

The bacterial counts were not sufficiently accurate to allow definite conclusions in regard to bacterial multiplication. However, it may be noted that the bacterial

counts correlate with the cellulolytic activity of the bacteria.

The specialized cells and tissues composing multicellular organisms had to function in a harmonious and efficient manner for the total organisms to survive in the competition for the necessities of existence. The less capable organisms could not compete and survive. The stable pattern of the mixed ruminal microflora suggests a multicellular mechanism in the digestion of cellulose. No single microorganism could perform this function alone "in vivo". The complex nutrients required by the cellulolytic bacteria are not supplied by either the normal dairy ration or the bovine saliva. Symbionts, that utilize the simple sugars and fatty acids resulting from cellulose digestion, elaborate the nutrients required by the cellulose digestors. The crux of ruminal bacteria research is to determine whether the ultimate in efficiency has been reached in the symbiotic relationship between members of the ruminal microflora and between the total microflora and the ruminant; or whether man can interpose new conditions to increase, bacteriologically, the efficiency of milk production, the rate of growth, and the rate of weight gain of the ruminant.

SUMMARY

The effect of crystal violet, sodium azide, penicillin G, streptomycin, neomycin, and chloromycetin on the "in vitro" cellulolytic activity of the ruminal microflora was studied.

The crystal violet and the sodium azide experiment was carried out in 500 ml flasks and incubated for periods of 24 hours, 36 hours, and 48 hours. The normal rumen was simulated by maintaining anaerobiosis, by maintaining the incubation temperature at 40°C, supplying nutrients to the ruminal organisms, and by maintaining the pH at 6.2-6.8. Sodium azide inhibited cellulose digestion while crystal violet had little effect.

In the antibiotic study, the normal rumen was further simulated by providing a mechanism for the continual escape of the soluble fermentation by-products. It was observed that the lower concentrations of penicillin G stimulated cellulose digestion but 15 units/ml decreased cellulose digestion. Streptomycin in a concentration of 12.5 ugms/ml had little effect but 25 and 50 ugms/ml of streptomycin depressed cellulose digestion. Neomycin, in the concentrations employed, stimulated cellulose digestion, but an increase in antibiotic concentration decreased the stimulatory action. Chloromycetin significantly decreased cellulose digestion with the concentrations studied.

In comparing the effect of the antibiotics and the other inhibitory agents with their effect on bacteria generally, it was concluded that the cellulolytic fraction of the ruminal microflora was resistant to neomycin, resistant to penicillin G, resistant to crystal violet, moderately sensitive to streptomycin, sensitive to chloromycetin, and sensitive to sodium azide.

Bacterial counts were made on the rumen liquor before and after cellulose digestion. All the counts correlated with the cellulolytic activity of the microflora except the samples that were incubated with penicillin G. The three concentrations of penicillin G that were used depressed bacterial multiplication even though cellulose digestion was increased by two concentrations.

Antibiotic assays were made periodically to estimate the rate of disappearance. The assays showed that penicillin G. persisted in quantities about one-fourth of the original concentration after 40 minutes of incubation. Streptomycin showed a synergistic action with the rumen liquor. At the first assay, the portions with 50 ugms/ml showed effective concentration of 70 ugms/ml. The other streptomycin concentrations also showed values higher than the original concentrations. All the concentrations gradually decreased through the fermentation. Neomycin gradually disappeared and 50 per cent decrease occurred over a period of 25 hours. Chloromycetin disappeared rapidly. The six-hour assay

showed no antibiotic activity, therefore, it can be assumed that the concentrations following this period were less than 9 ugms/ml.

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