A STUDY OF GRAMICIDIN IN THE TREATMENT OF CHRONIC STREPTOCOCCIC BOVINE MASTITIS AND ITS BACTERICIDAL ACTION ON THE STREPTOCOCCI IN VITRO

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Introduction

Bovine mastitis is a disease which has been known for a long time. As might be expected, the acute form of the disease was the first to receive any considerable amount of attention and study. With the development of the science of Bacteriology, the chronic form of mastitis was recognized, and today its importance as a problem which confronts the dairyman is well realized.

Various workers have studied the incidence of chronic streptococcic bovine mastitis as well as the effect this infection has on the composition of the milk secreted by the infected gland. The effect on the total production of the diseased animals has also been carefully studied.

Rosell in 1931 (88) in a study of 1,222 cows in the province of Quebec, Canada, found that 34% of the animals were infected. Hadley in 1935 (39) studied the prevalence of the disease in Wisconsin and found that 30% of the cows were affected to some degree in about 50% of their quarters. Bryan (12) in a study of 322 herds in Michigan found streptococcic mastitis in 86% of the herds studied. Of the total number of milking animals examined 26.2% were found to be infected.

Shaw and Beam (98) found that the total production of infected animals was reduced by 22% and that the drop in butterfat produced was 24%. Minett and Martin (72) compared the production of healthy cows to that of infected ones. Among the Ayrshires the decrease in production was 10.8% and for the Fresians was higher, being 16.5% and 19.5% (in two herds).

Since 99.5% of infectious mastitis is caused by streptococci (11), it is apparent that this form of the disease is of major economic importance and also readily explains the tremendous amount of work which has been done in trying to find a cure for it.

Review of the Literature

An almost innumerable number of treatments have been studied at various times in order to cure or prevent bovine mastitis. A brief account of some of these treatments will be included here inasmuch as the success or failure of the treatments tried contributes toward a knowledge of the disease. In their reporting of treatments tried many of the workers do not indicate whether the animals treated were suffering from acute or chronic cases of mastitis. The treatments will be discussed under the very broad headings of: 1)Chemotherapy, 2)Vaccines, 3)Miscellaneous, which includes serum therapy, radiation and sanitary precautions that are recommended.

Chemotherapy

Among the first treatments to be used were infusions of various antiseptics into the affected quarters of the udder with the idea that these antiseptic solutions would have a bactericidal effect upon the streptococci infecting the tissue.

A water solution of boracic acid was recommended at an early date by Nocard and Mollereau (65). They made use of a 4% solution of boracic acid in water. Williams in 1901 (111) also mentions the infusion of the udder with boric acid. Thierry in 1903 (106) advised intramammary injections of a tepid solution of boric acid

in water and in 1904 (107) he suggested frequent milking and injections of antiseptics such as borate or fluorid of soda in treating inflammatory processes resulting in suppuration of the teats.

Gilruth in studies of contagious mammitis in New Zealand recommended in 1904 (37) the injection of a 4% solution of boric acid into the affected parts. He recognized that the secretion of milk would be diminished by this treatment but felt that if the treatment were not continued for too long a time the milk yield would return to nearly normal. In 1906 (38) he reported the use of sodium fluorid at the rate of 1 to 5 parts per thousand. The solution was introduced into the affected quarter, the quarter then massaged, and the solution allowed to remain in quarter for ten minutes and then drawn out. The treatment was repeated two or three times per day. Both sodium fluorid and boric acid injections failed to produce any pronounced bactericidal effect.

Cameron in 1906 (15) also reports having obtained the best results in treating contagious mammitis by using an injection of 1:20 boracic acid and water solution.

Bigoteau in 1909 (5) used injections of boricated water. This treatment was first reported by him in 1905 and since that date is said to have been successfully

used in France and elsewhere. The treatment consisted in injecting 120 - 180 grams of a 3% solution of boric acid into the sinus of each affected quarter. The solution was injected after milking the quarter dry and was at a temperature of 20 - 25 degrees centigrade. A purgative was given at the same time.

Meyer in 1910 (66) stated that the only effective treatment lay in giving an injection of an antiseptic solution at the very earliest stage of the disease; later injections did not prevent the destruction of the affected quarter. The nature of the antiseptic solution to be used was not specified.

Wall in 1910 combined the use of an external iodine ointment with repeated injections of boracic acid solutions (110).

Mezey and Koppanyi in 1910 (67) treated the udder abscesses resulting from a diplococcus infection with an ointment consisting of boric acid, silver nitrate and lanolin; the abscesses healed in twelve days.

In addition to udder infusions or injections of boric acid solutions many other substances have been used. Mayo in a bulletin issued by the Kansas Agricultural Experiment Station in 1895 (65) reports the treating of one infected cow with an injection of 0.1% solution of corrosive sublimate and her subsequent re-

covery. For ordinary cases of mastitis (apparently acute cases) he recommends the following treatment: 1)Removal of milk, 2)Careful dieting, 3) Rubbing the gland and 4) Local application of hot water followed by a lotion of camphor and hot lard. This type of treatment was very widely advocated and was usually accompanied by the administration of a purge.

Zschokke in 1900 (112) reported the use of a number of different treatments. Injections of potash 1:1000 parts and of itrol 1:4000 parts in lukewarm water were without effect in destroying the microorganisms. The injection of 50 grams of colloidal silver in a 1% aqueous solution into the jugular vein had no effect on curing mammitis. Better results were obtained by using a salve made of citrate of silver, 10 parts of camphor, and 88 parts of oil. This salve was thoroughly rubbed into the skin of the udder for a period of 8 days in the case of a cow infected with mammitis in all four quarters. Zschokke reports that the milk gradually became clearer and that the number of streptococci diminished until the milk was found to be normal at the end of sixteen days. Another salve which was used contained cantharides. When rubbed upon the skin of the udder it produced (in every case) a cure or at least a decided improvement. Milk production decreased or ceased entirely as a result of this treatment.

Williams in 1901 (111) recommended the injection of antiseptic solutions. Among those mentioned were corrosive sublimate, carbolic acid and boric acid. The author feels that this type of irrigation does not reach the upper gland and is irritating as well. He thinks that hydrogen peroxide is good but admits that he has never tried it or heard of any one else doing so. He speaks as follows of iodoform: "It seems clear to us that the best preparation possible for this use is iodoform, which, comparatively insoluble, would remain in the gland for some time liberating iodine and introducing no irritation, but acting as a local anodyne. It should be injected dissolved in one of the lighter fats, olive oil, liquid vaseline or butterfat." This sounds very promising as do so many of the reports of treatments of mastitis. The author then goes on to say, "We hope to give this remedy an early test". Bangs and others are referred to as extolling iodine and camphor while other workers are said to recommend turpentine. These are supposed to disinfect after being applied externally and absorbed by the skin.

Mayall in 1905 (64) reports success in the use of an ointment which consists of equal parts of lanolin and lard mixed with one half dram of calomel and carbolic acid for every two ounces of the base. This salve was apparently intended for the external lesions resulting from contagious mammitis.

Schultze in 1906 (95) based his treatment on the premise that most cases were due to an invasion of bacteria through the milk canals and his treatment followed the same route. The udder was first thoroughly milked out, and then cleaned with warm soapsuds and a 3% solution of creolin (apparently external). Injections were then made into the milk cisterns with solutions containing 5% creolin or camphorated oil. This was repeated three times daily and reportedly resulted in prompt improvement in most cases.

In 1907 Moretti (76) reported good results from pumping filtered air into the udder and upon giving hypodermic injections of chlorid of caffein combined with the oral administration of iodid of potash in 20 gram doses and the application of blisters to the outside of the udder. In the same year (75) he reported on the treatment of two acute cases of mastitis following hoof and mouth disease. The udder was infused with a 5% solution of creolin. Caffein was administered internally but no beneficial results were obtained.

Best in 1908 (4) treated a streptococcus infection in cows and goats. The udders were inflamed. The milk was drawn with a milk tube and the udder injected with a solution of alphonzone (1:1000). This is an inorganic peroxide with the following formula: $(COOH, CH_2, CH_2CO)O_2$. Bichloride of mercury (1:1500 or 1:2000) was also used.

The constitutional treatment consisted in giving hyposulphite of soda in one dram doses three times a day.

Another treatment for severe inflammation of the udder was proposed by Schmidt in 1909 (94). He infused the affected quarter with eight ounces of an equal mixture of alcohol and glycerin. If there was a possibility of restoring milk production to the quarter the mixture was diluted with one-third normal saline solution to avoid undue irritation. If two or more quarters were affected only one was treated. The animals usually survived but milk production was not always restored to the quarters involved.

Frost in 1915 (32) treated four herds in which there was infectious mastitis with methylene blue. Each cow was given sixty grains and this was followed by a dose of thirty grains the next night and morning. In all cases the milk became colored. Rapid recoveries were achieved in all herds, no quarters were lost and no hard milkers were produced. The methylene blue was also used in conjunction with turpentine.

In 1917 Frost reported the cure of five cases of mastitis by means of formalin (33). The formalin was given in 25 cc. doses in capsules twice a day for two weeks. There were no ill effects. The formalin treatment was alternated with turpentine and purgatives; and diuretics were given at the same time. Frost has also

used boric acid solutions, ozone and the mixture of equal parts of alcohol and glycerin (Schmidt of Denmark) without any result.

In the same year (1917) Bosshart used the formalin treatment recommended by Frost(7). He cut the dosage to what he deemed to be a safer amount and only administered 0.2 ounces in 0.5 pint of raw linseed oil two or three times daily, alternated with one ounce of turpentine in oil.

Pugh, an English veterinarian, treated 23 cows infected with streptococcic mastitis with one half ounce doses of formalin, given twice daily for ten days. In no case could he observe any beneficial effect which might be ascribed to the treatment (84).

Savage (1922) reports that the formalin treatment is of value, more so in an acute than in a chronic case (92). It is best when used early, according to him, and does as much for internal disinfecting as does anything else. Catala of the Puerto Rico Station in 1925 also recommends the formalin treatment accompanied by a purgative (18).

Lentz as late as 1930 claimed that the fundamental causes of streptococcic mastitis were mistakes in care and feeding (57). He did find that daily doses of 50

grams of calcidigal were useful.

Bischoff in 1930 (6) used udder infusions of yatren and found that this treatment neither destroyed nor inhibited mastitis streptococci nor did it exert any appreciable effect upon the retention of secretion.

Hucker and Lee in 1932 (45) made a comparative study of the effects of crystal violet, brilliant green, acriflavine and azamine. These were administered orally, by intravenous injection and by udder infusion. When administered orally or as an udder irrigant brilliant green, crystal violet and acrilfavine did not appear to be of much value. Azamine had little effect upon the udder flora when fed orally or injected intravenously. The udder irrigations with Azamine showed definite promise. A certain percentage of the cases treated with Azamine in the field were benefitted. Azamine itself is an azo dye of the pyridine series (tolyl-azo-diamino-pyridine hydrochloride) and was further studied by Frost in 1932 (34). Frost fed the drug and also used udder infusions. Chronic cases were treated by infusion and the aqueous solution of the drug was left in the udder 8 to 10 hours.

Gildow, Hansen and Cherrington of the Idaho Experiment Station in 1933 studied the effect of formalin (given in blackstrap molasses), colloidal carbon, ultra-

violet light and autogenous bacterins on chronic mastitis (35). Ultraviolet light gave promise of controlling the clinical symptoms of the disease. None of these treatments, either alone or in combination, was effective in eliminating the causative organism from the udder or even in regularly reducing the number of organisms or leucocytes. The work at the Idaho Station included the injection of methphene (in 6 cows) and trypachrin (in 4 cows) directly into the prepubic artery. This was done in order to get the antiseptic into immediate contact with the infection. The results with methphene and trypachrin did not prove to be of any measurable value. Similar results were obtained with Azamine.

The annual report of the New Jersey Agricultural Experiment Station for 1934 describes the feeding of colloidal iodine to infected cows (80). Thirty cubic centimeters were fed with the grain. There was no correction of the condition in lactating cows. Eight cows were treated while dry and of this group only one had freshened at the time of the report and did not have a recurrence of the trouble.

The next type of medication to be widely used was Entozon and other acridine derivatives. Acridine therapy was introduced into the therapeutics of bovine mastitis by Bugge in 1923 (93,102) who used Rivanol on a

few cases. Schnorf (1925) did a great deal of work with acridine derivatives and introduced Uberasan which is a derivative of Rivanol. Entozon is a mixture of nitroacridine lactate, amyl saccharine and sodium biborate (Schalm).

Seelemann and Siemonsen in 1933 used Rivanol and Entozon (29). Both had a bactericidal effect upon the streptococci and it was found necessary to treat infected quarters only once or twice. Trautwein et al. in 1934 used Entozon and found that 81% of the treated animals were free from infection after two to five treatments (29). Similar observations were made by Weisshaupt, Wagner, Seit, Bertling, Lefevere and Schmidt (93).

Rolle in 1934 showed that the causal organisms of mastitis were uninfluenced by Rivanol in a 1:1,000 dilution but were inhibited by Chinosol in a 1:10,000 dilution (52). Domagk in 1935 used prontosil (a coal tar derivative) in the treatment of streptococcic infections (21). In 1938 the New Hampshire Agricultural Experiment Station reported the use of a new drug in the treatment of mastitis. The name of the drug was Prontylin (powder form) and Prontysil (liquid form). This is apparently the Prontosil used by Domagk. Several cows were treated over a two week period without any apparent results (78).

Lefevere in 1935 treated cows with Entozon (56). The animals were first milked dry, 100 cc. of Entozon

solution was used as a rinse and then each quarter was infused with one liter of Entozon solution. This solution was allowed to act for five minutes in lactating cows and for 15 to 24 hours in dry cows. The cows were milked out completely three times a day and a second treatment was given eight hours later. Production temporarily decreased about 50%. The author feels that acridine therapy has an incontestable value in the treatment of bovine mastitis.

Steck at the Twelfth International Veterinary Congress in 1934 reported on a series of experiments carried on from 1929 to 1934 (102). According to his results Trypaflavine appeared superior to Rivanol, Entozon and Uberasan.

Roemmele in 1936 treated 826 animals with Entozon (87). Treatment during lactation was apparently of little value. Treatment shortly before the end of lactation or during the dry period was best. Entozon was used in dilutions of 1:500 or 1:800. The milk production was sometimes decreased and the recoveries were seldom permanent. The author thinks that reinfection might account for the latter point.

Helm in 1937 treated 33 cows infected with chronic mastitis with Entozon infusions (41). In only two cases was the treatment successful, although two to three infusions were made.

Stableforth and Scorgie in 1938 (100) studied Entozon

and Acriflavine and reached the conclusion that Acrifavine in a dilution of 1:10,000 was to be preferred.

Schalm in 1940 (93) treated eleven lactating and eleven dry cows having a total of 60 quarters shedding Streptococcus agalactiae. All 88 quarters were treated by infusion with Entozon. Streptococcus agalactiae disappeared from 90% of the quarters. It is thought that this treatment will prove to be of value in those herds which have a control program based on the segregation of infected animals.

During the last few years many workers have tried to use sulfanilamide in the treatment of mastitis (1,2,3,13, 36,40,42,51,53,58,70,71,86,104). The drug was fed in all cases and the dosage varied with the individual conducting the experiment. The results seem to be generally agreed upon. As long as the sulfanilamide could be recovered from the blood or from the milk of the animal there were few if any streptococci shed and the leucocyte count approached normal. No sooner was the feeding of the drug stopped then the streptococci reappeared in the milk and the leucocyte count rose. The bulk of the evidence to date indicates that sulfanilamide is no cure for chronic streptococci bovine mastitis even though it does relieve the clinical symptoms of acute cases. The drug is also highly toxic if used in excess and Bryan and Arnold

(13) recommend calcium gluconate as an antidote for sulfanilamide poisoning.

Vaccines

Vaccine treatment for mastitis was first tried in 1910 when Reid reported fairly successful results on using an autogenous vaccine (killed cultures). In the same year von Ostertag and Weichel were unable to immunize by using vaccines but were successful in obtaining an ummunity of short duration by intravenous injections (77). The cultures used were those of C. pyogenes.

In 1914 Payne treated three cases of streptococcic mammitis by injecting killed streptococci and staphylococci. Four doses were given at intervals of three days with good results (81).

Varley in 1921 used infected milk as a vaccine. Four cows were treated; two cows cleared up and two showed anaphylaxis (108).

In 1925 Carpenter used living suspensions of alpha hemolytic streptococci with good results (16). Moore in 1927 treated three cows with vaccines and the results were apparently beneficial (74).

In a report issued from the Storrs Experiment Station by Brigham, McAlpine and Anderson in 1929

the following conclusions were reached: 1) Autogenous bacterins even when given in small doses appear to exert a marked curative action in severe cases of mastitis and 2) a prophylactic vaccination at intervals of approximately three months has apparently been efficacious in at least two of the herds. To be effective, however, the bacterin must be applied to all of the animals in the herd (8).

The Michigan Experiment Station in 1929 and the Storrs Station in 1931 reported good results, one with living streptococcus vaccines and the other with autogenous herd bacterins (68, 19).

In 1931 Krage and Gipmann reported on an experiment begun in 1928. They feel that vaccine therapy is of use although their results were a bit confusing (55).

Seelemann in 1933 found vaccination to be very unsatisfactory, either as a cure or as a prophylactic measure. He did recomment Rivanol infusions combined with sound stable hygiene (97).

Bryan, Burlingame and Cavell in 1933 treated two herds with a gentian violet lacto-vaccine. This treatment stopped the spread of the disease and nine out of eleven infected cows ceased to eliminate streptococci or to give other signs of infection (9). Bryan found that autogenous vaccines and lacto-vaccines were of value in treating animals. The effectiveness

of the treatment varied with the extent of the disease and the response of the animal to the vaccination (10). The treatment was also useful in protecting non-infected animals.

Rosell and Miller in 1933 uses injections of autogenous bacterins and protein (milk) (90).

Klimmer and Haupt in 1934 reported using dead and living organisms to vaccinate. The final results showed that the vaccinated animals had a higher percentage of infection than did the controls (54).

Seddon and Rose in 1934 (96) used autogenous vaccines and concluded that : "1) The incidence of infection is not altered, 2) The percentage of animals which develop mastitis recognizable by the dairyman is lowered, 3) The severity of the cases is markedly reduced and 4) The liability to recurrent attacks is much lessened".

Plastridge et al. in 1934 reached the following conclusions regarding bacterins: "1) periodic injections of autogenous herd bacterins fail to bring about complete recovery of affected animals; 2) they reduce but slightly the rate of spread of infectious mastitis; and 3) they apparently aid somewhat in retarding the occurence of milk abnormal in appearance by recently infected animals." (82)

At the Twelfth International Veterinary Congress Steck reviewed the quention of vaccination and concluded that there is no proof of its utility (102). In 1936 Steck, Gygax and Dachius found that vaccination did not affect the course of the disease in any way (103). The Louisiana State University in a circular issued in 1935 stated that the value of the general run of vaccines had not yet been proven (62).

Edwards in 1936 concluded that while vaccines may be good it is uncertain that they bring about a complete cure, inasmuch as there is lacking a thorough laboratory check (30).

Starr, Prescott and Huffman in 1936 used vaccines and found more evidence of disease in the vaccinated animals than in the control group (101). Miller in the same year reported on work done by the Bureau of Animal Industry. Vaccination was not found to be successful in eliminating the infection (69).

Hucker and Hansen in 1937 found that vaccines were of very little therapeutic or prophylactic value even when combined with intramuscular injections of milk (46).

Bryan (1941) concludes that the proper use of bacterins and vaccines can have no harmful effect in a herd. The only way to determine whether they will be of any use is by trial (14).

Miscellaneous Treatments

Some of the treatments which should be mentioned here have already been referred to (ultra violet radiation, filtered air, etc.).

Frequent milking is a treatment which had been advocated by many, particularly as a relief for acute cases (17, 111). Williams recommended that if the milk was too clotted to be milked out, injections of a 1% soda solution to which was added 3 to 5% hydrogen peroxide be made to facilitate the milking (111).

Eggink in 1912 reported using an antistreptococcus serum with good results (31).

Hucker and Reed (1937) fed irradiated yeast to cows. This was found to have no significant effect upon the resistance to infection though some of the infected yeast-fed cows showed an improvement over the infected cows not fed yeast (47).

Sanitation as a prophylactic measure is of tremendous importance. Inasmuch as no one treatment has proven to be a reliable cure for the disease it is necessary to use whatever other means are available to prevent its spread.

Franck in 1875 and Nocard and Mollereau in 1887

recognized the values to be derived from segregating the infected animals (83). DeBruin in 1905 recommended using clean straw and preventing cows' tails from coming into contact with the filth(20). Ramella in 1914 dipped the teats of cows suffering from foot and mouth disease into a 5% solution of lysoform to prevent the development of mastitis (85). On twelve farms supplying certified milk to Brooklyn, New York in 1916 it was required that after milking the teats be dipped in a weak solution of pyxol, wescol or hycol. No new cases of mastitis were reported from herds following this procedure (73).

Plastridge and associates in 1936 reported that the segregation of infected animals reduced the rate of spread of mastitis 50 to 100%. Segregation combined with gradual replacement of first calf heifers resulted in complete elimination of the disease (83). Stableforth, Edwards and Minett (1935) also advise evacuating infected animals or, if that is not possible, to milk them last (99).

As a result of work done by Slanetz at the New Hampshire Experiment Station it is concluded that the detection, segregation and replacement of infected animals are the best methods of preventing and eradicating streptococcus mastitis (79). Little (1940) has been able to maintain several herds free of mastitis by elimination of infected animals. In another herd where the infected animals were not eliminated but were milked last, new

cases developed during the period of observation (61).

Bryan (1941) recommends the testing of the herd by physical examination of the udder and microscopic examination of the milk. All infected animals, including those not yet in the advanced stages of mastitis, should be isolated at the end of the milking line and subsequently eliminated from the herd. Replacement cows should be tested before being admitted to the herd and sanitary stable conditions maintained at all times (14).

The importance of sanitation is further emphasized by the work of Sanders (1940). He was able to transmit mastitis to susceptible cows by first permitting flies to feed on infective material and then upon residual milk on the ends of the teats (91).

The review of the various treatments used in mastitis which is included here is by no means complete. The object was to present some of the treatments so that the type of work done might be understood. Munch-Petersen (77) has reviewed the entire subject of mastitis, including the treatment, until the end of 1935. All the material included here and considerably more will be found there. The summary of the work done from the end of 1935 until the present has been made as complete as possible.

Gramicidin

Gramicidin was first isolated by R. J. Dubos in 1939 (22,23). It is a cell-free extract obtained from the autolysates of an aerobic, sporulating strain of a soil bacillus of the Bacillus mesentericus type. The active principle obtained is not volatile, does not dialyze through collodion membranes, is very stable at alkaline reactions but rapidly inactivated at reactions more acid than pH 5.5 even at room temperature. The substance can be precipitated at a pH of 4.2-4.4; the precipitate when redissolved in a neutral medium will exhibit the lytic activity of the original solution. Gramicidin has the following effect upon gram-positive organisms: 1) lyze the cells, 2) bactericidal effect, 3) inhibit growth, 4) inhibit dehydrogenase activity. The extract had no effect upon gram-negative organisms.

In addition to its action in vitro, gramicidin has been used to protect white mice against infection when artificially exposed to large numbers of virulent pneumococci (24). It will also exert a curative effect if administered to the mice some time after they have been infected with the pneumococci. The mechanism which protects the mice is the same as that which determines the action of the gramicidin in vitro. The specificity of the pneumococci has no effect upon the degree of protection afforded or upon the minimal effective dose of the agent. The lysis which occurs is thought to be

a secondary process which follows a primary injury done to the cell by the bactericidal agent (23).

In further work with gramicidin Dubos and Cattaneo (25), and Hotchkiss and Dubos (43,44) report the further purification of the extract as well as presenting some of chemical characteristics. The purified preparation is protein-free, and does not give a positive reaction in any of the tests for protein, carbohydrate, fat or sterol. It is soluble and stable in alcohol, acetone, dioxane, pyridine and glacial acetic acid but insoluble in water, chloroform, sulfuric ether, petrolic ether, benzol or toluol. The purified protein-free material exhibits about one hundred times more activity than that used in the earlier experiments.

The purification of the ether extract has resulted in the isolation of two other substances, namely, graminic and gramidinic adids. These two acids do not exhibit such marked bactericidal powers as does gramicidin.

Gramicidin contains 62.7% carbon, 7.5% hydrogen and 13.9% nitrogen. The molecular weight as determined in camphor is 1400 and the following empirical formula is suggested: $C_{74}H_{106}N_{14}O_{14}$ or formulae differing by C_5H_7NO.

MacLeod, Mirick and Curnen studied the toxicity of gramicidin for dogs (63). All the animals which received 0.3 mg. or more per kilogram of body weight daily showed

well-marked acute or chronic changes in the liver, spleen, kidneys, heart and lungs. Those animals which did not die during the ten day course of treatment were subsequently sacrificed. On autopsy the animals which received a daily dose of 0.2 mg. per kilogram of body weight (or less) showed only a slight fatty degeneration of the liver.

Little, Dubos and Hotchkiss first used gramicidin in the treatment of bovine mastitis (59). The infected quarters were rinsed out with a dilute gramicidin solution and then 800 - 900 cc. of an aqueous solution containing 60 - 240 mg. per liter was infused into each quarter. A transient elevation of temperature was noted and there resulted an acute swelling of the udder which subsided by the next milking. The results obtained were encouraging.

In a subsequent experiment Little, Dubos and Hotchkiss (60) compared the action of gramicidin with novoxil and acriflavine. Novoxil is a 5% or 10% preparation of a pure oxide pf silver in mineral oil. Weirether et al. (60) were the only ones to have used novoxil in the treatment of mastitis previous to this.

The gramicidin was administered in aqueous solution as well as mixed with various kinds of oils. Of all the oils used the best was found to be heavy mineral oil. The object in using oil was to try to reduce the irritation of the secretory tissue as much as possible so that

repeated treatments could be given within a shorter period of time, as well as to avoid exciting the already existing irritation.

The results obtained were as follows: The three therapeutic agents were used to treat 53 quarters of 21 cows. The streptococci were apparently destroyed in 34 quarters (64%). Of 32 quarters treated with gramicidin the streptococci were eliminated from 21 (65%). Eleven of these quarters required only one treatment and eleven quarters did not respond to the treatment at all. Seventeen quarters were injected with novoxil and the streptococci were eliminated from 10 (58%). Only one quarter not previously treated with some other agent responded to a single treatment with novoxil. Seven quarters were treated with infusions of acriflavine and the streptococci were eliminated.from three (42%).

Dubos also reports the isolation of a substance which he calls tyrocidine (28). This agent is inhibitive for both gram-positive and gram-negative organisms. Some of the gramicidin used in the treatment of mastitis described above contained this fraction.

Other extracts of organisms have been reported to have bactericidal powers. Waksman and Wodruff have isolated actinomycin from Actinomyces antibioticus nov. sp. (109). Stokes and Woodward have also obtained alcoholic extracts of bactericidal substances from soil organisms (105).

Experimental

The experimental work with gramicidin consisted of both in vitro and in vivo experiments. The in vitro experiments were designed to study the action of gramicidin when brought into contact with various fluid dairy products as diluents as well as its effect on naturally infected milk. The in vivo work consisted of the treatment of infected cows under varying conditions with infusions of gramicidin solution.

In vitro

In all of the experiments conducted under this heading pure cultures of Lancefield Group B streptococci were used. The cultures were isolated from the milk of an infected animal and typed serologically according to the Lancefield Precipitin Test (macromethod). The organisms were grown on tryptose agar and in tryptose broth and all references to media are to these two. The cultures were incubated at 37 degrees centigrade.

The gramicidin was dissolved in alcohol and then diluted with the selected substance. In all cases the final solution contained, in addition to the gramicidin, 50 cc. of 95% alcohol and 50 cc. of the diluent. The gramicidin used was supplied by Parke, Davis and Co.

The diluents used were: distilled water, 5%

lactose solution, sterile whey, commercial buttermilk, pasteurized milk and pasteurized skim milk. The procedure was the same regardless of the diluent used and was as follows:

The gramicidin solution was made up by dissolving 10 mg. of the extract in 50 cc. of 95% alcohol and then diluting with 50 cc. of the selected diluent. The final concentration of gramicidin in all the solutions used was 0.1 mg. per cc.

An 18-24 hour broth culture of the streptoccus was centrifuged at 3000 RPM for 30 minutes. The supernatant fluid was poured off and the organisms resuspended in 10 cc. of sterile broth. The number of organisms so suspended was kept between 20 and 60 million per cc.

One cc. portions of the resuspended organisms from each of four test tubes was plated out and to the 9 cc. remaining in each tube the following was added:

To tube 1 - 1 cc. distilled water
To tube 2 - 1 cc. alcohol mixture (½ water)
To tube 3 - ½ cc. gramicidin solution & ½ cc. water
To tube 4 - 1 cc. gramicidin solution (0.1 mg.)

Tube 2, in all cases, contained proportions of alcohol and diluent equivalent to that in the tubes of gramicidin solution but did not contain any gramicidin.

The tubes were then incubated in a 37 degree C. waterbath and samples were plated out at ten minute intervals.

Inasmuch as each of the four tubes contained only 9 cc. of suspended organisms (after removing one cc. for plating) the initial count obtained (0 time) was multiplied by 0.9 to correct for the dilution which occured when the gramicidin solutions, etc. were added. The results obtained are summarized in Table 1. A comparison of these results shows that gramicidin was more bactericidal when diluted with water than when diluted with fluid dairy products.

To determine whether or not gramicidin would completely sterilize a broth solution of streptococci an aqueous solution of gramicidin was added to a broth suspension of streptococci. The final concentration of gramicidin was 0.01 mg. per cc. of suspension. The suspension of streptococci was obtained by centrifuging an 18 -24 hour broth culture and then resuspending the organisms in sterile broth. Plate counts were made at ten minute intervals up to 90 minutes and the flask then held in the incubator overnight. The following morning the flask was not sterile as indicated by the plate count. The colonies found on these plates were larger than those regularly obtained and had irregular margins in contrast to the normal smooth margins. The counts obtained are shown in Table 2.

Table 1 - The survival of group B streptococci in broth upon exposure to gramicidin diluted with distilled water, 5% lactose solution, whey, buttermilk, milk and skim milk as indicated by the bacterial plate count in thousands.

Tube No.*	Time of Exposure in Minutes			
	0	10	20	30
		Water Diluent		
1	31,500	35,500	56,000	30,000
2	33,300	31,000	72,000	20,000
3	60,300	4,200	257	67
4	28,800	1,630	200	68
		5% Lactose Diluent		
1	40,950	40,000	42,000	30,000
2	28,350	36,500	38,000	31,000
3	31,500	15,450	12,550	14,100
4	38,700	4,200	2,630	1,067
		Whey Diluent		
1	52,200	74,500	57,500	77,000
2	50,400	58,500	84,000	58,000
3	50,400	45,500	14,950	5,550
4	56,250	2,900	895	500
		Buttermilk Diluent		
1	18,900	23,500	26,500	42,500
2	19,800	21,000	27,000	16,000
3	21,600	12,500	12,950	11,700
4	26,550	9,800	8,350	9,950
		Milk Diluent		
1	27,000	28,000	34,000	30,000
2	26,100	25,000	30,000	29.500
3	22,950	32,500	18,700	15,900
4	30,600	25,000	12,300	5,300
		Skim Milk Diluent		
1	44,550	34,000	37,500	49,000
2	33,750	36,000	34,000	35,000
3	34,200	31,000	23,000	20,550
4	34,200	27,500	18,000	13,350

*In all cases these tubes contained:
Tube 1 - 1cc. of distilled water
Tube 2 - ½cc. of alcohol and ½cc. of diluent used
Tube 3 - 0.05 mg. of gramicidin
Tube 4 - 0.1 mg. of gramicidin

The counts appearing under "0" were all corrected for dilution.

Table 2 - The survival of group B streptococci in broth upon exposure to gramicidin.

Time of Exposure	No. of organisms per cc.
0	41,666,666*
10 minutes	160,000
20 "	10,000
30 "	1,800
40 "	142
50 "	57
60 "	31
70 "	34
80 "	30
90 "	30
18½ hours	19

*corrected for dilution

The next step was to determine the effect of gramicidin upon the bacteria in milk from an infected animal which was eliminating streptococci in her milk. The standard plate count was used to measure the effect. To four tubes, each containing 9 cc. of freshly drawn infected milk, the following was added:

To tube 1 - 1 cc. distilled water
To tube 2 - 1 cc. alcohol mixture (½ water)
To tube 3 - ½ cc. gramicidin solution plus ½ cc. water
To tube 4 - 1 cc. gramicidin solution (0.1 mg.)

The tubes were then held in a 37 degree C. waterbath and plated out at ten minute intervals. The results obtained are shown in Table 3. Gram-negative organisms normally present in milk might account for most of those bacteria surviving.

In addition to measuring the effect of gramicidin upon the bacteria in normally infected milk by the standard plate count, the effect upon the reduction time of methylene blue and resazurin was also determined. The infected milk was first held for 24 hours at room temperature and then to two series of five tubes, each tube containing 9 cc. of milk, the following was added:

Tube 1 - 1 cc. water
Tube 2 - 1 cc. alcohol mixture (½ water)
Tube 3 - 0.05 mg. of gramicidin
Tube 4 - 0.10 mg. of gramicidin
Tube 5 - 0.20 mg. of gramicidin

The tubes were held in a 37 degree C. waterbath for 30 minutes. Methylene blue was then added to one series and Resazurin to the other. The results are shown in Table 4.

Table 3 - Effect of gramicidin upon the standard plate count of milk from an infected cow.

Substance Added	Time of Exposure in Minutes 0	10	20	30
1*	5,715	5,900	6,050	6,900
2*	5,715	5,550	6,450	4,950
3*	5,715	1,750	895	800
4*	5,715	890	565	570

* c.f. footnote Table 1.

Table 4 - Effect of gramicidin upon the methylene blue and resazurin reduction times of infected milk.

Substance Added	Reduction Time in Minutes Methylene Blue	Resazurin
water	1	7½
water & alcohol	2½	19
0.05 mg. gramicidin	2	10
0.10 mg. gramicidin	2	13
0.20 mg. gramicidin	4½	37

In Vivo

This work involved the treatment of cows infected with chronic streptococcic mastitis. The cows treated represented animals which had been infected for varying lengths of time as well as animals in various stages of lactation. One acute case was treated.

In all of the chronic cases the quarters treated were first completely milked out and then infused by gravity pressure with an alcoholic solution of gramicidin which had been diluted with sterile tap water just prior to the infusion. The solution was heated to body temperature before use and the apparatus contained enough material to infuse four quarters, using separate milking tubes as indicated in the photograph. The pictured apparatus was not used in treating the first case (No. 13) because of the large volumes infused.

Milk samples were collected aseptically three times a week for one week prior to treatment and for as long a time as possible following treatment. All the animals treated had in the past been tested at monthly intervals for mastitis by means of the microscopic test. The milk samples were subjected to the following tests: standard plate count, microscopic examination of incubated milk for streptococci, leucocyte count, percentage of chlorides and the thybromal test. Because of the

Photograph of infusion apparatus used in treatment.

difficulty of obtaining samples suitable for these tests from the cows at the Kellogg Farm only the microscopic test was applied to the milk samples from the treated cows. In all cases the microscopic test was used to determine the presence or absence of the streptococci of mastitis.

A brief discussion of each case treated will show the effect of the various dosages employed on the streptococci as well as upon the bacteriological quality and on the total production of milk.

Guernsey No. 13 was the first cow treated. She was fourteen years old and had been infected ever since 1932. The udder was heavily indurated and one quarter was going "blind". Lancefield Group B streptococci were isolated from the milk. The udder was completely milked out and each quarter was infused with 700 cc. of a gramicidin rinse containing 100 mg. of gramicidin per liter. The rinse was immediately milked out; only about one half of the amount infused being recovered. Each quarter was then infused with one liter of solution containing 300 mg. of gramicidin. Seven hours later this was milked out. In the interim the cow's temperature had risen from 101 degrees to 102.6 degrees F., the animal stood shivering and went off feed. The first milk drawn after treatment was gargety and very bloody. After two weeks the milk was no longer bloody and had only a few flakes in it.

The leucocyte count was also returning to normal after having risen. Immediately after treatment milk production fell to about 25% of normal but slowly returned to normal. Sixteen days after treatment the herdsman considered the milk fit to use. No streptococci were found in the milk 87 days after treatment.

The second cow treated, Holstein No. 421, had become infected two months prior to treatment after having been put into a stanchion previously occupied by an infected animal. Group B streptococci were isolated from the milk of the two infected quarters. No rinse was used in the treatment and the dose per quarter consisted of 100 mg. of gramicidin dissolved in 10 cc. of 95% alcohol and diluted with 100 cc. of sterile tap water. The solution was infused late in the afternoon, allowed to remain in the udder overnight and then milked out the next morning. Only two quarters were treated. Ther was no visible reaction in the cow. At no time was the milk bloody although that drawn from the treated quarters was cheesy in appearance for a few days and had a high leucocyte count. Five days after treatment the milk was normal in appearance except for a few flakes. The cow was dried up 19 days after treatment and no streptococci were recovered from the milk during that time. Upon freshening one treated quarter was still negative (68 days after treatment), the other was eliminating streptococci and one untreated quarter was eliminating staphylococci and streptococci.

The following cows were treated at the Kellogg Farm: Guernsey No. 26 was dry at the time of treatment. She had been infected for 13 months. Each quarter, after being completely milked out, was infused with 100 cc. of solution containing 150 mg. of gramicidin. The solution was allowed to remain in the udder overnight and milked out the following morning. Three of the treated quarters were negative following treatment and have remained so for 58 days. The fourth quarter was given a second treatment of 150 mg. seventeen days after the first treatment and has been negative ever since (41 days). All the quarters were negative when the cow freshened and have been so for one month.

Guernsey No. 53 had been infected for 5 months prior to treatment and was in the later stages of lactation. Each quarter, after being milked out, was infused with 100 cc. of solution containing 150 mg. of gramicidin which was allowed to remain in the udder overnight and then milked out. The milk was gargety for about one week following treatment and had a high leucocyte count. There was no blood present in the milk at any time. All quarters were negative on test after treatment and have remained so to the present time (35 days). Milk production fell immediately after treatment but gradually returned to normal.

Guernsey No. 74 had been infected in two quarters for 8 months prior to treatment and was also in the later stages of lactation. Each infected quarter was infused with 100 cc. of solution containing 150 mg. of gramicidin. This was allowed to remain in the quarter overnight. One of the treated quarters cleared up but the other remained suspicious and was given a second treatment of 50 mg. of gramicidin 35 days after the first treatment. The milk was gargety but not bloody for a week following treatment and had a high leucocyte count. Production was also temporarily decreased. The quarter receiving only one treatment has now been negative for 58 days; the other for 23 days.

Guernsey No. 113 had become infected in all four quarters three weeks prior to treatment and was at the beginning of her lactation period. All four quarters were infused, each with 100 cc. of a solution containing 150 mg. of gramicidin which was allowed to remain in the udder overnight. The milk was at no time bloody and was gargety for only a few days. Production was decreased about 50% immediately following treatment but rapidly returned to normal. No streptococci had been recovered 41 days following treatment.

Two non-infected control animals, Guernseys Nos. 70 and 73, were used to determine whether gramicidin exerted any toxic effect upon the udder. One quarter in each cow was infused with 100 cc. of a water and alcohol solution

containing no gramicidin. The water and alcohol were in the proportions used in treatment. There was no change in either the physical appearance of the cows or the milk. The infusion of another quarter in each cow with 100 cc. of solution containing 150 mg. of gramicidin resulted in gargety milk from those quarters. In the case of cow 70 the milk remained gargety for three days and in the case of cow 73 the milk remained gargety for five days. Still another quarter of each cow was infused with 100 cc. of solution containing 50 mg. of gramicidin. The milk from the one quarter of cow 70 was gargety for two days and from cow 73 for four days. In all cases these infusions had been allowed to remain in the quarters overnight; the udder being milked out the following morning. The last quarter was infused with about 40 cc. of an alcohol and oil suspension which contained 150 mg. of gramicidin. The suspension was withdrawn after two and one half hours in the quarter. The milk from the treated quarter in each cow was gargety for about six days following this treatment. One of the previously treated quarters in each cow (the one receiving no gramicidin) was then infused with 100 cc. of solution containing 150 mg. of gramicidin. The solution was milked out after 2 hours and the milk was gargety for several days after treatment.

The one acute case treated was Ayrshire No. 157. This animal exhibited all of the clinical symptoms of an

acute mastitis with systemic illness and was shedding streptococci from all quarters. One hundred cc. of a solution containing 150 mg. of gramicidin was infused by pressure into each of the four quarters and allowed to remain in the udder overnight. Immediately after infusion the cow's temperature rose to 106.1 and she was in great distress. Two of the quarters ceased to eliminate streptococci and two subsequent treatments, using the same dosage in all quarters,had no effect, either in eliminating the clinical symptoms or the streptococci. The animal's reaction to the last two treatments was not as violent as to the first. During the entire period of treatment the milk contained blood, pus, and was very gargety just as it had been before treatment.

Table 5 - Summarized account of the effect of treatment with gramicidin upon cows infected with chronic streptococcocic mastitis.

Cow #	Time Infected	Stage of Lactation	mg. of gramicidin per quarter	Reaction	Effect on Production	Days Negative
113	3 weeks	Beginning	150	Milk gargety one week	Temporary decrease	41
421	3 months	End	100	Milk gargety five days	Temporary decrease	68 1 quarter still +
53	5 months	End	150	Milk gargety one week	Temporary decrease	35
74	8 months	End	150	Milk gargety one week	Temporary decrease	58-1 quarter 23-1 quarter
26	13 months	Dry	150	Dry	Dry	58-3 quarter 41-1 quarter
13	9 years	End	300	Milk bloody and gargety two weeks	Temporary decrease	87*

* Slaughtered 101 days after treatment. No streptococci recovered from udder tissue.

Table 6 - Summarized account of the reaction of non-infected cows to udder infusions of gramicidin.

Treatment	Reaction Cow No. 70	Cow No. 73
Infused LF quarter with 100 cc. of alcohol and water mixture (12½ cc. alcohol). Left in overnight.	None	None
Infused RF quarter with 50 mg. of gramicidin in 100 cc. of solution. Left in overnight	Milk gargety two days	Milk gargety four days
Infused LR quarter with 150 mg. of gramicidin in 100 cc. of solution. Left in overnight.	Milk gargety three days	Milk gargety five days
Infused LF quarter with 150 mg. of gramicidin in 100 cc. of solution. Left in two hours.	Milk gargety two days	Milk gargety two days
Infused RR quarter with 150 mg. of gramicidin in 7.5 cc. alcohol, 10 cc. water and 25 cc. oil. Left in 2½ hours.	Milk gargety six days	Milk gargety six days

Summary

The work previously discussed shows very clearly the bactericidal power of gramicidin solutions in vitro. One tenth milligram of the extract or a final concentration of 0.01 mg. per cc. was extremely bactericidal for Group B streptococci even when the gramicidin was diluted with various fluid dairy products. In no case was complete sterilization obtained.

The treatment of infected animals by udder infusion with 150 mg. of gramicidin in 100 cc. of an aqueous suspension left in the quarter overnight was effective in eliminating the streptococci as indicated by the direct microscopic examination of incubated milk samples. A total of twenty infected quarters was treated and nineteen ceased to eliminate streptococci in the milk and have remained negative to date (95%). One quarter failed to clear up (5%) though the infection of one untreated quarter in the same animal with staphylococci during the period of treatment might indicate reinfection. Seventeen of the now negative quarters (89%) responded to one treatment and two quarters (11%) required two treatments. The one quarter still remaining positive has not yet been treated a second time.

The reaction of the non-infected control animals as well as the infected animals to the treatment indicates

that the gramicidin is irritating to the glandular tissue. The effect varied with the individual cows. When the solution was allowed to remain in the udder for shorter periods of time (2 hours) the lessened irritation, as shown by the reaction of the cow and the quality of the milk produced following treatment, was not appreciable. The use of an oil suspension of gramicidin in the control animals was not any the less but more irritating than an equal amount of gramicidin in a water suspension.

In all the cows treated, both infected and non-infected, treatment was followed by a marked decrease in milk production and then a gradual return to normal production. The leucocyte count and the percentage of chlorides increased immediately following treatment and then returned to normal within a week or ten days. The standard plate count of milk from treated quarters was lowered and the milk was temporarily abnormal following treatment.

The one acute case treated did not respond satisfactorily to the treatment and the violent reaction which followed the treatment did not appear to warrant its use in such cases.

The chronic cases treated indicate that gramicidin might be of great value in the treatment of chronic streptococcic mastitis.

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