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TESTS OF COMPOUNDS WHICH
MIGHT BE USED TO PREVENT
NAVEL AND SURGICAL
INFECTIONS IN YOUNG LAMBS

Thesis for the Degree of M. S.
MICHIGAN STATE COLLEGE

Jack D. Tiner
1946

THESIS

This is to certify that the

thesis entitled

Tests of Compounds which Might be Used
to Prevent Navel and Surgical Infections in
Young Lambs.
presented by

Jack D. Tiner

has been accepted towards fulfillment
of the requirements for

M. S. degree in Animal Pathology

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Date 7-30-46

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PREVENT NAVEL AND SURGICAL INFECTIONS
IN YOUNG LAMBS

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by

JACK D. TINER

A THESIS

Submitted to the Graduate School of Michigan
State College of Agriculture and Applied
Science in partial fulfilment of the
requirements for the degree of

MASTER OF SCIENCE

Department of Animal Pathology

1946

THESIS

7/26/46
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ACKNOWLEDGEMENT

The writer is indebted to Dr. Frank Thorp, Jr. and especially to Dr. W. L. Mallmann for much fundamental preliminary knowledge and many helpful suggestions. The tireless and sympathetic aid of Dr. Thorp on more than one occasion doubtless extended the performing of the experiments presented here further than one person alone could have carried them.

Sincere thanks are offered to the many others who contributed ideas of value in the conduct of this investigation and the preparation of this report. Dr. E. S. Feenstra, Dr. C. F. Clark, and Mr. Donald A. Schmidt deserve more credit than merely mentioning their names gives.

Thanks are expressed to Dr. R. F. Langham for taking all photographs.

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INTRODUCTION

Statistical studies on the causes of death in lambs are almost non-existent. Common knowledge and data from the breeding and experimental flocks of Michigan State College for the year 1943 indicate that a significant number of lambs die each year from navel and docking infections. Since no work had been done to indicate the relative efficiency of various skin disinfectants as an aid in preventing these losses, the present study was undertaken.

HISTORICAL REVIEW OF EXPERIMENTAL SKIN DISINFECTION

The earliest account of the recovery of organisms from skin after the application of a disinfectant to which this writer had access was one by Welch (1892). "Scrapings from the epidermis and beneath the nails were made with a sterilized knife constructed for the purpose, and were transferred to tubes of liquified agar which were then rolled or poured into Petri's dishes." The danger of bacteriostasis was recognized and hands treated with bichloride of mercury were subjected to ammonium sulphide to precipitate the mercury. By thus neutralizing the disinfectant Welch showed that a previous investigator, Furbringer, was not getting the degree of sterilization which had been reported.

A search for organisms in deeper layers of the skin was also made by Welch. An area on a human subject was thoroughly disinfected with potassium permanganate and oxalic acid so that surface scrapings showed no growth. Sterile silk threads were then drawn through the skin from one to five times and dropped into melted agar. A white staphylococcus grew in several colonies along the side of the thread. Also, the stitches from laparotomy wounds were removed by aseptic methods and dropped into melted agar. Fifty to sixty colonies of the white staphylococcus grew around each.

Grossich (1908) has been frequently credited with introducing tincture of iodine as a skin disinfectant, but his priority was disputed by Gershanfeld and Miller (1932) in their review of the literature. Several persons who used iodine prior to this date are mentioned. Gershanfeld and Miller state that "As a disinfectant for the skin, particularly

for the treatment of injuries and for the field of operation, iodine came prominently to the attention of everyone during the year 1905." Tinker and Prince (1911) showed that tincture of iodine was not entirely reliable. They rubbed Bacillus subtilis into the skin of the finger and applied tincture of iodine after allowing the culture to dry for six hours. Sterile threads were drawn vigorously across the skin and dropped into bouillon. Thirty minutes later iodine was again applied and the threads drawn across the area and cultured. Additional scrapings were made with a sterile scalpel and dropped into bouillon culture medium. A "prompt and positive" growth was obtained in every case.

Robb (1913) stated that Bovee employed somewhat similar methods and was able to report sterilization of the skin. This was attributed by Robb to the fact that "the cultures were all taken from the skin still coated with iodine." Removing the iodine with 10 per cent potassium iodide enabled the recovery of Bacillus subtilis, coli, and Staphylococcus albus from the skin of dogs. Silk threads drawn through the area revealed B. subtilis in one of the two trials.

Turner and Catto (1911) tested the extent of surgical disinfection by removing a thin strip of skin immediately after the first incision. This was taken to the laboratory in a sterile test tube and cultivated in nutrient broth for 72 hours. The culture was examined every 24 hours, and if turbidity was observed, a hanging drop slide was made. All specimens not showing growth at the end of 72 hours were routinely examined macroscopically and microscopically. When microorganisms were found they were transferred to agar, studied, and identified.

Bonney and Browning (1918) made cultures of surgical areas on patients prepared for operation with a combination of crystal violet and brilliant green. The skin was deeply scratched with a sharp needle and the surface of an agar plate inoculated. The needle was then resterilized and the process repeated until four streaks were obtained. The results enabled them to report favorably on the mixture. A fallacy in their method, however, was demonstrated by Colebrook (1941):

"Experiment I. A circular area 6 cm. in diameter on the thigh of a normal man was sampled in two ways: a. By making four scratches two inches in length which almost drew blood. The scalpel point used was then streaked across a blood agar plate after each scratch. No colonies could be detected by naked eye or by low power microscope after 48 hours incubation. b. The same area was vigorously rubbed with a gauze swab moistened in a sterile broth, the swab being immediately shaken in 20 cc sterile broth and the bacteria in this enumerated by subcultures of an aliquot part. This count showed that 5,000 organisms were present on the area swabbed.

A conclusion based on method a would clearly have been misleading."

In advocating the use of picric acid, Farr (1931) suggested that antiseptics be tested by scraping and culturing the edge of the wound immediately after the first incision. A complete cross section of the skin would thus be obtained. By doing this he had observed growth in 11 of 27 trials.

Scott and Hill (1925) rubbed a sterile moist swab on the skin and then on an agar plate. The disinfectant, aqueous mercurochrome, was applied and the culturing process repeated. Since 100 per cent results were obtained, bacteriostasis was obviously present.

Tinker and Sutton (1926) tested on human skin the then commonly used antiseptics. The compounds were bichloride of mercury, tincture of iodine, trinitrophenol, mercurochrome, chloramine T, chlorinated lime, and acriflavine. The method employed is not very clearly stated. Apparently a sporulated culture of Bacillus subtilis was applied to the skin, allowed to dry, treated with antiseptic, moistened by sterile distilled water, and scraped deeply with a sterile scalpel. Loops of the scrapings "deeply stained with antiseptic" were used to make cultures. Penetration was tested by excising a 3 mm. strip of skin from a patient prepared for operation. Many different regions of the body were represented, but how they were cultured is not stated. No growth was obtained only with acriflavine, and this was later questioned by Leonard (1930).

Reddish and Drake (1928) used rabbits to compare mercurochrome-220 soluble with tincture of iodine. The abdominal skin was shaved, washed with warm water, and allowed to dry. "The operations were carried out with ordinary aseptic technique, but no special precautions were taken against contamination." Staphylococcus aureus was applied with a sterile cotton swab over the shaven surface and allowed to dry. The rabbit was removed from the table and placed in a cage. Two hours later (5 tests) and 24 hours later (47 tests) squares were marked off and antiseptics applied to the center of each. An effort was made to apply in the same manner each time. One area treated with distilled water was used as a control. Five minutes after the antiseptics had been applied the excess was removed with a sterile moist swab. The control area was also swabbed. One square centimeter in the center of each area was scraped with a sterile scalpel until capillary bleeding occurred. All scrapings were re-

moved in 1 cc. of sterile water in a petri dish. Fifteen cc. of cooled melted agar was poured to make a plate. If no growth was observed in 24 hours, Staphylococcus aureus was streaked on the plate, and if it produced colonies, bacteriostasis was considered to be not present. No unseeded and untreated area was cultured to establish the necessity for seeding the skin with Staphylococcus aureus instead of using merely the skin flora.

A completely new method was introduced by Simmons (1928). The skin of the abdomens of a series of white mice was shaven and contaminated with a 10 day old sporulated culture of Bacillus anthracis and painted liberally with antiseptic. Ten minutes later (2 tests) and 2 hours later (7 tests) the skin from the center of the area was excised, inverted, and the wound sutured together with horsehair sutures. If the animal died, cultures were made from the heart blood. Surviving animals were observed for eight weeks.

Simmons used the skin of rabbits in a manner similar to the methods of other investigators which have been described. An area was dry shaven, and cultures of Staphylococcus aureus, Streptococcus pyogenes, Streptococcus scarlatinae, Escherichia coli, and Clostridium welchii applied. After being allowed to dry for an hour, 5 cm. squares were marked off and each painted liberally with an antiseptic. After 5, 10, and 20 minute intervals (30 and 60 minutes for Cl. welchii) cotton swabs moistened in sterile broth were rubbed over the painted areas and cultures made on a plate of nutrient or blood agar and a flask containing 100 cc. of broth. A second set of cultures was then made from a smaller portion of the same area. Deep skin cultures were made by scrap-

ing a still smaller portion until capillary bleeding occurred and plating the scrapings in 15 cc. of melted agar. Superficial and deep cultures were made for each of the time intervals used. Simmons secured best results with U. S. P. tincture of iodine, stating that it destroyed all bacteria in every case.

Rodriguez (1928) compared iodine with mercurochrome on the natural flora of the oral mucosa and obtained very good results with 3.5 per cent tincture of iodine and 1.75 per cent iodine in glycerin.

Roberts (1929) took cultures by holding a sterile swab of standard size moistened in sterile media against the treated and control areas. There was no rubbing and no more pressure than necessary was used to hold the swab in place. At the end of exactly thirty seconds the swabs were rinsed in a tube of nutrient media and discarded. The tubes were then incubated at 37° C. for five days. Antiseptics were tested on the skin of rabbits and humans by this technic. This would obviously not give any idea of the depth of the disinfection. This procedure can merely show that a film of disinfectant was placed on top of the skin, and perhaps on top of the bacteria as well.

Raziss, Severac, and Moetsch (1930) used a technic based on that of Roberts. The abdominal skin of rabbits was depilated with barium sulphide and the area washed for three minutes with soap containing Metaphen. Afterward Metaphen 1-1,000 was applied to the skin and removed with alcohol and ether. The area in question was then rubbed 30 seconds with a sterile cotton swab moistened in sterile broth. The tip of the swab was then clipped and allowed to fall into a test tube of sterile bouillon. They concluded that "this procedure effected complete sterilization of the abdomen as evidenced by cultures."

The publication of 100 per cent results drew some criticism. Leonard (1930) in a letter to the editor of the American Medical Association Journal questioned the validity of Tinker and Sutton's results with acriflavine and those of Raziss, Severac, and Moetsch with metaphen. He stated that: "One hundred per cent results involving the use of biologic material such as living tissue or bacteria always invites close inspection on the part of those who have been unable to duplicate such results by their own experimental methods." He further pointed out that acriflavine will inhibit growth of spores and bacteria in high dilution, and that metaphen according to Raziss et al. was more than bacteriostatic enough to prevent growth in the dilutions used.

White and Hill (1930) preceded to demonstrate that Raziss and co-workers were actually dealing with bacteriostasis by repeating their experiments, and inoculating the tubes in which no growth had been obtained with Staphylococcus aureus. The staphylococci failed to grow, thus demonstrating that enough metaphen had been carried over to produce bacteriostasis. When swabs were dropped into 900 cc. of media dilution was such that microorganisms grew.

Kelser and Mohri (1932) made comparative tests of "Mercurochrome-220 Soluble" and tincture of iodine on the normal skin flora of the horse. An area 6 by 9 inches on the side of an animal was dry shaven and rectangles $1\frac{1}{2}$ by 2 inches were marked off. The disinfectants were rubbed on. At the end of ten minutes moist sterile swabs were rubbed briskly over the area, dropped into sterile test tubes, carried to the laboratory, and streaked on blood agar plates. Good percentages of growth were obtained in spite of the obvious risk of bacteriostasis.

In the same paper an apparatus for washing the organisms to free them from bacteriostatic quantities of the disinfectant was described. It consisted of Berkefeld filters with an attachment for forcing a backflow of distilled water. Unfortunately, they used it only for test tube cultures of Clostridium tetani. They pointed out that some of the organisms stick in the pores of the filter. Whether the method would be practical for washing skin scrapings would have to be determined by actual trial.

Birkhaug (1933) compared phenyl-mercuric-nitrate, mercurochrome, and metaphen using rabbits. The animals were strapped on operating tables and the abdomen depilated with a semi-liquid mixture of 1 part barium sulphide and 2 parts flour. After rinsing with water 25 squares with about 20 mm. sides were marked off with a red wax pencil. Cultures of Staphylococcus aureus, Bacillus subtilis (sporulated), Streptococcus hemolyticus, and Escherichia coli were smeared on and dried. The disinfectant was then applied. Exactly three minutes later an area approximately 3 by 3 by 2 mm. was pulled up with forceps, cut off, and deposited in 100 cc. of Douglass broth. Ninety-two per cent sterility was obtained with metaphen and 96 per cent with phenyl-mercuric-nitrate.

Norton (1920) showed that in handwashing neither antiseptic nor plain soaps were of any value other than for mechanical cleansing. However, it was not until 1938 that Price published a method for measuring the quantitative effect that this mechanical cleansing had on the bacterial flora of the hands. By washing in a series of sterile basins and plating the water, Price derived a mathematical formula for the rate of removal which was found to decrease logarithmically. He was able to estimate with accuracy the numbers of bacteria on the forearms and hands of

various individuals. The terms "transient" and "resident" bacteria were introduced. It was demonstrated that persistent exposure to pathogenic organisms will incorporate them into the individual's flora as Welch had shown (1892).

Pohle and Stuart (1940) and Sears et al. (1941) used Price's procedure and found it quite reliable.

Bass (1939) prepared rabbits in the same manner as Birkhaug. However, he did not add organisms to the skin, stating that "in no case was it ever sterilized by depilation." Mercurials, tincture of iodine, and picric acid were tested. Seventy per cent ethyl alcohol served as somewhat of a control. The antiseptics were placed in sterile test tubes, each containing a sterile swab. The soaked swabs were rubbed on the area for 25 seconds, and at the end of three minutes a piece of skin was snipped off and dropped into a test tube containing 100 cc. of infusion broth. This was incubated at 37° C, and at the end of 48 hours, if no growth was observed, 50-1,000 organisms from untreated skin were dropped in to prove that there was no bacteriostasis.

Barnes (1942) tested a synthetic detergent, cetyl-trimethyl-ammonium bromide by marking out an area on the forearm 4 by 6 inches. It was first swabbed a minute with a cotton swab. This was dropped into 100 cc. of broth. One cc. of this was plated in 10 cc. of nutrient agar and resulting colonies counted. The detergent was then applied by rubbing three minutes with moistened swabs. These were rubbed briskly so as to obtain a good lather. A one minute rinsing with water under the tap was used to remove the disinfectant. Plates were then made as before and colonies counted. Barnes cited Price's results as sufficient evidence that

swabbing did not remove an appreciable amount of the total flora and used as a control previously untouched areas. The effect of an equal amount of rubbing using ordinary soap followed by rinsing was not determined.

In an effort to estimate with reliability the true value of a disinfectant against pathogenic organisms, Numgester and Kempf (1942) employed an infection prevention test in mice. Cultures of organisms of "high and constant virulence for mice" were rubbed on the tail, the tail was dipped into an antiseptic, and a half inch piece was removed and inserted into the peritoneal cavity. The same pathogenic organisms were recovered with a high degree of frequency from the heart blood of those animals which died of the ensuing peritonitis. Various disinfectants showed different abilities to protect animals. Alcoholic iodine two per cent was the only antiseptic which gave 100 per cent survival, and Merthiolate the only one which failed completely. Cultures were also made directly from a second portion of the tail. The percentage of growth obtained from these was slightly lower than the number of mice infected.

A similar procedure was developed by Sarber (1942). The abdominal skin was contaminated with Streptococcus hemolyticus, disinfected with various substances, and then inserted into the peritoneal cavity. Hemolytic streptococci were recovered from all mice that died.

Gershenfeld and Witlin, (1941) tested disinfectants on the skin flora of rabbits. They were able to show that "in not one instance was there complete sterility", but unfortunately their method allowed no studies as to the relative merits of the various compounds.

The question of the location of bacteria on or in the skin has always confronted workers in skin disinfection. Welch (1892) was probably

the first to investigate this problem.

Price (1938) found that the transient organisms were "relatively scarce on clean, unexposed skin These bacteria lie free on the skin, or are loosely attached by grease and other fats along with dirt. Many collect under the nails. . . The resident flora is entirely on the skin, attached by adhesion, adsorption, or possibly in some special way." Apparently he was unable to demonstrate organisms in sebaceous and sweat glands.

Lovell (1945) incubated human skin samples in a moist atmosphere and at the end of six hours fixed and sectioned them. With specially stained slides, masses of staphylococci were demonstrable in the sebaceous glands.

In the literature to date there seems to be no report of anyone's being able to demonstrate a bacterial flora in sweat glands.

METHODS AND RESULTS

The disinfectants used in these experiments were as follows:

Tincture of Iodine USP XII.

Liquor Iodi Fortis USP XII or Lugol's Solution.

Iodine Suspensoid, Merck and Co. Inc., Rahway, N. J. A 4 per cent solution will be referred to in this paper as colloidal iodine. In some instances it was used in combination with a wetting agent, Naccanol, National Aniline Chemical Co., New York, N. Y., in order to reduce surface tension and enhance penetration among densely matted wool fibers.

Liquor Cresolis Saponatus USP XI, Jensen-Salsbery Laboratories, Kansas City, Mo. A 2 per cent solution was given a limited trial.

Roccal, Winthrop Chemical Co. Inc., New York 13, N. Y. A quarternary ammonium compound.

Hyamine 1622, Rohm and Haas Co., 222 Washington Square, Philadelphia, Pa. A quarternary ammonium compound, obtained through the kindness of Dr. W. L. Mallmann. The purity of the sample used in making up the solutions was unknown, and the concentrations consequently could not be determined.

Seventy per cent ethyl alcohol.

Tincture of Phemerol, Parke Davis and Co., Detroit, Michigan. A quarternary ammonium salt. The tinted form of the tincture was employed.

Zephiren Chloride, Winthrop Chemical Co. A quarternary ammonium compound with the same structural formula as Roccal, but marketed as a stainless tincture.

At the outset the immediate problem was to find a method of showing whether it would be advisable to replace the generally recommended Tincture of Iodine USP with some other compound. Since the worth of a laboratory test is largely related to the degree to which natural conditions are simulated, the initial trials became an empirical search for a method that would approximate field usage and yield quantitative data.

Sheep wool is unique in that it contains lanolin, a fat like secretion from the sebaceous glands. The question of what effect it might have on the efficiency of the disinfectants had to be answered. An attempt to study this directly was made by seeding tryptose agar* plates with a rapidly growing *Staphylococcus* isolated from a lamb dying of a navel infection. A coat of sterile lanolin was then poured over the surface and a drop or two of the compound in question was placed on top of the lanolin after it had hardened. The plates were incubated for 48 hours and the extent of the zone of inhibition was measured. Trials indicated that colloidal iodine was quite efficient and that an aqueous solution of Hyamine was of little if any value for penetrating a heavy coat of lanolin. Tincture of iodine was considered not comparable since it persistently etched through the wool fat and diffused throughout the agar below, thus allowing no growth over large areas of the plate.

The methods of Price (1938) seemed to have some promise of being useful in evaluating the disinfection of sheep skin and wool. Accordingly, a sample of wool was clipped from a sheep in the barn with sterile scissors and placed in a sterile jar. One gram samples were weighed out and exposed to tincture of iodine and colloidal iodine for the intervals of 30 or 60 seconds. They were then rinsed in 500 cc. of distilled water to which 10 grams of sodium thiosulfate had been added before sterilizing in the autoclave. Samples were then transferred to two-quart jars containing one gram of soap which was dissolved and sterilized in one liter of distilled water. Two agar platings of 0.5 cc. each were made from the wash

*Bacto Tryptose dehydrated, Difco, Detroit, Mich.

water and incubated 48 hours before counting. On some of the plates an excessive growth of spreaders made counting difficult.

In order to surpress as many variables as possible until more was known of the method and its possibilities, it was decided to substitute wool which had been sterilized and seeded with the Staphylococcus mentioned above by immersing in inoculated broth, incubating for 24 hours at 37° C., draining, and drying. One change was made in the procedure: The shaking was done with a Miller Paint Mixer* for three minutes in this and all succeeding trials. The data in Tables I and II indicate the results obtained. When the application of iodine was not followed by rinsing in sodium thiosulfate, 100 per cent results were obtained. A ten second immersion in iodine with a 40 minute delay in neutralization did not achieve a complete kill. Aqueous hyamine neutralized by soapy water failed to significantly reduce the numbers of bacteria.

The Staphylococci on the seeded wool died out in approximately ten weeks and it was decided to try measuring the effect of the disinfectants on skin samples taken from young lambs coming to autopsy. These lambs represented several breeds and had died of various conditions as indicated in Table IX and constituted a more or less random sampling of the lamb crop. A rough estimate was made of the total number of bacteria per lamb. This was done by determining the average number that were removed from a square centimeter (Table XII) and multiplying by the area of the whole skin as calculated by direct measurement when spread out. The results of such computations on ten lambs are recorded in Table III. The variation in the total number of organisms per lamb might be termed ex-

*Miller Mfg. Co., 3238 Bryn Mawr Ave., Chicago.

treme -- from 432 million to in excess of 43 billion. Distilled water was found to be as effective as soapy water for removing bacteria from skin and wool and was used in these and in all subsequent experiments. It was found that the skin samples could be stored in the freezing compartment of the electric refrigerator at a temperature of -3 to -5° C. with no appreciable quantitative change in the bacterial flora over periods of several months.

The data recorded in Tables VI and VII were obtained by using the procedure outlined in Figure 1. Small skin samples of measured area were immersed in 30-50 cc. of disinfectant for ten seconds, allowed to drain for fifty seconds, and rinsed in 500 cc. of a sterile solution of a neutralizing agent for thirty seconds. Sodium thiosulfate was used in the case of iodine and 0.2 per cent soap in the case of the quaternary ammonium compounds. This was followed by washing for three minutes. Counts for the "control" listed in Table VI were obtained by dropping skin samples directly into the washing jar and shaking. That such counts were too high is shown by Table IV in which percentages of organisms lost in disinfecting and rinsing varied from 56.4 to 93.7. Approximate percentages of the flora removed by the first three minutes washing and rinsing was determined and recorded in Table V. They appeared high enough that no further washing was considered necessary. Use of soapy water apparently gave no significant increase in efficiency.

The altered method outlined in Figure 2 was used to obtain the results in Tables VIII and IX upon which the conclusions of this paper are largely based. Except in the case of Lamb 7989 (Table VII), the neutralizing agent was replaced by 500 cc. of sterile distilled water. The con-

trol runs were subjected to an equal amount of agitation in volumes of sterile distilled water equaling the amounts of disinfectant and water that the skin samples were exposed to. More uniform skin sample sizes were obtained by measuring the area of a large piece of skin, for example 50 sq. cm., dividing it into perhaps 70 small pieces which were made as uniform as possible, and then taking three of these at random to make samples of about $50/70 \times 3 = 2.15$ sq. cm. for each trial.

In order to show the relationship of the compounds extensively tested to some of those that are very commonly used by the veterinary profession, some data were obtained on Liquor Cresolis Saponatus USP XI and 70 per cent ethyl alcohol.

An additional experiment was done to determine the extent of any neutralizing action which oxalated blood might exert on tincture of iodine and colloidal iodine. The procedure is depicted in Figure 3, and the results recorded in Table IX. The 5 minute stop in oxalated sheep blood between the rinsing and washing jars is the only change from the procedure outlined in Figure 2.

In order to observe the location of bacteria on lamb skin, samples were taken from near the naval of recently dead newborn lambs, fixed in Zenker's solution, embedded, sectioned, and stained with Gram Weigert stain. Photomicrographs (Figures 4 and 5) were made of some bacterial forms among the wool fibers. The red blood cell present in Figure 4 is interesting to note. It hints as to the nature of the medium in which bacteria establish themselves on the skin of a newborn lamb -- a mixture of fetal fluids and blood.

During the performance of the above tests the observation was made that areas of the skin stained as long as a week previously with iodine had extremely low numbers of bacteria. It appeared that the duration of disinfection might offer some basis for the comparison of disinfectants. Accordingly a group of five sheep was placed in the barn, areas marked off on their sides, control samples taken, and 24 hours later disinfectants applied. The first control samples represented 1/5 gram per area per sheep. A control patch was interspersed among the disinfected areas. The day following disinfection the samples listed for September 15 in Table X were taken. The experiment was run a second time 28 days later, using different areas on the same sheep. Samples were taken just before and shortly after the disinfectants were applied. Results of both experiments are recorded in Table X in which the logarithm of the number of organisms is plotted as a function of the time in days. The iodine compounds achieved the most immediate and lasting suppression of the bacterial flora. When the samples were still wet with the solutions it was necessary to dry them in a vacuum. The areas treated with the tinctures were apparently dry within 24 hours, but the aqueous compounds produced wet areas which dried out more slowly. No effort was made to reach a constant weight. The samples were considered dry when the petri dishes containing them no longer became cold after being removed from the vacuum chamber, warmed, and returned to the vacuum. Neither was it determined whether ordinary wool, e. g. control samples would suffer a change in weight if placed in a vacuum. These factors would probably not affect the results significantly, but should be checked were the method to be much more extensively used.

When lambskin was placed in Roccal, a visible precipitate often immediately formed. It was easily seen by all observers who happened to be present. Its presence was confirmed by use of the photelometer*. A liter of distilled water and a liter of Roccal 1-1,000 were checked for percentage of light transmission. Washings from the control sample which had just been used a few minutes previously to obtain the bacterial count for October 13, 1945 (plotted in Table X) were then added to each in 10 cc. amounts and the percentage transmission again checked. Results are recorded in Table XI. The increased light absorption on the part of the Roccal solution indicates the presence of the precipitate.

The mean and standard deviations of a few series of samples were determined. Table XIII contains a series of eight consecutive control areas from Lamb 8030 and Table XIV nine consecutive areas used in determining the total bacterial count for Lamb 7989.

*Sheard-Sanford type.



Figure 1. Apparatus for disinfectant tests with rinse in neutralizing agent.



Figure 2. Apparatus used in disinfectant tests with rinse in distilled water.



Figure 3. Apparatus used for disinfectant tests including sterile oxalated blood for study of its neutralizing effect.



Figure 4. Photomicrograph of section of lamb skin showing wool fibers, bacterial forms, and a red blood cell. x 838



Figure 5. Photomicrograph of section of lamb skin showing wool fibers and bacterial forms. x 838.

Table I. ONE GRAM SAMPLES OF STERILE WOOL SEEDED
WITH STAPHYLOCOCCI AND EXPOSED TO TINC-
TURE OF IODINE AND SODIUM THIOSULFATE

Minutes Exposed to Iodine	Minutes in $\text{Na}_2\text{S}_2\text{O}_3$	Colonies per Plating .5 cc.
0	0	24,946
$\frac{1}{4}$	2	1,746
$\frac{1}{2}$	2	3,095
1	2	2,908
2	2	621
3	2	986
5	2	751

Table II. ONE GRAM SAMPLES OF STERILE WOOL SEEDED
WITH STAPHYLOCOCCI AND EXPOSED TO:

Minutes Exposed	Minutes in $\text{Na}_2\text{S}_2\text{O}_3$	Colonies per cc. Plating from Soapy H_2O
a. TINCTURE OF IODINE FOLLOWED BY SODIUM THIOSULFATE		
0	0	39,375
1	0	0
1	(3 in H_2O)	0
1	$\frac{1}{2}$	14,957
3	$\frac{1}{2}$	4,865
10	$\frac{1}{2}$	2,487
20	$\frac{1}{2}$	1,365
40	$\frac{1}{2}$	145
b. COLLOIDAL IODINE FOLLOWED BY SODIUM THIOSULFATE		
0	0	39,375
1	0	0
1	$\frac{1}{2}$	3,415
3	$\frac{1}{2}$	5,123
10	$\frac{1}{2}$	7,600
20	$\frac{1}{2}$	1,337
c. AQUEOUS HYAMINE, 1-1,000 FOLLOWED BY SOAPY WATER		
	Minutes in Soapy H_2O	
0	0	39,375
1	$\frac{1}{2}$	Innumerable
3	$\frac{1}{2}$	38,000
10	$\frac{1}{2}$	43,000
20	$\frac{1}{2}$	36,000
40	$\frac{1}{2}$	(Plated from distilled H_2O) - Innumerable

Table III. TOTAL BACTERIAL COUNT AND MISCELLANEOUS INFORMATION ON LAMBS USED

Date of Death	Breed & Autopsy Number	Diagnosis	Age	Weight in pounds	Area 2 in Cm ²	Sampling De-organisms lay in Days in Millions
2/22/45	Shropshire 7928	Undetermined	Born dead	8	2,285	1 563
2/26/45	Shropshire 7932	Undetermined	12 hrs.	5	2,245	1 27,400
3/2/45	Rambouillet 7944	<u>Cl. perfringens</u> infection	33 days	25	. . .	0 (214.00 per Cm. ²)
3/2/45	Shropshire 7946	Internal hemorrhage	1 day	9	2,359	0 17,500
3/16/45	Cotswold 7982	Internal hemorrhage	2 days	8	2,166	1 45,320
3/19/45	Shropshire 7988	Pneumonia	9 days	12.5	2,640	4 586
3/19/45	Shropshire 7990	Pneumonia	9 days	15	3,127	36 2,840
3/19/45	Hampshire 7989	Internal hemorrhage	4 days	9	2,428	3-13 10,320
4/4/45	Hampshire 8024	Pneumonia, open foramen ovale	25 days	19	2,451	1-22 432
4/6/45	Shropshire 8030	Broken ribs	5 days	6.5	1,551	2-19 11,590
4/6/45	Shropshire 8031	Pneumonia, open foramen ovale	23 days	20	2,327	19 545
5/1/45	Hampshire 8069	Pneumonia	14 days	15	3,027	1 37,200
5/2/45	Oxford 8085	Pneumonia	15 days	14	1,795	1 4,098

Table IV. "MECHANICAL" LOSS OF BACTERIA IN DISINFECTING AND RINSING

Lamb	Total No. Organisms per Cm^2 - Without Previous Washing	No. per Cm^2 Indicated by Control Plates	% Lost
7989	4.26×10^6	1.86×10^6	56.4
7990	907,000	104,500	88.5
8024	177,000	28,010	84.3
8069	1.23×10^7	765,000	93.7
8030	7.46×10^6	1.23×10^6	83.5

Table V. EFFECTIVENESS OF WASHING PROCEDURE IN REMOV-
ING BACTERIA FROM SKIN OF YOUNG LAMB

Lamb 8030:		<u>Distilled Water</u>				
Minutes		3	6	9	12	15
No. Cm^2		1.09×10^7	299,000	34,860	11,860	11,300
		87.1%				
1.13 $\times 10^7$ = No. per cm^2 removed by first 15 min. washing						
Total No. per Cm^2		1.13 $\times 10^7$ of which 97.1 removed in first 3 min. Subtract approx. 83.5% lost in disin- fectant and rinsing to leave approx. 13.6% of flora used in actual test.				
Lamb 8030:		<u>Soapy Water</u>				
Minutes		3	6	9	12	15
No. Cm^2		3.42×10^6	28,000	4,000	14,660	3,110
		98.7%				
3.47 $\times 10^6$ = No. per cm^2 removed by first 15 min. washing						
Total No. per Cm^2		3.47 $\times 10^6$ of which 98.7% removed in first 3 min. subtract approx. 83.5% lost in disin- fectant and rinsing to leave approx. 15.2% of flora for actual test of disinfectant.				

Table VI. EFFECT ON LAMBSKIN FLORA OF ONE MINUTE EXPOSURE TO VARIOUS
DISINFECTANTS FOLLOWED BY NEUTRALIZATION, "CONTROL" COUNTS
TOO HIGH -- SURVIVAL PERCENTAGE TOO LOW

Lamb		"Control"	Tr. I Na ₂ S ₂ O ₃	Col. I Na ₂ S ₂ O ₃	Col. I & Nac. Na ₂ S ₂ O ₃	Roccal Soap
7932	#Samples	4	2	2		
	#Cm ²	1.2x10 ⁷	280,000	2.4x10 ⁶		
	%Survival	100.0	2.3	19.7		
7944	#Samples	4	1	1		
	#Cm ²	214,000	53,000	49,000		
	%Survival	100.0	2.47	23.0		
7982	#Samples	5	1	1		
	#Cm ²	2.0x10 ⁷	11,830	1.1x10 ⁶		
	%Survival	100.0	0.01	5.6		
7988	#Samples	7	3	3	3	2
	#Cm ²	2.2x10 ⁶	356,000	576,000	780,000	965,700
	%Survival	100.0	16.0	26.0	35.1	43.4

Table VII. EFFECT ON LAMBSKIN FLORA OF ONE MINUTE EXPOSURE TO
VARIOUS DISINFECTANTS FOLLOWED BY NEUTRALIZING AGENT

Lamb		Cont. H ₂ O	Tr. I Na ₂ S ₂ O ₃	Col. I Na ₂ S ₂ O ₃	Col. I & Nac. Na ₂ S ₂ O ₃	Roccal Soap
7989	#Samples	5	7	4	8	5
	#Cm ²	2.0x10 ⁶	585,000	1.12x10 ⁶	1.56x10 ⁶	1.08x10 ⁶
	%Survival	100.0	29.1	55.5	77.5	53.8

Table VIII. EFFECT ON LAMBSKIN FLORA OF ONE MINUTE EXPOSURE TO
VARIOUS DISINFECTANTS

Lamb		Cont.	Tr. I	Col. I	Col. I & Nac.	Lugol Iod.	Roccal	70% Ethyl Alcohol	Liquor Cresolis Sapona- tus 2%
7990	#Samples	3	5	5	3		3		
	#Cm ²	104,200	1,500	3,010	972		9,100		
	%Survival	100.0	1.44	2.89	0.93		8.72		
8024	#Samples	4	4	4	4				
	#Cm ²	23,400	1,685	1,500	375				
	%Survival	100.0	7.2	6.4	1.6				
8069	#Samples	15	12	12	12	12	10	4	4
	#Cm ²	735,000	9,650	7,300	7,100	10,650	21,800	78,200	71,300
	%Survival	100.0	1.31	0.99	0.97	1.45	2.97	10.64	9.7
8030	#Samples	8	8	8		8			
	#Cm ²	1.99x10 ⁶	11,950	21,200		15,350			
	%Survival	100.0	0.60	1.06		0.77			

Table IX. EFFECT ON LAMBSKIN FLORA OF ONE MINUTE EXPOSURE TO
VARIOUS DISINFECTANTS

Lamb		Cont.	Tr. I	Col. I	Col. I & Nac.	Lugol Iod.	Roccal
8069	#Samples	15	12	12	12	12	10
	#Cm ²	735,000	9,650	7,300	7,100	10,650	21,800
	%Survival	100.0	1.31	0.99	0.97	1.45	2.97
+ ADDITIONAL 5 MINUTE STOP IN OXALATED BLOOD							
			Tr. I	Col. I			
	#Samples		3	5			
	#Cm ²		23,400	28,300			
	%Survival		3.18	3.85			

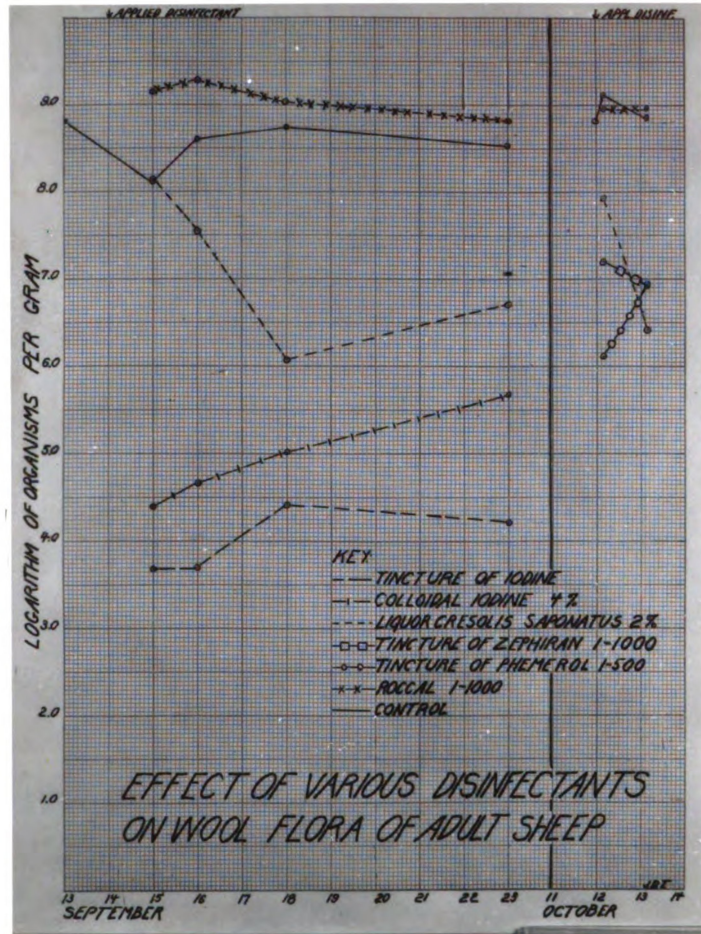


Table X. EFFECT OF VARIOUS DISINFECTANTS
ON WOOL FLORA OF ADULT SHEEP

Table XI. PRECIPITATION OF AQUEOUS ROCCAL BY WOOL WASHINGS

	1 liter Water Distilled	1 liter Roccal 1-1,000
Photometer	100	100
Washings obtained by shaking 5 grams sheep wool in 1,000 cc. distilled water added at rate of 10 cc. between photometer readings:		
	98.6	98
	97.2	96
	96	94.4

Table XII. AVERAGE NUMBER OF ORGANISMS PER CM² SHOWING VARIATION IN A SERIES OF LAMBS

Lamb Number	Organisms in Thousands
7928	246
7932	12,200
7944	214
7946	7,430
7982	20,000
7988	2,220
7990	907
7989	4,260
8030	7,460
8031	234
8069	12,200
8085	<u>2,588</u>
	12) <u>70,069</u>
Mean:	5,839 Standard deviation: 5,985

Table XIII. A SERIES OF CONTROL SAMPLES SHOWING VARIATION

Lamb 8030

Organisms per Cm^2 in thousands

855
3,380
1,004
1,720
1,390
2,500
1,890
2,180

8) 14,919

Mean: 1,865 Standard deviation: 775

Table XIV. VARIATION IN TOTAL BACTERIAL COUNT PER SAMPLE

Lamb 7989

Organisms per Cm^2 in thousands

4,630
2,580
2,860
1,540
2,150
3,540
10,600
3,950
3,360

9) 35,210

Mean: 3,912 Standard deviation: 2,552

DISCUSSION

Since the time of Lister various substances have been introduced as skin disinfectants. More than once the initial claims for a new product have implied that actual sterilization was accomplished. Evidence from experimental results often seemed to substantiate these claims. Closer examination has, in every instance of which the writer is aware, revealed that only varying degrees of disinfection had been accomplished. Perhaps it is time to recognize the apparent reality and instead of seeking an agent which will give absolute sterility, effort should be made to develop germicides which are very highly efficient in reducing the skin flora to a minimum.

In the past, conclusions that any agent is 100 per cent germicidal have invariably been due to a faulty method of evaluating its action on the skin. Before attempting any further work in this direction a careful examination of the procedures of other investigators and the potentialities of each method was considered necessary.

Two approaches to the problem of evaluating the action of disinfectants on the skin have been used. By one, a pure culture of the organism, usually a pathogen, is applied, allowed to dry, and treated with the compound in question. Various techniques are then employed to recover the surviving organisms. The second method uses the normal skin flora. In both, a most difficult problem seemed to be to separate recovered organisms and antiseptic so that bacteriostasis did not give a false impression of germicidal value. Not too adequate fundamental knowledge as to when bacteria are really "dead" increases the difficulty of

reaching an agreement on how this separation may be accomplished.

Table II shows 100 per cent disinfection with iodine compounds when the seeded wool sample was transferred without rinsing directly from the disinfectant to the washing jar. When transferring from tincture of iodine a deep iodine color was produced in the latter. This seemed to indicate that the carrying over of any appreciable quantity of iodine into the solution from which the plating is to be done is to be carefully guarded against.

The data in Tables VI and VII indicate that a neutralizing agent which is specific for a disinfectant is able to "revive" supposedly "killed" organisms. The work of Sears, et al. (1941) contains evidence that they too could have been "reviving" some organisms when they followed 3.5 per cent alcoholic iodine with sodium thiosulfate solution in experimental pre-operative hand scrubbing. Welch, (1892) stated that a large percentage of the flora on supposedly disinfected skin could be recovered if the bichloride of mercury was followed by ammonium sulfide.

Bachem (1913) mentions that the usual method of applying tincture of iodine as a skin disinfectant at that date, in Germany at least, was to allow it to dry and remove it within a minute with sodium thiosulfate. To the best of the writer's knowledge, Tables VI and VII present the first evidence that this procedure is not experimentally justifiable. In performing the experiment it was noticed that frequently the iodine stain in the wool fibers was visible even after the percentage of survivors had been markedly raised by the thiosulfate solution. It is interesting to note that the color produced by tincture of iodine seemed to be more completely removed by the thiosulfate solution, whereas colloidal iodine

which was consistently much more susceptible to neutralization in or on the bacteria produced the more lasting stain in wool fibers.

The data presented can leave very little question that solutions of iodine are among the best skin disinfectants available at the present if they are not followed by neutralization. Just which iodine compound should be used depends largely on the task at hand. Tincture of iodine, because of its alcoholic content and resulting lowered surface tension is able to diffuse very rapidly among wool fibers and foreign material. However, there is as yet no experimental evidence to indicate that there is any justification for its choice over the more economically prepared aqueous Lugol's solution. Colloidal iodine, according to Anderson (1939), has unusually high penetrating power and consequently it may be superior to the other two for certain purposes. Nonetheless, it was noticed that when colloidal iodine was applied to the wool of adult sheep the stains remaining a few days later were highly irregular. Apparently the colloidal particles did not disperse among the wool fibers as readily as the aqueous iodine solution in which these particles were suspended, and consequently were left concentrated in the areas of original application. The results of this particular experiment (Table X) indicated that it did not suppress the flora in the disinfected areas quite as lastingly as tincture of iodine.

When Naccanol was added to colloidal iodine an apparently stable mixture with lowered surface tension resulted. It gave promising results (Table VIII) as a disinfectant. However, the neutralizing effect of the sodium thiosulfate solution appeared to be markedly raised by the presence of the Naccanol (Tables VI and VII). Since the trial with oxalated blood

indicated that blood and probably other body fluids might have some neutralizing effect on iodine compounds, it was not possible to reach any conclusions as to the relative worth of the colloidal iodine -- Naccanol mixture.

There is no evidence as to what might result if the iodine content of the usually prepared solutions were varied. Work on the question of what might approximate the optimum strength of the various iodine compounds and in fact all disinfectants is indicated. The writer is indebted to Dr. N. A. Fattu of the Department of Psychology and Philosophy, Michigan State College, for calling to his attention the dosage-mortality curves which were employed by C. I. Bliss in determining the strengths of insecticides to be used. More references on the subject are found in Bliss (1935) and Broadbent and Bliss (1936).

Quarternary ammonium compounds have been given their first controlled trial on the skin of sheep. Although the percentages of survival recorded from lamb skin flora seem favorably low in the trials using Roccal, they are not dependable. In the performance of the experiment it was noticed that when lambskin samples were immersed in aqueous Roccal a cloudy white precipitate was often immediately apparent. Several trials in which there was seemingly 100 per cent survival were not averaged in because of the possibility of mistaken use of distilled water. In the light of the work on adult sheep it seems probable that actually no such error was committed. The sustained rise in the count in Roccal treated areas can probably be attributed to the fact that the moisture left after the quarternary ammonium salt was inactivated facilitated bacterial reproduction. Confirmation that some substance on sheep does precipitate

quaternary ammonium compounds was obtained with the photometer readings recorded in Table XI. Convincing evidence has thus been secured that aqueous Roccal is not useful for disinfecting ovine skin.

The work of Nungester and Kempf indicates that Tincture of Phenol 1-500 is decidedly more effective than 70 per cent alcohol or an acetone and alcohol mixture. The experiments reported here, however, offer no means of determining what percentage of bacterial mortality was due to the action of the alcohol and/or acetone in the tincture.

Some disinfecting action was observed with the 2 per cent solution of Liquor Cresolis Saponatus USP XI. The very limited trial on lamb skin indicated that 90 per cent of the organisms were killed by a one minute exposure. The experiment on adult sheep suggests, however, that it is a rather slowly acting compound. Table X shows that the number of organisms declined for two days or more. The low point seems to be around 2,000,000 organisms per gram. As compared with the original count this may seem to be a significant reduction. Nevertheless, when one considers that the wool was still wet with the solution 24 hours after application and had a characteristic cresol odor, and had to be dried in a vacuum before samples could be weighed, and that its organisms represented a sampling of the environmental flora, cresol may not be as satisfactory for barn and stable disinfection as the U. S. Dept. of Agriculture Farmer's Bulletins 926 and 954 (Dorset 1918 and Pope 1918) seem to imply.

The writer and those with whom he has associated during the performance of these tests feel that the results obtained are quite a reliable basis for drawing conclusions as to the relative values for use on lamb skins of the types of disinfectants in question. Experience showed

that some lambs had a flora which was more evenly distributed than others, and also, some seemed relatively free from the surface spreaders which tended to make counting difficult. It was with these skins, selected by trial and error, that the data upon which the conclusions are based were obtained. The results of a series of consecutive samplings, recorded in terms of organisms per square centimeter are listed in Table XIII for Lamb 7989 (total number bacteria per Cm.²) and in Table XIV for Lamb 8030 (the figures listed were among those used for controls for experiment). These may be considered, in the light of the greater variation found in most of the others as almost ideal for the purpose of testing disinfectants. Usually the standard deviation exceeded the mean.

The fraction of the skin flora actually used to test the disinfectants is surprisingly small. Table IV indicates that from 56.4 to 93.7 per cent of the total flora was washed off before the jar from which the plating was to be done was reached. Since the washing procedure failed to remove perhaps three per cent of the organisms, an additional though small fraction was lost by not being removed from the skin. Table V indicates that in the case of Lamb 8030 approximately 13.6 to 15.2 per cent of the total flora was used in the test.

Extensive comparison with other commonly used substances was not made because the supply of skin samples stored from the lambing season became exhausted. The performance of such tests would probably have little bearing on the problem at hand. The compounds which seemed most valuable on the basis of Dr. Mallmann's years of experience were compared in a fairly thorough manner with the tincture of iodine now in use.

SUMMARY AND CONCLUSIONS

Experimental work comparing disinfectants under conditions simulating actual use in veterinary medicine has been quite limited. The present study was undertaken to compare quaternary ammonium and iodine preparations for possible use in disinfecting ovine skin.

The compounds given extensive testing were as follows:

Tincture of Iodine, U.S.P. XII
Colloidal Iodine
Colloidal Iodine with 1 per cent Naccanol added to
reduce surface tension
Lugol's Iodine, U.S.P. XII
Roccal

A new method for testing skin disinfectants was devised. The technic involved the use of skin taken from lambs just prior to autopsy. Samples of known area possessing the natural skin flora were treated with the compounds for one minute intervals, rinsed, and then washed in sterile distilled water. Platings with tryptose agar were made in order to calculate the number of organisms per square centimeter. The percentage of survival was determined by comparison with control samples subjected to equal volumes of sterile distilled water. The number of surviving microorganisms could be raised markedly if the rinsing was done in a neutralizing agent specific for the disinfectant.

Best disinfection was obtained with the iodine preparations. Oxalated blood counteracted to a slight degree the effect of iodine compounds. Naccanol and colloidal iodine do not seem chemically incompatible. As yet we do not wish to advocate the replacement of tincture of iodine as a skin disinfectant.

A test was also made of the relative efficiencies of some of the compounds in depressing the numbers of microorganisms composing the flora of the wool of adult sheep. Tincture of iodine was most effective for this purpose.

Evidence has been obtained that aqueous Roccal is precipitated by some substance on adult sheep and had no value as a disinfectant for unwashed wool.

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