A BIOCHEMICAL AND MICROBIOLOGICAL INVESTIGATION
OF DISCOLORATION IN SALTED HIDES

by

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[Signature]

Head of the Department of Zoology
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INTRODUCTION

In Canada the leather industries have long been established on a considerable scale, mainly because the large number of cattle raised and slaughtered provide a ready supply of hides. There are many large tanneries throughout the Dominion and the boot and shoe factories alone have an annual output valued at more than thirty-two million dollars.

The hides and skins from which leather is manufactured are obtained usually from animals killed for food. They would soon undergo putrefactive decay if not treated in some way to preserve them until ready for tanning processes and hence they are preserved by means of that operation known as curing.

The tanner receives pelts which have been cured by different methods. Pelts may be:

1. green -- fresh from the animal;
2. green-salted -- salt has been rubbed on the flesh side;
3. dried -- stretched on boards and dried;
4. dry-salted -- rubbed with salt and dried.

Pelts are divided on the basis of a size
classification into hides, kips and skins. Those pelts from the larger animals such as the cow, camel, horse and walrus are known as hides. They yield the heavy leather used for shoe soles, belting, harnesses and other strong leather products. When hides are cut into splits they give a lighter leather which is used for shoe uppers, for bags and cases, and for automobile seat covers. Kips are the untanned skins of calves or small cattle. The finer leather products are manufactured from them. Skins are the external coverings of other small animals from which leather may be derived.

In most slaughter houses the hides are quickly and skillfully removed from the animal soon after killing and sent to the curing room. Here, they are spread out hair side down and are trimmed of the tail, shanks and pates in an operation taking only a very few minutes for each hide. (Plates 1,2). The hides are placed hair side down and each is covered with a heavy sprinkling of salt. (Plate 3). They are piled in such a manner that the slope is towards the centre of the stack. In this way the brine formed is retained and the cure is materially aided.

The time necessary for a thorough cure is at least thirty days. The hides are then taken up and after the salt has been partially removed they are folded into bundles for shipment.
When the hides are taken up, preparatory to bundling for shipment, a marked red discoloration of the flesh side is often apparent. In addition, the hides may be quite slimy, the flesh soft, and in extreme cases 'hair slippage' may have occurred. This condition of the hides is commonly referred to as 'salt burn', 'salt stain', or 'red heat'. Hides exhibiting 'salt burn' are considered inferior because leather made from them is spotty in texture and less durable than leather from normal hides. They are thus classed along with hides showing cuts, brand marks, grub holes, botfly sores and other undesirable features. It is probable that many hides with some degree of 'salt burn' escape the notice of inspectors.

It is of economic importance to eliminate if possible, these abnormal conditions in the hides and thus produce leather of superior quality. 'Salt burn' is quite prevalent in hides during curing in various establishments in the city of Vancouver. The present investigation was undertaken in an attempt to ascertain the causes of the red pigmentation or discoloration in hides and to determine a suitable cure.

A survey of the literature indicated that a considerable amount of investigation had been carried out with regard to discoloration of hides and fish. Some of the more pertinent references are listed chronologically in the following pages.
SURVEY OF THE LITERATURE

As early as the year 332 B.C. the occurrence of blood colored bread was noted in the memoirs of Alexander the Great at the siege of Tyre (16). In 1819 inhabitants of the Province of Padua noted that many of their food articles were discolored red. One of the most characteristic colorings was that which occurred repeatedly in salted codfish. The reddening in salted codfish appears to be very similar to that in salted hides.

Dr. W.G. Farlow (29) studied the reddening of salted codfish in some detail. About 1880 he examined bacteriologically scrapings from red discolored codfish and from the floor and utensils in the plant where they were canned. He believed that the reddening was caused by a fungus named Clathrocystis roseo-percina. Farlow also makes reference to an organism grouped in fours and whose fours are surrounded by a thin hyaline envelope. He named this Sarcina morrhuae. Farlow did not attribute the reddening to this organism. He also noted that the above contaminants were present in sea salt from Cadiz.

In 1880 V.A. Poulsen (66) found and isolated an organism from the mud on the seashore at Copenhagen which
which he called Sarcina littoralis and which he believed to be identical to the Sarcina morrhuae of Farlow.

In 1884 E. Bertherand (10) reported an organism discovered by Mégnin and named Cornothecium bertherandi. Mégnin attributed the reddening of fish to this organism.

In 1886 Gayon and Carles (30) possessed a bacillus and a micrococcus which when mixed produced red coloration on cod. Reference is made to the ability of these organisms to exist on moist crystals of salt.

E. Mauriac (55) in 1886 showed experimentally that reddened fish did not cause food poisoning. He believed the origin of the red to be in the solar salt used for preserving and recommended that mined salt be used.

In 1887 two workers, Ewart (28) and Layet (49), attributed reddening of fish to the presence of microorganisms.

A. Edington (24) in 1887 stated that the cause of the red coloration was a non-motile rod. His work has not been confirmed and he did not cultivate organisms producing red on salted codfish or on salted artificial medium.

Le Dantec (50) in 1891 located on red codfish algae, bacilli and cocci. He made reference to a
a sarcinal organism which occurred in groups of fours and which was found when the fish was in the decayed state. In his extensive bacteriological inquiry he also located a red bacillus which was motile and which had a terminal spore. It produced colonies which were pale red in the centre and darker at the margin. He stated that sterilized codfish reddened less readily than did fresh codfish. Le Dantec noted that the red color was more intense on the salted side of the cod.

K. Hoye (45) about 1906 emphasized the presence of red bacteria on salted hides. He isolated an oval coccus, encapsulated and often appearing as a diplococcus. He states that this organism and others were found in numerous samples of sea salt.

In 1911 T.D. Beckwith (5) reported an organism, *Diplococcus gadidarum*, with which he produced reddening on tubes of shredded fish. The organism was an aerobe and would not grow if the salt concentration were over 20%.

In this same year A.W. Bitting (11) reported that a spore forming bacterial rod and cocci occurring as tetrads or diplococci could be associated with the reddening of salted fish. These organisms were nonmotile, strictly aerobic and encapsulated. Growth was delayed by 5% and 10% concentrations of sodium chloride and inhibited by 15%, 20%, and 25% concentrations. Bitting produced
red discoloration on codfish by inoculating with this organism.

Among first reported works on the red discoloration of salted hides is that of Becker (4) in 1912. He cultivated from reddened areas on the hides a strictly aerobic micrococcus I.1 to 1.5 \( \mu \) in diameter. In addition he isolated a species of *Torula* with cells approximately 6 to 7.5 \( \mu \) in diameter.

Romano and Baldraco (710 reported that hides salted with salt to which a small amount of sodium fluoride was added showed little bacterial growth. The leather produced from hides so treated was of a high quality.

In 1914 K.F. Kellerman (47) reported on two cocci isolated from red codfish. He believed one of them to be identical with the *Micrococcus littoralis* of Poulsen, the *Sarcina littoralis* of Poulsen, the *Glathrocystis roseopercina* of Farlow and the *Diplococcus gadidarum* of Beckwith. A culture of his organism was allowed to dry out in 1907. It was revived in 1911. Again it was allowed to dry out and was revived in 1914. It had retained its characteristics. Truly an example of long retained vitality.

In 1919 E. Le Fevre and L.A. Round (51) made a report upon some halophilic bacteria. Organisms were isolated from the scum on the surface of brine.
from cucumber pickles. These organisms required an appreciable concentration of sodium chloride for development. They showed a great resistance to drying for thoroughly dried out cultures kept in the laboratory for one year were easily revived by adding salt broth to the tubes.

In 1922 W.W. Brown (12) reported obtaining two organisms which were strictly aerobic and whose size, shape, and motility were influenced by the salt concentration. He named them Spirocheta halophilica and Bacterium halophilica. Their habitat is sea salt.

Also in 1922 the work of Harrison and Kennedy (38) on discoloration in codfish was published. It is a classic on the problem.

Cloake (20) in 1923 attributed pigmentation of hides to bacterial action.

Sturges and Heidemann (79,80) in 1923 and 1924 reported isolation of salt loving organisms from meat curing solutions.

About 1925 from an anonymous Russian source (84) there was a report of red spots noted on raw salted calf-skins. These were found to be co-extensive with damaged spots in the leather derived from these red spotted calf-skins. The spots were believed caused by aerobic,
proteolytic bacteria.

Chen and Fellers (18) in 1926 showed that immersion in sodium hypochlorite (NaOCl) containing 0.6% available chlorine for five minutes reduced materially the number of bacteria present on fish and enhanced the keeping qualities.

A.H. Gee (33) in a paper published in 1927 on spoilage of haddock mentions bacteria which will flourish in salt concentrations up to saturation but makes no reference to pigment produced by these organisms.

In 1927 L. Tattevin (81) published a report on the microflora of sea water, sea salts and brines. He concluded that the organisms involved in the reddening of fish were those to which reference has been made in the present summary. He states that the development of red color is the initial sign of rapid microbial increase of all kinds.

Clayton and Gibbs (19) in 1927 suggested the use of fish-broth-gelatin medium and fish-broth-rice-agar medium in the study of halophils.

In 1928 F. Stather (73) applied the term 'heating spots' to the red spots on the flesh of the hide. The organisms which he isolated from these spots were stimulated in growth by low concentrations of salt but inhibited by higher concentrations. The bacteria he isolated were the more common types usually found on normal hides.
Lloyd, Marriott, and Robertson (53) did extensive study on 'red heat' in salted hides in 1929. Using the medium recommended by Clayton and Gibbs and a salt concentration of not less than 16% and not over 30%, they isolated bacteria which produced both red and yellow pigment. These bacteria were usually seen in cubes or packets of eight. At 20°C. and at 100% humidity a definite growth of red organisms was obtained on infected hides in five days. The time under corresponding conditions for the yellow producing organisms was four days. At the same temperature but at 80% humidity neither organism shows as growth for sixteen days. A reduction in humidity checks growth very greatly. At 50% humidity or less no growth appeared even after three weeks. This (drying of the hides) is not a satisfactory practical method of preventing bacterial growth for the more water removed by evaporation or brining or both the less satisfactory the hide is for subsequent work. Professor McLaughlin (60,61) finds that the more moisture taken from hide the lower is its power to take up water when put into soak or even lime liquor. It is the conclusion of Lloyd, Marriott and Robertson that for the sole leather manufacturer hides cured with common salt containing 0.25% of sodium hydrogen sulphate will not be decomposed to any extent by bacterial action.

Harrison and Sadler (39) in 1929 made a detailed report on discoloration of halibut.
F.G. Harrison (40) noted that discoloration was most marked in the slime of dead fish. This slime was rich in nitrogen and thus excellent bacterial food.

M. Bergmann (9) found in 1930 that a spreading red coloration is produced on salted hides by mixed cultures of putrefactive bacteria. Seven kinds of spreading bacteria were found and grown in pure culture. Some of these organisms were halophils and nearly all liquefied gelatin. The optimum hydrogen ion concentration for the typical red bacteria was pH9. Where 'salt burn' had occurred on the hide, the minerals Ca and Fe and the radicals SO$_4^-$ and P$_2$O$_5^-$ were present in greater concentrations than on the normal hide. The addition of soda to skin salt inhibits the salt stains but favors the red coloration.

At one time Bergmann and his co-workers (8) believed that chromogenic dust organisms which were capable of developing in salt concentrations of up to 10% were responsible for the 'red heat'. Later conclusions by Stather (75) show that though both organisms may play a part in the production of red coloration on salted hides, the halophils added in salting play the larger part.

Baas-Becking (1) in 1931 discussed a red color found in salt marshes, in salt and in brines. He concluded that it was due to either iron oxide or to the action of pink, red or purple bacteria.
Hanzawa and Takeda (37) in 1931 examined reddened salted codfish chemically and microbiologically. They found that indol and skatol were present in small quantity. Torula wehmeri, Micrococcus albus-translucens, Micrococcus lutulentus and Bacterium zopfi were isolated from the reddened codfish. Reddening of the salted codfish was caused by Torula wehmeri which produced red pigment on culture medium. The other isolated organisms did not produce color. Indol is detected in pure cultures of Torula wehmeri and Bacterium zopfi.

In a paper published in 1931 M.E. Robertson (67) credits the red growth to a coccus, sarcinal in type and arrangement. She makes use of an adaptation of the Clayton-Gibbs medium. Although it might be possible for organisms other than halophils to cause coloration, she shows definitely that none of the following organisms gave any sign of growth in broth of a salt concentration in excess of 8% sodium chloride: (1) Sarcina lutea; (2) Sarcina aurantiaca; (3) Sarcina rosea; (4) Micrococcus tetragens; (5) Actinomyces; (6) Bacillus proteus; (7) Bacillus pyocyaneus; (8) Bacillus flourescens liquefaciens.

In a paper published the next year Dr. Robertson (69) states that extensive red discolorations are sometimes found to have developed on hides coming to England from abroad, especially if they have been long in transit or have
had to cross tropical seas. She states that hides arriving at the tannery looking clean and white may sometimes develop a red discoloration if stored where it is warm and moist. The cause is attributed to organisms present in the salt used in curing which are similar to those organisms which caused a reddening of fish. Growth of the organisms was checked by the addition of a little sodium fluoride or sodium silicofluoride to the known infected salt. There is no change in the leather because of this treatment.

Stather and Liebscher (76) showed that in advanced stages of reddening in the hides, the epidermis was often destroyed and at such spots the hair slipped. They found that the organisms which produced the reddening were capable of splitting fats and that consequently infected hides produced leather with an uneven grain.

That bacteria actually are present normally on hides and that their action will cause putrefaction unless checked was proved by the experiment of Allen Rogers (70). Thirty steer hides free from manure were sampled as they dropped from the carcass. Various samples of hides were incubated at temperatures from 12°C. to 52°C. In each case the hide was allowed to remain in the incubator until marked putrefaction occurred as it did in all cases. This experiment established definitely that bacteria naturally occurring in these hides can act upon the hide and cause spoilage. In no case was a red discoloration produced by these normal hide flora.
Stuart, Frey and James (77) using a hide-peptone-salt broth to which they added polished rice studied many salts used with hides. They found that thirty-four of thirty-five samples of crude solar-evaporated salts and twenty-five out of thirty-nine open pan evaporated grainer salts were contaminated with red chromagens. All kiln dried solar salts and all mine salts were found to be free from contamination with these organisms. No correlation was found between the pH of salts and the presence of chromogenic organisms. Cultural studies of red chromogenic growths on media of high concentrations of salt showed them to consist usually of a highly mixed bacterial flora.

A.G. Lochhead (54) in 1934 isolated a red halophil from Argentine hides. From Canadian hides he obtained two organisms. One from salted hides was similar to the Serratia salinaris of Harrison and Kennedy. The other from buffalo hides he called Serratia cutiruba. Both of these halophilic organisms owing to their proteolytic activity are considered capable of greater damage to hides than the red sarcinal types which are non-liquefying and which may also be present on Canadian hides. Non-chromogenic bacteria (halophilic) were also isolated from discolored hides. These develop at a lower salt concentration range than the red organisms and are probably less active in causing injury to fibre in well salted hides.

In 1934 R. Bedford (7) showed that when the skin
of halibut is seeded with certain marine chromogenic bacteria that produce pink, orange and yellow pigments in pure culture and that ordinarily occur on the skin of dead halibut, it becomes pink or yellow as well as sour in ten days at 0 C.

E.K. Petrova (64) in 1934 isolated a coccus from salts of various sources. The coccus forms a red pigment and in general resembles other pigmented halophils previously described.

L.S. Stuart (78) describes an organism with a peculiar spreading growth manifested by crateriform indentations of the agar with which no well developed stainable bacterial cells could be associated. The organism is heterotrophic, since it grows in both organic and inorganic substances. It produces hydrogen sulphide from a basal nutrient solution containing free sulphur. It grows in a large variety of vegetative cell types varying with the medium from cocci and oval shaped rods to long straight sided rods, thread forms and granulated thread forms.

In 1935 Robertson, Marriott and Humphreys (68) found that addition of sodium fluoride and sodium silicon fluoride to the sodium chloride used for curing the hides inhibited 'red heat' and in general gave better preservation than sodium chloride alone. Large scale experiments on curing of hides and skins with sodium chloride and sodium fluoride showed that the leather made from hides and skins
so treated is of as good quality as that from hides or skins cured with sodium chloride alone. Experiments were carried out to show whether small amounts of fluorides or silico-fluorides in curing salts for hides or skins might be carried over into gelatin made from tannery scrap arising from such material. It was determined that gelatins made from hide scraps subjected to the fluoride treatment did not appear to contain any more fluoride than the normal gelatins.

Patwardhan and Subramaniasastry (63) in 1935 made a report on salt stains on South Indian hides and skins. They described the conditions of curing in Malabar which lead to stain formation. Their experimental evidence indicated that stains are not due to direct bacterial action on the skin, that the presence of iron in the cures leads to stain formation, and that stains are caused by the precipitation in the hide of iron as iron sulphide.

Also in 1935, E.K. Petrova (65) reported a highly pleomorphic organism isolated from 'red' salted fish. It seemed to be identical with Micrococcus roseus, a contaminant of the most important U.S.S.R. salt beds. It resembled bacteria described by other workers as producing reddening.

In 1938 Kurochkin and Emelianchik found that in the Aral sea region, as in the Caspian sea region the causative agent of red coloration of salted fish was Serratia salinaria.
Serratia salinaria occurs in salt recovered from the Djaksy-Klych lake and used for salting the fish. All salt recently recovered from salt lakes of S.E. Russia contains Serratia salinaria.

A paper published in 1939 by Chembard and Castellei (17) draws attention to defects in the preservation of skins with salt.

The following tabulation shows the conclusions reached by various workers with regard to the organism producing red pigmentation and whether or not it originated from solar salt.

<table>
<thead>
<tr>
<th>Author</th>
<th>Organism producing redness</th>
<th>Halophilic (growing in salt at least 10% cause salt medium)</th>
<th>Solar salt</th>
</tr>
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<tbody>
<tr>
<td>Baas-Becking</td>
<td>red bacterium</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Beckwith</td>
<td>Diplococcus gadil-</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>arum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bergmann</td>
<td>mixed organisms</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Bitting</td>
<td>coccus</td>
<td>no</td>
<td>inconcl.</td>
</tr>
<tr>
<td>Browne</td>
<td>Spirocheta halophil-</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>ica</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carles and Gayon</td>
<td>coccus and bacillus</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>Edington</td>
<td>Bacillus rubescens</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Farlow</td>
<td>Clathrocystis roseo-persicina</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>Hanzawa and Takeda</td>
<td>Torula wehmeri</td>
<td>yes</td>
<td>?</td>
</tr>
<tr>
<td>Author</td>
<td>Organism producing Halophilic redness (growing in salt at least 10% salt medium)</td>
<td>Solar cause</td>
<td></td>
</tr>
<tr>
<td>-------------------------------</td>
<td>---------------------------------------------------------------------------------</td>
<td>-------------</td>
<td></td>
</tr>
<tr>
<td>Harrison and Kennedy</td>
<td>mutable form</td>
<td>yes</td>
<td></td>
</tr>
<tr>
<td>Hoye</td>
<td>sarcina</td>
<td>yes</td>
<td></td>
</tr>
<tr>
<td>Kellerman</td>
<td><em>Micrococcus littoralis</em></td>
<td>yes</td>
<td></td>
</tr>
<tr>
<td>Kurochkin and Emelianchik</td>
<td><em>Serratia salinaria</em></td>
<td>yes</td>
<td></td>
</tr>
<tr>
<td>Layet</td>
<td>sarcina</td>
<td>no</td>
<td></td>
</tr>
<tr>
<td>Le Dantec</td>
<td>red bacillus</td>
<td>no</td>
<td></td>
</tr>
<tr>
<td>Lloyd, Marriott and Robertson</td>
<td>coccus -packets of eight</td>
<td>yes</td>
<td></td>
</tr>
<tr>
<td>Petrova</td>
<td>coccus</td>
<td>yes</td>
<td></td>
</tr>
<tr>
<td>Stuart, Frey and James</td>
<td>mixed microbial flora</td>
<td>yes</td>
<td></td>
</tr>
</tbody>
</table>

(?) - not stated
(inconcl.) - inconclusive

Most of the investigators listed above agree that red discoloration in fish and in hides is produced by an organism or organisms which flourish in media containing a considerable concentration of salt. In the majority of cases evidence indicates strongly that the source of these organisms is salt produced by evaporation of sea water.
CAUSATIVE AGENT OF DISCOLORATION IN SALTED HIDES

A preliminary inspection of hides affected with discoloration ('salt burn') indicated strongly that this condition was the result of microbial action. Many hides were slimy and soft, with considerable red discoloration on the flesh (muscle) side. This characteristic appearance of the hides, together with cited information from the literature, formed evidence enough to warrant a bacteriological investigation of the problem.

(a) Isolation of the Organism

On the assumption that 'salt burn' was caused by bacterial action an attempt was made to isolate the causative organism. Two factors which facilitated isolation were readily evident. The organism or organisms present on the hides flourished in strong saline solutions and it or they produced under certain conditions a characteristic pink-red pigment.

At Burns & Co.'s plant in Vancouver 'salt burn' is usually first noticed when a hide pack is 'pulled' for shipment to the tanner. To obtain samples of bacterial flora present on hides exhibiting 'salt burn', tubes of sterile nutrient 3% NaCl broth, sterile tongue depressors and sterile Petri plates were taken to the hides' basement
of Burns & Co. during the 'pulling' of a pack. With the tongue depressors, scrapings from a number of different hides were placed in the tubes of broth. Samples of the salt used in curing and also samples of salt which had not come into contact with the hides were taken. Pieces of infected hide were placed in the Petri plates. During the investigation, scrapings and infected hide pieces were collected at regular intervals from Burns & Co. and from other local packing houses.

In the isolation procedure the first media used were:

(I) Nutrient salt broth -- consisting of

- Bapco beef extract 1.5 gms.
- Peptone 2.5
- Water 500 cc.
- NaCl different concentrations
- pH adjusted to 7.2

(2) Nutrient salt agar -- this was prepared by adding sufficient agar to the above broth to make a 1.5% solution;

(3) Nutrient gelatin -- this was prepared by adding sufficient gelatin to the nutrient salt broth to make a 12% solution.

To obtain pure pure cultures spread plates containing nutrient 3% salt agar were made from the original broth cultures, the salt samples and the hide pieces. The
morphology of organisms from single colonies from each of these spread plates was studied. Then sub-cultures were made from these specific colonies. Microscopic preparations were made and studied. The organisms isolated from the salt samples, the broth cultures and the hide pieces were compared with organisms taken directly from infected hide pieces. In all cases the organisms appeared very similar. To further establish the similarity of these organisms their growth characteristics were determined.

(b) Growth Characteristics of the Organism.

(i) Optimum Sodium Chloride Concentration.

The maximum and optimum salt concentrations in which the organism would grow were determined. This was accomplished by preparing spread plates in which the salt concentrations varied from 3% NaCl to 21% NaCl with 3% intervals. (i.e. 3-6-9% etc.) This experiment was performed in triplicate and it was found that the organism survived, but grew very slowly in a salt concentration as high as 21%. The optimum salinity for growth appeared to be 8%.

It was necessary in all cases to prevent evaporation of water and consequent salt crystallization by binding the plates with parafilm or sealing them with sealing wax. (Figure I)

The organism exhibited peculiar characteristics. It appeared to be highly pleomorphic, for small gram-negative rods and cocci were obtained from broth cultures at low salt
concentrations, whereas in the higher salt concentrations or upon agar plates, or after a period of about ten days from subculture on other media, it assumed a characteristic tetracoccal arrangement. This tetracoccal arrangement seemed to be assumed when growth conditions were slightly unfavorable.

(ii) Optimum Temperature.

To determine the optimum temperature for growth cultures were started on agar plates containing 8% NaCl. These were incubated at temperatures varying from 5°C to 40°C. Cultures flourished at all temperatures from 10°C to 35°C, but they appeared to grow best at 35°C for the first seventy-two hours. For longer periods best results were obtained at 30°C. (Figure 2) A maximum-minimum thermometer was placed in the middle of a typical hide pack and left for a period of one month. In this time the temperature varied from 9°C to 21°C, with the mean average estimated at about 15°C.

(iii) Optimum Hydrogen Ion Concentration.

For an optimum hydrogen ion determination, plates of nutrient 8% salt agar were prepared and the pH of the plates was varied from 6.2 to 8.8. The optimum for growth of the organism was pH 8.0 to 8.2. (Figure 3) With regard to the actual pH of the hide when under conditions similar
to those in the pack, results obtained with the Beckmann pH meter indicated that the fleshy surface of the hide was quite alkaline (readings ranged from pH 8.2 to pH 8.8 on various pieces of hide).

(iv) Cellular Morphology with Regard to pH Change.

When grown on nutrient 8% salt agar the isolated organism was present in diplococcal and tetracoccal forms, was encapsulated and stained gram-negative. Some spores were produced by the organism on this medium. Colonies on plates at this hydrogen ion concentration were pale, serrated, mucous and moist. In more alkaline medium, i.e. up to a pH of 8, growth characteristics were the same except that no spores were present. Lack of presence of spores suggests that this pH is more nearly optimum than other pH ranges.

In the study of the morphology of the organism the following techniques were applied.

(I) Gram-staining: A microscopical preparation is made. This is flooded with methyl violet for 1 minute, washed in water, flooded with Gram's iodine solution for 1 minute, washed in water, decolorized with 95% alcohol(approximately 25 seconds with this organism), washed in water, and counterstained with aqueous fuchsin for 10 seconds. The preparation is washed in water and dried.
(2) Specific capsular staining.

(a) India Ink method (34). One drop of a broth culture of the organism is mixed with one drop of India Ink and this is smeared on a slide, fixed with methyl alcohol and counterstained. The slide is stained a dark grey and the interior of the cell is stained by the counterstain. Around the cell is a clear zone—the unstained capsule.

(b) Copper sulphate method (44). A microscopical preparation is made and dried. It is flooded with aqueous fuchsin and steamed for ten to thirty seconds, drained, flooded with 20% solution of copper sulphate, drained, and dried between blotters.

(3) Spore staining. A film is prepared, flooded with carbol-fuchsin and steamed for five minutes, washed in water and decolorized lightly with 95% alcohol. The slide is flooded with Loffler's methylene blue for two minutes, washed and dried.

(v) Rate of Growth.

The rate of growth in nutrient salt broth was determined by sampling a series of tubes at various time intervals and estimating the number of bacteria present. The number of bacteria was determined by two methods:

(I) Slide count method - A piece of parafilm, 2.5 cm. by 5 cm. is taken and a 1 cm. square cut from its center. The parafilm is placed on a clean slide and caused to adhere
gently. From a well mixed suspension of the organism 0.01 cc. is delivered into the square. The slide is dried thoroughly in air. The piece of parafilm is stripped off the slide, the smear is fixed by heat and stained with gentian violet. The area of the field covered by oil immersion was calculated by means of a calibrated slide. The bacteria were counted in a number of fields (25 fields) and from this the number of bacteria in one cc. of the suspension was calculated.

(2) Plate count method - Dilutions were made from 1 cc. of well mixed #1 McFarland suspensions. Three poured plates were made from each of the dilutions. These were incubated for 48 hours. Colonies were counted and the number of bacteria/cc. of original suspension was estimated.

The information obtained by use of these methods was used to plot the number of bacteria produced against the time elapsed. (Figure 4)

The above experiments were carried out with organisms isolated from infected hides, salt in contact with these hides and salt not in contact with the hides. The results obtained with regard to morphology, pH, sodium chloride and temperature optimums, and rate of growth were the same; it was concluded that organisms isolated from each of these three sources were identical. Further experimental work, i.e., pigment production and biochemical activity of the organisms, confirmed this view. This

#1 McFarland - a turbidity comparison standard. 10 cc. of a solution of 99 cc. of 1% H_2SO_4 and 1 cc. of 1% BaCl_2.
particular organism is a coccus and usually occurs in groups of four or packets of eight. It stains gram-negative and flourishes best in a medium of 8% sodium chloride with a pH of 8.2. Its optimum temperature is from 30°C to 35°C.

Although it is probable that there are other halophilic organisms present on the hides this specific organism was the dominant organism in samples obtained during this investigation.

(c) Pigment Production by the Organism.

The amount of pigment produced by the organism on the media used in the determination of its growth characteristics was quite small. The colonies on nutrient salt agar were slightly yellow or colorless. Reculture on sterile pieces of hide from these colonies resulted in production of a light pink pigment if the atmosphere was kept moist. The pigment was produced in from one to three weeks (depending on the percentage humidity) at 30°C.

With many chromogenic bacteria pigment production is good on potato medium, however in this case growth and pigment production were negligible. In an endeavor to find a more suitable medium for pigment production several artificial media were made up and the growth of the organism on these was studied.
(i) Artificial Beef Infusion Medium.

Portions of hide were taken and ground up in a meat grinder. These were then digested in 500 cc. of water for twelve hours over a small flame. The broth obtained was filtered off, the sodium chloride concentration was made up to 8% and the pH adjusted to 8. Some of the broth was made into agar by adding sufficient agar to make a 1.5% medium.

On this medium (agar) the amount of pigment produced was small. Results otherwise were very similar to those obtained with nutrient-salt agar.

(ii) Clayton-Gibbs Medium.

The next medium to be tried was the Clayton-Gibbs fish-broth-rice-flour medium. Several variations of the original were prepared. The best results with regard to pigmentation were obtained when a medium of the following composition was used.

One hundred gms. of sockeye salmon scraps were placed in a litre of water and allowed to digest for 48 hours. The resultant broth was filtered off and 8% NaCl added. Nutrient agar (1.5%) and enough rice flour to make a solid paste when warm were added to some of the broth. To make agar plates, the agar was poured into Petri plates, rice flour added, and the whole allowed to solidify. Plates were prepared and inoculated at pH
values from 7.0 to 8.4.

The red growth was most abundant at a pH of 8.2 and when the humidity was one-hundred percent. Growth was spreading and slimy in nature.

(iii) Silica Gel Medium.

This medium has only recently been introduced in the study of halophilic organisms. As stated by Moore (57) in 1940: 'The cultivation of that group known as the halophils has claimed the attention of industrial bacteriologists for some time. Various types of media have been proposed but none have proved completely satisfactory. Rice starch paste, hide and fish digest made solid with agar, coagulated eggs, and the classic nutrient agar containing three molar sodium chloride have been used by many workers with indifferent results. The most serious criticisms of these media are: (1) slow, scanty growth; (2) colonies small and hard to see; (3) high pouring temperatures of the agar mixtures; (4) peptization of the agar; (5) difficulty in clarifying; (6) irregularities in results.'

The silica gel medium consists of two solutions:

Solution A

\[(NH_4)_2SO_4\] 1.0 gms.

KH_2PO_4 0.2

K_2HPO_4 0.8

Glucose 20.0
Cystine 1.0 gms.  
Peptone 5.0  
FeSO₄ 0.001  
Bismuth citrate 0.15  
HCl concentrated 20.0 ml.  
NaCl saturated solution 500.0

**Solution B**  
95 gms. Na₂SiO₃ (Bakers 40 Be) in 500 ml. 1.5% NaCl solution.

The formation of the gel is carried out by the following procedure: Ten ml. of the acid solution are pipetted into a small beaker, a few drops of bromthymol-blue indicator added, and the silicate run in slowly from a burette until the color of the indicator just begins to change. The change occurs at approximately pH 7.2. The mixture is then quickly poured into a Petri dish containing the sample and allowed to gel. These silica gels grow progressively more alkaline on standing, so that if they are poured at a pH of 7.0 or 7.2 they finally change towards pH 8.0 which is more optimum for the growth of halophils.

The silica gel medium is based on that suggested by Hanks and Weintraub (36) in 1936. The time required for growth of halophilic organisms on this medium is much less than that required in agar. Further Moore (57) found that it has a greater clarity and stability with high salt.
concentrations than had normal agar medium. In the present work the medium was made up to the specifications of the formula given on pages 28 and 29 except that it was minus cystine which could not be obtained at the time.

Experimental work with this medium was limited. Red growth was obtained on plates when a considerable number of organisms was used. Some of the plates did not solidify very well and little growth occurred on them. It is possible that the absence of cystine might have been responsible for the comparatively poor growth and pigment production obtained with this medium.

In general with these media, (i, ii, iii,) pigment was produced most abundantly upon primary subculture of the organism from the hide. Upon subculture from artificial medium to artificial medium pigment production fell off greatly. There were indications that pigment production might vary in bacilli of the same culture for even upon isolation from single colonies and reseeding of these on agar plates some of the colonies were highly colored, whereas others exhibited little color and some were completely colorless.

From the results of this investigation and those of other workers (83) it would seem that a specific organism only produces pigment in the presence of oxygen.
and at an optimum temperature for pigment production. The oxygen factor might explain the greater production of pigment in the outer folds of hides in a pack, where more air is available than in the more central regions of the pack.

In the case of some organisms, the optimum temperature for growth may be considerably higher than the optimum temperature for pigment production. This is well illustrated by the organism under study, for it produces considerable pigment in the hide pack where normal temperature is 15°C, yet from experimental determination, its optimum for growth is between 30°C and 35°C. At this temperature (35°C), pigment production is scanty.

In experimental work with this organism, pigment production was heaviest on Clayton-Gibbs medium at a pH of 8.2, a temperature of 25°C, and 100% humidity.

(d) Bio-chemical Activity of the Organism.

The growth of the organism in various alcohols, saccharides, and on gelatin was studied.

Action on:

<table>
<thead>
<tr>
<th></th>
<th>Amount of Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>18 hrs.</td>
</tr>
<tr>
<td>(I) Hexahydrlic alcohols</td>
<td></td>
</tr>
<tr>
<td>a. dulcitol</td>
<td>none</td>
</tr>
<tr>
<td>b. inositol</td>
<td>&quot;</td>
</tr>
<tr>
<td>c. mannitol</td>
<td>&quot;</td>
</tr>
</tbody>
</table>
(2) Monosaccharides
   a. Pentoses
      xylose
   b. Hexoses
      dextrose

(3) Polysaccharides
   a. sucrose
   b. maltose

(4) Sclero-protein
   gelatin

<table>
<thead>
<tr>
<th></th>
<th>18 hrs.</th>
<th>24 hrs.</th>
<th>7 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amount of Growth</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>xylose</td>
<td>none</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>dextrose</td>
<td>acid production in all cases no gas production.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sucrose</td>
<td>none</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>maltose</td>
<td>acid</td>
<td>acid</td>
<td>acid</td>
</tr>
<tr>
<td>gelatin</td>
<td>liquefaction</td>
<td>complete liquefaction</td>
<td></td>
</tr>
</tbody>
</table>

The fermentation of the sugars by this organism is important only in classification, but emphasis must be placed on the liquefaction of gelatin. The significance of such rapid gelatin liquefaction will be discussed later.

SOURCE OF THE ORGANISM

The isolated organism was obtained from: (1) hides showing red pigmentation (Plate 4); (2) salt used in curing these hides (Plate 5); and (3) salt which had not been in contact with the hides (Plate 6). The sum total of the
evidence available at this stage indicated that the salt used in curing was the source of the organism under study. With the idea of confirming this theory samples of salt were obtained from the salt company supplying Burns & Co. with curing salt. An organism with properties identical to those exhibited by the organism isolated from (1), (2), and (3) above (page 32) was obtained from five out of six of these samples.

The salt distributed for commercial use by the company supplying Burns & Co. is obtained by evaporation of sea water in large ponds near San Francisco, Calif. Salt deposited in the evaporating ponds is harvested by hand or by machinery methods. It is stacked in large piles in the ponds and is then hauled to the storage piles for further purification. This treatment consists of preliminary crushing, then washing with a pure salt brine, after which the salt goes to storage piles for final draining and drying preparatory to shipment. The salt produced by this process is not of sufficiently high quality to be sold as table salt, so it is distributed as a rough commercial product. Crops are usually harvested as soon as dry so therefore they are rarely exposed to the sun for a time longer than that sufficient for moisture evaporation.

These data are presented to show that at no time is the salt processed to destroy bacteria. The salt sprinkled on the hides in the Burns & Co. hide basement contains
essentially the same microorganisms as it contains when it is harvested in California. The work of Stuart, Frey and James (77) shows conclusively that 98% of crude evaporated sea salts contain red chromogenic organisms. In one case in the present investigation the pigment producing organism retained the ability to grow after having been left on dried salt and nutrient agar for more than six months. In view of this fact it is probable that many salt borne organisms remain alive from time of harvest of salt in California to time of distribution in Vancouver. This point was confirmed by isolation of the organism from salt taken from the distribution center in Vancouver (page 33).

ACTION OF THE ORGANISM ON HIDE

The hide consists of two principal layers, the epidermis and the dermis (corium or true skin). Leather is manufactured from the dermis, the epidermis is removed during preliminary processing. The corium is composed principally of interlacing bundles of connective tissue which are cemented together by a substance more soluble than the fibres themselves. These fibre bundles are loosely interwoven in the middle portion of the skin but become compact again near the muscle. The outermost layer is very close and compact.
The tissue connecting the skin to the animal will not be dealt with because it is removed in the process known as 'fleshing' and any changes in it by bacterial action would not affect the finished leather.

The whole of the epidermis including the hair is separated from the corium by an exceedingly fine membrane called the 'hyaline' or 'glassy' layer. This forms the very thin surface of tanned leather, which is of a structure different from the rest of the corium. According to Kaye (46) the layer of skin at the level of the hair roots is structurally somewhat weak because of the presence of such large cellular structures as the hair roots and the sweat glands, and also because the subdivision of the coarse collagen fibre bundles of the corium as they merge into the finer ones of the grain layer occurs in this region. Lloyd, Jordan and Robertson have noted that bacteria and proteolytic enzymes attack collagen fibres more readily at their cut ends or point of division than along the intact surface of the fibres. Thus decomposition is liable to be most advanced on the cut edge or where a brand has been marked. Where growth has been extensive and of long duration the cells composing the hair follicles may be decomposed and hair slippage will result. Hides so attacked yield leather with a very inferior grain. When destruction of the glands and hair roots of the epidermis occurs, the hyaline layer is weakened with the result that in leather
manufactured from a partly purified hide or skin, the connecting layer between the grain and the corium is frequently loose. The fibres may be so weakened that in extreme cases the grain layer can be ripped from the underlying corium.

(a) Substances Available for Nourishment.

With regard to the actual chemical substances present it has been found by analysis (70) that samples of various parts of hides have the following composition:

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Belly</th>
<th>Butt</th>
<th>Shoulder</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mucin</td>
<td>1.24%</td>
<td>4.81%</td>
<td>2.29%</td>
</tr>
<tr>
<td>Coagulable protein</td>
<td>4.30</td>
<td>4.14</td>
<td>5.16</td>
</tr>
<tr>
<td>Elastin</td>
<td>19.43</td>
<td>12.31</td>
<td>16.74</td>
</tr>
<tr>
<td>Keratin</td>
<td>25.73</td>
<td>19.91</td>
<td>36.15</td>
</tr>
<tr>
<td>Collagen</td>
<td>51.46</td>
<td>58.83</td>
<td>39.66</td>
</tr>
</tbody>
</table>

Thus the substances present in the skin and available to the action of bacteria are mucin, coagulable protein, elastin, keratin and collagen.

The term mucin designates those glycoproteins which occur in tissue. The glycoproteins are usually classified under the title 'conjugated proteins'. Glycoproteins (52) may be considered as compounds of the protein molecule with a substance or substances containing a
carbohydrate group other than a nucleic acid. The glycoproteins yield upon decomposition protein and carbohydrate derivatives notably glucosamine $\text{CH}_2\text{OH}((\text{CHOH})_3\text{CH}(\text{NH}_2)\text{CHO}$ and galactosamine with the same empirical formula.

Elastin, like keratin and collagen, is a member of the albuminoid group. Albuminoids differ from all other proteins whether simple, conjugated or derived in that they are soluble in all neutral solvents. Elastin then, in common with keratin and collagen is an insoluble body and gives the Xanthoproteic and Millon color reactions. It differs from keratin principally in the fact that it may be digested by enzymes and that it contains a small amount of sulphur.

Keratins as a group are insoluble in the usual protein solvents. They all respond to the Xanthoproteic and Millon reactions and all are characterized by containing large amounts of sulphur. Keratin is a scleroprotein. This class includes such substances as gelatin, chondrin, elastin and keratin. The organism under study acted vigorously upon gelatin medium (page 32). It follows then that it might also digest keratin, a protein of the same class. The hide surface upon which the organism acts is approximatley 25% keratin. In hides in an advanced stage of digestion the characteristic odor of $\text{H}_2\text{S}$ is often noticed. This might be produced by the action of the organism upon keratin which contains considerable sulphur.
Collagen is the principal solid constituent of white fibrous connective tissue. It differs from keratin in that it contains less sulphur. One of its chief characteristics is the property of being hydrolyzed by boiling water or acid with the formation of gelatin. Emmett and Gies (27) state that under these conditions there is an intramolecular rearrangement of collagen and the resultant gelatin is consequently not the product of hydrolysis. The liberation of ammonia from the collagen during the process apparently confirms this view. Collagen gives Millon's reaction as well as the Xanthoproteic and Biuret tests.

(b) Nature and Extent of Action.

To determine quantitatively the degree of decomposition of the hide by the organism would entail difficult laboratory procedure. The rate of digestion may be estimated by measuring the action of the salt organism on a laboratory medium somewhat similar in composition to the hide.

When proteins are decomposed by the action of enzymes or bacteria, amino acids are formed. If the amount of amino acid present in a solution is determined before and after the action of bacteria, the decomposition (i.e., conversion of protein to amino acid) may be determined. The
amount of amino-acid nitrogen present in a solution varies as the amount of amino-acid present. In practice the amino acid nitrogen rather than the amino acid is measured.

(i) Digestion by the Organism.

The following experiment is not intended to show the amount of digestion in the hides by the organism but is intended to show comparable action on an artificial laboratory medium.

Experimental procedure:

A Difco nutrient-beef-salt broth (Difco nutrient beef is three parts Bacto-beef extract and two parts Bactopeptone) was used for the digestion determinations. A five-day-old culture of the organism in the Difco nutrient-beef-salt medium was prepared. To each of seven test tubes containing exactly ten cc. of the medium, one cc. of the five-day-old culture was added. The first tube was incubated immediately at 5°C to serve as a control. The others were incubated at 35°C. These were taken from the 35°C incubator and placed at 5°C at intervals of 2, 4, 14, 18, 36, 96, and 168 hours.

Then each tube was titrated by means of the Sorenson formol titration to determine production of amino-acid nitrogen.
Experimental results:

<table>
<thead>
<tr>
<th>Tube</th>
<th>Time of incub. at 30°C.</th>
<th>Cc. of NaOH required</th>
<th>Amino-acid N present</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0 hrs.</td>
<td>1.7 cc.</td>
<td>.0047 gms.</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>1.8</td>
<td>.0050</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>1.8</td>
<td>.0050</td>
</tr>
<tr>
<td>3</td>
<td>14</td>
<td>2.0</td>
<td>.0056</td>
</tr>
<tr>
<td>4</td>
<td>18</td>
<td>2.0</td>
<td>.0056</td>
</tr>
<tr>
<td>5</td>
<td>36</td>
<td>2.1</td>
<td>.0059</td>
</tr>
<tr>
<td>6</td>
<td>96</td>
<td>2.2</td>
<td>.0062</td>
</tr>
<tr>
<td>7</td>
<td>168</td>
<td>2.4</td>
<td>.0067</td>
</tr>
</tbody>
</table>

This table shows that when conditions for growth are favorable, considerable decomposition takes place even in a relatively short time. The results are expressed graphically (Figure 5). If the graph of the amino-acid nitrogen is inverted, the result is the decrease in amount of protein present. When this is superimposed upon a typical growth graph, a chart is obtained which shows decomposition of protein and multiplication of bacteria with reference to time (Figure 6).

The Henriques-Sørenson formol titration method (42) for amino-acid nitrogen determination was used to determine the amount of amino-acid nitrogen present.

Principle: A solution containing amino-acids is nearly neutral in reaction. If formaldehyde be added, however,
the following reaction takes place with the formation of methylene derivatives which are more strongly acid in reaction due to the destruction of the amino groups. The carboxyl groups may then be titrated using phenolphthalein as indicator.

\[
\text{R-CH-NH}_2 + \text{C-OH} + H^+ \rightarrow \text{R-CH-N=CH}_2 + H_2O + \text{COOH}
\]

The acidity as shown by the titration is a measure of the amount of amino-acid present. Ammonia likewise reacts with formaldehyde in a similar manner as shown in the following equation:

\[
4 \text{NH}_4\text{Cl} + 6 \text{CH}_2\text{O} \rightarrow \text{N}_4(\text{CH}_2)_6 + 6 \text{H}_2\text{O} + 4\text{HCl}
\]

Hence the formol titration method in the presence of ammonia gives results which include both amino-acid and ammonia nitrogen. It must be borne in mind that polypeptides and still more complex protein derivatives likewise react with formol to a certain degree so that the results do not strictly represent 'amino-acid nitrogen'.

Procedure: The solution to be analyzed is made neutral to litmus and is treated with formaldehyde. (As a standard of comparison the litmus paper used for neutralization is contrasted with a similar piece dipped in a phosphate solution having a neutral reaction, M/15 KH$_2$PO$_4$ and M/15 Na$_2$HPO$_4$, in the proportion 40 : 60.)

Final titration: For the final titration a volume of ten cc. of unknown is used. A control solution is run containing ten cc. of boiled distilled water and ten cc. of the
formaldehyde mixture. (The formaldehyde solution is freshly prepared for each set of determinations as follows: to 50 cc. of commercial formaldehyde, formal 30-40%, add 1 cc. of the phenolphthalein solution. Then 0.2N. alkali is added until the mixture acquires a faint red color. The volume of HCHO used will vary with the volume of the solution to be analyzed - approximately 5 cc. of the formalin solution to each 10 cc. of the unknown solution.

The control solution is colored so that its tint matches that of the solution to be titrated.

First stage: To this control is added about half the volume of 0.2N. alkali which will be used in the titration of the solution under investigation and it is then titrated with 0.2 N. acid to a faint red.

This is done in order that the final volume of the control and the unknown solutions shall be approximately the same when the process is complete.

Second stage: An additional drop of 0.2 N. alkali is added which imparts a distinct red to the solution.

The solution to be analyzed is now titrated to the color produced in the second stage of the control. The formaldehyde mixture is now added, 5cc. for each 10cc. of the solution, and the mixture again titrated to the second stage with 0.2N. alkali. (This is best accomplished by adding alkali until the color is deeper than that of the control, then acid again until lighter and finally alkali to the desired color.)
Third stage: Two drops of the 0.2 N. alkali are now added to the control solution which assumes a deep red color. To the solution under examination, 0.2 N. alkali is now added until it assumes a color corresponding to the third stage of the control. This completes the titration.

Calculations: The calculations are similar to those which pertain to any acidimetry procedure. Each cc. of an 0.2 N. alkali or acid solution is equivalent to 0.0028 gm. of nitrogen. (From amount of 0.2 N. NaOH used subtract the amount of 0.2 N. required for the control.)

(ii) Digestion by the Extracellular Enzyme.

To determine if an extracellular enzyme was produced by the organism the following experiment was carried out.

Procedure: A five day culture of the organism in 100 cc. of nutrient salt broth was filtered through a Seitz filter. (The Seitz filter allows passage of an enzyme if present but completely retards passage of bacterial cells.) Three Petri plates containing sterile hide pieces were prepared. To No. 1, 0.5 cc. of filtrate was added. To No. 2, 0.5 cc. of broth and bacteria (control). To No. 3, 0.5 cc of broth (control).

To the remainder of the filtrate 0.5% of phenol was added
Experimental results: Plate No. 2 showed considerable growth and pigment production whereas the other two plates showed no change. From this it was concluded that the extracellular enzyme, if present, caused no production of pigment on the sterilized hide.

To determine the quantitative action of the extracellular enzyme, if present, an experiment similar to that used in the quantitative determination of the action of the organism (page 39) was carried out.

Procedure: Tubes were inoculated with the Seitz filter filtrate and incubated for various time intervals, then titrated for amino-acid nitrogen production by the Henriques-Sørenson method.

Experimental results: Action of the extracellular enzyme on Difco nutrient beef salt broth.

<table>
<thead>
<tr>
<th>Tube</th>
<th>Time of incub. at 30°C.</th>
<th>Cc. of NaOH required.</th>
<th>amino-acid N present.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0 hrs.</td>
<td>I.7 cc.</td>
<td>.0047 gms.</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>I.7</td>
<td>.0047</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>I.8</td>
<td>.0050</td>
</tr>
<tr>
<td>3</td>
<td>14</td>
<td>I.8</td>
<td>.0050</td>
</tr>
<tr>
<td>4</td>
<td>18</td>
<td>I.8</td>
<td>.0050</td>
</tr>
<tr>
<td>5</td>
<td>36</td>
<td>I.8</td>
<td>.0050</td>
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<tr>
<td>6</td>
<td>96</td>
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<tr>
<td>7</td>
<td>192</td>
<td>2.0</td>
<td>.0056</td>
</tr>
</tbody>
</table>
The results were plotted graphically (Figure 7). They show conclusively that the organism produces an extracellular enzyme which would probably cause considerable digestion of hide components.

(iii) Histology of Decomposition.

Histological sections were made from two sources, hide fresh from an animal and hide which had been subjected to considerable action by bacteria. No significant change in structure due to bacterial action was noted when sections from the two sources were compared. In this experiment the hide pieces used were from different animals. Thus there was some difference in structure. To avoid this difficulty, a portion of calfskin was taken and divided into two pieces. One of these pieces was inoculated heavily with organisms and sprinkled with salt, the other was sprinkled with uncontaminated salt (control) and both were incubated at 30°C for thirty days. In this time period (thirty days) the action of the bacteria on the hide was not extensive enough to cause structural changes noticeable in histological sections. Study of preparations made from hide where bacterial action was of longer duration than thirty days indicated that decomposition commenced on the cut edges of the collagen fibres.
PREVENTION OF DISCOLORATION AND DECOMPOSITION IN HIDES

(a) Use of Chemicals.

Hides have been treated in many ways to delay or prevent entirely the action of the salt borne organism. In a number of packing plants in Canada and in the United States hides are dipped in a ZnCl$_2$ solution prior to being stacked for curing. This treatment appears to prevent growth of the great majority of air and dust bacteria, and to some degree growth of the salt bacteria. The use of various other chemicals with the salt appears to be of value in preventing growth of the salt organism. Those which give best results are NaHSO$_3$, NaHSO$_4$, Na$_2$CO$_3$, NaF and Na$_2$SiF$_6$. With NaF and Na$_2$SiF$_6$ concentrations of 0.5% to 1.0% are sufficient to prevent decomposition by bacteria. HCHO is of value if used sparingly enough so as not to injure the hide. Experiments using H$_2$O$_2$ as a germicide showed that it did not greatly retard growth of the organism. The hides are usually quite alkaline and destruction of spores by H$_2$O$_2$ is not as great when the organism is in alkaline medium as when it is in acid medium. (22).
(b) Use of Ultra-violet Irradiation.

The pigment producing organism is found in sea salt that has been improperly cured by under exposure to sunlight. It is probable, then that the organism might be destroyed by sunlight and more especially by ultra-violet light. Irradiation of bacteria is a comparatively recent field of investigation. No reference is made in the literature to the application of ultra-violet light to infected hides or to impure salt.

On one specimen of hide from which bacterial cultures were isolated anthrax bacilli were located. It is thus possible that the salt borne organism parallels the anthrax bacillus in some of its growth and death characteristics. Becquerel (6) reported bactericidal action with anthrax bacilli by ultra-violet light in two or three minutes at room temperature. (It is to be noted that the time factor will vary as the strength of irradiation varies.) Bang (2) found that an increase in temperature, with constant light intensity, lowered the time to kill certain bacteria. Hill and Eidinow (43) showed that both excessive heat and excessive cold lower the resistance of organisms to the ultra-viol- et. With respect to pH, Bayne-Jones and Van der Lingen (3) reported that the killing effect of ultra-violet light
remains constant throughout the range pH 5.5 to 8.0.

In the present investigation experiments were undertaken to determine the effect of sunlight and ultraviolet light on the salt borne organism.

Effect of sunlight on the organism: Contaminated broth cultures, agar plates and pieces of hide were exposed to the direct action of July sunlight for varying periods of time. Lethality occurred for the organisms on the hide when exposed for six hours at a mean average temperature of 28°C. There was considerable evaporation of water with resultant drying but this should not be a factor in lethality for experiment (page 34) showed that the organism retains its vitality when left on dried medium. No complete lethal effect was obtained with the broth cultures or with agar spread plate cultures when exposed for eight hours to sunlight. This is probably due to the fact that the glass in the test tubes containing the broth, and the lid of the Petri plate in the case of the agar filtered the lethal rays from the sunlight.

Effect of ultra-violet light on the organism: In these experiments some difficulty was encountered with regard to culture procedure and the type and time of exposure. The ultra-violet lamp available was a General Electric S I Sunlamp. The chart supplied by this company indicates that the quartz glass allows passage of radiations of the
following wavelengths: 2664, 2700, 2753, 2804, 2894, 2967, 3024, 3132, 3342, and from 3650 to 5461 Å inclusive. Irradiation was carried out at distances of ten and twenty cms. from the edge of this lamp.

The agar plate technique whereby a suspension of bacteria is washed over the agar and then certain portions irradiated affords a simple procedure but is probably subject to error because of unequal distribution of the bacteria. A more accurate method of determination is the suspension method. In this the bacteria are suspended in a salt solution and irradiated for the period desired, then plated out. Concentrations may be varied by means of dilutions. If radiation were carried out through the glass of a test tube it would be necessary to use specially constructed quartz glass tubes to obtain radiation in the bands mentioned above. However by radiating through the top of a test tube and exposing only small quantities it is probable that this difficulty would be overcome sufficiently for present practical determinations.

Vlé's makes reference to a source of error in the suspension method because of scattering of light. Nevertheless most workers agree that the 'suspension method' is the most suitable technique for comparative work.

Using *Bacillus coli*, a very careful radiometric study of the effects of ultra-violet was made by Coblentz and Fulton (21). Their data indicated that the effective
spectral range varied from about 3650 Å to the shortest used. It is concluded that the shortest wavelengths have the most violent lethal action. They state that wavelengths less than 2800 Å are estimated to be at least ten times more active in killing than wavelengths greater than 3050 Å. Their results also showed that continuous and intermittent exposures are equally effective.

Gates (31, 32) is responsible for the development of efficient and practical methods resulting in consistent qualitative results. His suggested agar plate method whereby the bacteria for exposure are washed over hardened agar surface and after exposure the surface is covered with a thin layer of agar reduces the colony spread and thus promotes accuracy of colony counting.

B.M. Duggar(23) makes reference to the work of Ehrismann and Neethling (25) who state that the highest sensitivity for Bacillus prodigiosus occurs at 2801 Angstrom units.

Ultra-violet radiation has been used successfully to eliminate bacteria from the drinking water supplies of some towns(72) and also in partial sterilization of swimming pools. It seems logical to conclude in the light of this evidence that ultra-violet irradiation would be of practical value in the elimination of the salt borne organism if it had a lethal effect upon it. To determine the effect of ultra-violet light upon the organism when suspended in
nutrient salt broth the following experiments were carried out.

**Experimental procedure**: A five-day culture of the organism in ten cc. of nutrient salt broth was prepared. From this culture five, one cc. quantities were taken and a 1:10 dilution made of each. One cc. of the 1:10 dilution was placed in a Petri plate, nutrient salt agar added and the plate incubated. This served as a control. From each of the other 1:10 dilutions, one cc. was placed in a test tube and irradiated. The test tube was placed so that the ultra-violet rays passed down the open end of the tube and were not filtered by the glass. After irradiation, the broth in the tube was poured into a Petri plate, agar added, swished around and the whole was incubated at 30°C. for 48 hours. Then the colonies on the plates were counted. Irradiation was carried out at 10 cms. and 20 cms. distance from the edge of the ultra-violet lamp.

**Experimental results**:

<table>
<thead>
<tr>
<th>Tube</th>
<th>Time of irradiation</th>
<th>Organisms/cc. of 1:10 dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>not irradiated</td>
<td>925</td>
</tr>
<tr>
<td>1</td>
<td>2 mins.</td>
<td>551</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>318</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>74</td>
</tr>
<tr>
<td>4</td>
<td>8</td>
<td>33</td>
</tr>
</tbody>
</table>
Irradiation distance - 10 cms.

<table>
<thead>
<tr>
<th>Tube</th>
<th>Time of irradiation</th>
<th>Organisms/cc. of 1:10 dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>not irradiated</td>
<td>1102</td>
</tr>
<tr>
<td>1</td>
<td>2 mins.</td>
<td>441</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>72</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>15</td>
</tr>
<tr>
<td>4</td>
<td>8</td>
<td>4</td>
</tr>
</tbody>
</table>

From these results the killing effect of the ultra-violet light appeared to vary inversely as the square of the distance. (Figure 8).

Exposure of hide infected with the contaminant to the action of ultra-violet light at a distance of ten cms. resulted in 100% lethality if exposure was for ten minutes. However, considerable drying of the hide occurred in this time so the practical value of direct application of ultra-violet light to hides is questionable.

Irradiation of the salt used or heating of the salt at a temperature and for a time sufficient to kill bacteria present, would be of value in the elimination of the salt borne organism. If the same salt is to be used over and over in the curing processes it should be re-irradiated or re-heated.
SUMMARY

Many hides, calfskins in particular, if cured with solar salt (salt produced by sea water evaporation) show a red discolouration on the flesh side. This discolouration is a visible indication that bacterial action is occurring which may result in considerable decomposition of the hide. Leather produced from hides in which only a small amount of decomposition has occurred is apt to have a spotty texture and a faulty grain. In extreme cases of decomposition, the fibres may be so weakened that the grain layer may be torn completely from the underlying corium.

Only a small group of bacteria, 'the halophils' can exist in the salt concentrations used in curing. The organism responsible for discolouration is found in the salt used in curing the hides. This organism is a coccus and usually occurs in groups of four or packets of eight. It stains gram-negative and flourishes best in a medium of 8% salt concentration and a pH of 8.2. Its optimum temperature is from 30°C to 35°C, and at this temperature range it liquefies gelatin rapidly. When grown on a suitable medium, the organism may produce considerable red pigment. It produces an extracellular enzyme which causes rapid
decomposition of protein.

The natural habitat of the organism is sea salt. Mined salts or kiln dried salts do not contain the 'pigment producing red salt organism'. Contamination of hides could be avoided if the curing room were sterilized and care taken to ensure that the curing salt were not of solar origin. Many chemicals have been used with the curing salt for prevention of growth of the organism. Those most effective in this respect are NaF and Na₂SiF₆. Exposure of the sea salt to sunlight or ultra-violet light, or heating the salt would prove of value in the destruction of the organism.
BIBLIOGRAPHY


71. Romano, and Baldrac. (1914). Coll. 517.


Figure - II

Optimum Temperature.

Maximum Growth
Many Colonies

New Colonies
Minimum Growth

Temperature In Deg. Cent.
Figure 1.

Optimum NaCl Concentration

Maximum Growth. Many Colonies.

Minimum Growth. Few Colonies.

% NaCl In The Nutrient Agar

Number Of Bacteria
Figure III.

Optimum pH.

Maximum Growth.
Many Colonies
8 Days

Minimum Growth.
Few Colonies
4 Days

Temp. 30°C.

pH Of The Medium.

Number Of Bacteria

12 14 16 18 20 22 24 26 28 30
Figure IV

Rate of Growth.

Maximum Growth. Many Colonies.

Few Colonies. Minimum Growth.

Number of Bacteria.

Time In Days.
Figure V.

Digestion By The Organism.

Production Of Amino-Acid Nitrogen.

Amino-Acid N in Grams.

0.0062

0.0057

0.0052

0
10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170

Time In Hours
Figure - VI

Protein Decomposition - Bacterial Growth

Maximum Protein

Protein Decomposition

Bacterial Growth

Maximum Growth

Minimum Protein

Minimum Growth

Time In Days

0 1 2 3 4 5 6 7 8 9
Figure VII

Extracellular Enzyme Action

Amino Acid % in Grams:
- 0.0057
- 0.0055
- 0.0053
- 0.0051
- 0.0049

Time In Hours:

0 20 40 60 80 100 120 140 160 180
Figure VIII

Lethal Effect Of Ultra Violet Light

Number Of Bacteria per c.c.

Time Of Irradiation In Minutes

Irradiation Distance 200 cm
Removal of Hide from Animal.
Plate 2.

Hide on Trimming Platform in Curing Basement.
Plate 3.

Curing Salt and a Hide Stack.
Plate 4.


Organism Isolated from Discolored Hide.

Plate 5.

Organism Isolated from Curing Salt on Hide.
Plate 6.


Organism Isolated from 'Fresh' Salt.