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TESTS FOR INCIPIENT PUTREFACTION OF MEAT

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Tests for Incipient Putrefaction of Meat*

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Bacteriology Section

INTRODUCTION

In view of the fact that meat is one of the most important articles in the diet of man, it is evident that the marketing of meat should be under strict supervision in order that it may be in the best possible sanitary condition when it reaches the consumer. This is particularly true when it is considered that meat is an ideal medium for the growth of microorganisms. Toxins and decomposition products formed by these microorganisms are undoubtedly more or less harmful to the human being. For these reasons, it is important that some rapid test be available, which can be applied to determine whether or not meat has been handled under sanitary conditions and to determine whether the meat is strictly fresh or whether it is in the beginning stages of putrefaction. It is the purpose of this work to study some of the tests which can and have been applied to meat and to determine, if possible, one to meet the above requirements.

Review of Literature

In this discussion, it will be impossible to review all of the papers which have been published pertaining to the subject because the entire fields of protein decomposition and nitrogen metabolism of microorganisms are concerned. The more important papers dealing directly with meat and a few of those concerned with the other two fields will be discussed under the following heads:

a. Bacterial count. A limited number of workers have attempted to apply the plate count to the investigation of the sanitary condition of meat. Marxer (1), 1903, suggested a standard of 1,000,000 bacteria per gram for the purpose of condemnation. His studies were all made on hamburger steak.

Weinzirl and Newton (2-3), 1914, published two papers giving reports of the bacteriological study of hamburger steak. They found that the standard of 1,000,000 per gram established by Marxer was too low, and advanced 10,000,000 as being a fairer limit. They compared the bacterial count with Eber's (4) ammonia test and with the organoleptic test. In their conclusion appears the following statement, "There is no close agreement between the number of bacteria present and the degree

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and ammonia producing abilities of a number of organisms using peptone and meat peptone solutions as media. He found that neither alone was a good index of decomposition because it was possible to get an accumulation of amino acids with a very slight production of ammonia, or conversely a decomposition of the amino acids as fast as formed with a subsequent high concentration of ammonia.

Strohecker (21), in 1920, stated that the determination of amino acids by the "ninhydrin" reaction was very unsatisfactory. He found that oxygen-consumed tests, nitrate reduction tests, and methylen blue reduction tests were much more applicable.

Waksman and Lomanitz (22), 1925, made an extended study of the chemistry of the decomposition of amino acids and of proteins. With regard to amino compounds they said, "The determination of amino nitrogen as an index of decomposition of proteins should be used, however, only when the particular processes carried on by the different organisms are properly understood. . . . The amino compounds are therefore formed and decomposed; they will accumulate in the medium only if the particular organism is unable to use these compounds or the larger part of them, formed from the protein; they will disappear only when the organism attacks them as readily as it does the protein or when another organism is present that attacks these compounds as soon as they are formed by the other organisms from the protein molecule."

Ammonia tests, generally speaking, have met with more approval than the amino acid tests because ammonia is an end product while amino acids are intermediate products. Eber (4) proposed a qualitative test for ammonia in 1893. He claimed this test to be a good criterion of the point of beginning putrefaction. The test proved worthless because it was not delicate enough. Meat developed sufficient odor to condemn it at about the same time or before the ammonia test was positive. The test proved to be an indicator of advanced, rather than one of beginning or incipient, putrefaction.

Richardson (23), in the chapter on "Meat and meat products" in Allen's *Commercial Organic Analysis* said, "Nitrogen in ammonium salts, together with other substances easily decomposed by means of weak alkalies probably affords the best available chemical methods at the present time for the detection of decomposition in flesh foods."

Falk, Baumann, and McGuire (24), 1919, made determinations of total nitrogen, non-protein nitrogen, ammonia nitrogen, total creatinine nitrogen, and purine nitrogen to determine if any of them would be indicative of meat spoilage. The results of these tests showed ammonia nitrogen to be the only one which consistently increased with spoilage. In a continuation of this work, Falk and McGuire (25) found that in order to interpret the results it was necessary to know the history of the sample examined. They discovered that the amount of ammonia present in a given sample of meat was much greater at the time of spoilage if the meat had been kept at a low temperature than if it had been kept at a higher temperature. This difference they attributed to autolysis.

Among others who have found the ammonia test valuable are Van Driest (16) and Kendall and Walker (26).

Sears (20), as stated above, discovered that both the amino acid and

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the ammonia tests must be run on the same sample to be of any value. DeBord (18) concluded that, "The ammonia found in bacterial cultures is not a reliable index of bacterial proteolysis."

Waksman and Lomanitz (22) found that the ammonia test was the most valuable of the chemical methods; however, they also stated that, "Ammonia accumulation can serve as a good index of proteolysis only when no available carbohydrates are present. In the presence of the latter, this method should be supplemented by the study of another process, either the disappearance of the original protein, or the formation of amino nitrogen. . . . The term is only relative and should not be made to mean any more than it does." This sparing action of carbohydrates has been demonstrated by numerous other investigators. Since carbohydrates are present in the ordinary sample of meat it can only be concluded that the ammonia test is comparatively unreliable.

Eber (27), in addition to his ammonia test, worked on a hydrogen sulfid test intended to give similar results. In this test, he partially decomposed the meat with dilute sulfuric acid and measured the amount of hydrogen sulfid given off by means of a strip of filter paper soaked in lead nitrate solution. Eber died before the completion of his work and the test was never applied enough to determine its value. A method was elaborated by Fellers, Shostrom, and Clark (28), in 1924, for the determination of hydrogen sulfid in bacterial cultures and in certain canned foods. They found that in general the non-proteolytic types of bacteria including the cocci, aerobic sporing rods, yeasts, and inactive water and soil bacteria fail to liberate hydrogen sulfid from proteins. Under the conditions of the experiment, no hydrogen sulfid was liberated from decomposed salmon or shrimp.

Clough (29), 1922, made a study of the formation of indol and skatol during the decomposition of salmon. He concluded that, "Although the determination of indol cannot supplant odor and physical appearance in the examination of canned salmon, it is nevertheless of value and affords considerable information as to the previous history of the sample."

As a generalization, it may be stated that attempts to apply chemical methods for the determination of incipient putrefaction have met with moderate success. Some of these methods are of value but good results are limited by so many factors that their use as simple, practical tests is questionable. Many of the methods indicate advanced rather than incipient putrefaction. None of them indicate gross contamination of the meat by careless handling, unless sufficient time has elapsed after contamination to allow active putrefaction to set in.

c. Biological Tests. Certain German investigators have attempted to apply biological tests based on the number and kinds of microorganisms present, as well as on the products of their activity. The first report was published by Tillmans and Mildner (30) in 1916, the second by Strohecker (21) in 1920, and the third by Tillmans, Strohecker and Schutze (31) in 1921. They found three tests which were of value in showing the first stages of decomposition. These were the oxygen-consumed test, the nitrate reduction test, and the methylen blue reduction test. From data published it would appear that these tests are worthy of further investigation. They are based however, on the number and activity of the common aerobic organisms present,

rather than on the number and activity of the specific organisms responsible for the greater part of putrefaction. Thus it would appear that they must be subject to many of the same criticisms which have been applied to the bacterial count.

Brauer (32) found that incipient decomposition in sausages, preserved foods, and other substances could be detected by the inoculation of glucose fermentation tubes with a small portion of the sample and noting whether gas was formed or not. This is in opposition to the statement of Hoffstadt (8) that the count of dextrose fermenters is unreliable as a standard for meat analysis.

d. Organisms Concerned in Putrefaction. Numerous investigators have ascribed the phenomenon of putrefaction to aerobic organisms. Chief among these aerobic organisms is *Bacillus (Proteus) vulgaris.* Most later investigators have been unable to find any aerobic organism capable of causing putrefaction. Bienstock (33) attributed it to *Bacillus putrificus.* He defined true putrefaction as the bacterial decomposition of albuminous matter accompanied by the formation and elimination of foul-smelling substances.

Tissier (34) in a recent review of the subject, based on twenty years work by Tissier and Martelly, found that the main role in putrefaction was played by anaerobes but that certain aerobes prepared the way for them and destroyed the intermediate products of decomposition remaining after their action.

Rettger (35) has shown repeatedly that only anaerobes can initiate putrefaction. He distinguishes between digestion or decomposition as caused by the aerobes, like *Bacillus (Proteus) vulgaris* and *Bacillus subtilis,* and true putrefaction. The former is very much slower and is not accompanied by foul odors. He has found that hydrogen sulfid is formed early in putrefaction as caused by the anaerobes. On the other hand, he has found little or no indol, skatol, or phenol formed. The rate of putrefaction, according to his work, depends somewhat upon the aerobes associated with the proteolytic anaerobes.

Hunter (7, 36-38) found that the decomposition of salmon was due chiefly to the organisms described in the literature as water, sewage, and soil organisms. He added however that, "The bacteria responsible for the decomposition of the fish are not, in the strict sense, putrefactive and there is no rapid digestion of the flesh of the fish in the cultures. * * * This makes it apparent that the flesh of fish is more susceptible to decomposition by ordinary water bacteria than the flesh of higher animals." He found that no contamination with anaerobes took place during the handling of fish.

Hoffstadt (8-9) concluded that spoilage of ground beef was due to the anaerobes present. She isolated three types of anaerobes—proteolytic, medially proteolytic, and saccharolytic; and found that the type of spoilage was due to the type of anaerobe present. She stated that, "The presence of proteolytic anaerobes indicates a definite way by which the keeping qualities of meat can be predicted."

Weinzirl (39), 1924, confirmed the work of Bienstock, Rettger, and others and showed that anaerobes do cause putrefaction. He proved, by the use of a modified Weinzirl Anaerobic Spore test (40), that these anaerobes are present. In his conclusions, he suggested the desirability of a test based on the anaerobes present; "Aerobic and facul-

tative bacteria assist in the spoilage of meat, but the putrefactive odor is mainly due to anaerobes. The aerobic bacterial count is, therefore, only indirectly an indicator of putrefaction. * * * A test for anaerobes in meat, comparable to the *Bacillus coli* test in water analysis, appears highly desirable."

Part I.

A Study of Organisms Isolated from Fresh Hamburger Steak and from Hamburger Steak in the First Stages of Putrefaction

Experimental Data

In this investigation, it seemed expedient to attempt to base a test for incipient putrefaction of meat upon the results of the physiological activity of the organisms present.

To do this, it was necessary to know something of the type of flora in the meat at this stage. A series of organisms isolated from comparatively fresh meat and another series isolated from meat in the early stages of putrefaction were studies in order to note what types developed during the so-called stage of incipient putrefaction. Only aerobes were included in this study.

Hamburger steak was chosen for these investigations because it was easily obtained and worked with, and because it is one of the meat products which is most frequently grossly contaminated and which needs close supervision. For the first series of organisms, a sample was collected from a local market. This sample was ground in the presence of the investigator and was made of meat which appeared comparatively good and fresh. The sample was plated immediately on standard meat infusion agar. The organisms were freed from the meat by grinding in sterile sand, according to the method of Weinzirl and Newton (3). A count of 3,000,000 bacteria per gram was obtained. Two plates of the 1 to 100,000 dilution were selected which contained 27 and 44 colonies respectively. Transplants were made from all of the colonies on these plates. Due to the fact that many of the colonies were very small and also, undoubtedly, to the fact that many of them would not grow readily on ordinary media, only 51 cultures were obtained. No attempts were made to eliminate duplicates because the main result to be obtained was the proportion of the different physiological types present.

The second series of organisms was isolated from a sample of hamburger steak purchased from the same market, under as nearly identical conditions as possible. The sample was stored at 20° Centigrade for a period of two days and was then plated according to the same method. A moderately putrid odor was given off by the meat at the time of examination. A count of 178,000,000 was obtained. Attempts were made to transplant 77 colonies, which represented one-half of those present on a 1 to 1,000,000 dilution. From these, 62 cultures were obtained.

A microscopic examination was made of each culture in order to classify them according to morphologic groups. The results, together

Table I.—A study of organisms isolated from fresh meat.

Number	Hydrogen Sulfid	Ammonia	Glucose Acid	Glucose Gas	Meth. Blue Reduced	Nitrates Reduced	Indol	Morphology
1	−	+	2	−	+	+	−	Coccus
2	−	±	−	−	−	−	−	Coccus
3	−	−	−	−	±	−	−	Coccus
4	2	±	1	1	+	+	−	Coccus
5	2	±	4	−	+	+	−	Coccus
6	−	−	−	−	±	±	−	Coccus
7	3	+	1	−	+	±	−	Coccus
8	2	+	2	−	+	+	−	Coccus
9	−	−	3	−	±	+	−	Coccus
10	−	+	3	−	±	+	−	Coccus
11	−	−	−	−	−	−	−	Coccus
12	−	±	1	−	+	±	−	Coccus
13	6	+	1	−	+	+	−	Coccus
14	3	±	3	−	+	±	−	Coccus
15	2	±	1	1	+	+	−	Coccus
16	−	+	3	−	+	+	−	Coccus
17	2	±	1	1	+	±	−	Coccus
18	−	±	2	−	±	+	−	Coccus
19	6	+	−	−	−	±	−	Coccus
20	−	±	2	−	−	+	−	Coccus
21	−	+	3	−	±	±	−	Coccus
22	6	+	1	1	+	+	−	Bi-polar rod

Table I.—Continued

Number	Hydrogen Sulfid	Ammonia	Glucose Acid	Glucose Gas	Meth. Blue Reduced	Nitrates Reduced	Indol	Morphology
23	−	−	3	−	−	±	−	Coccus
24	4	±	3	−	±	+	−	Coccus
25	2	±	1	1	±	+	−	Bi-polar rod
26	−	−	−	−	−	−	−	Coccus
27	−	+	1	−	±	−	−	Coccus
28	−	+	2	−	+	+	−	Coccus
29	3	±	1	−	±	+	−	Involution rod
30	6	+	1	−	+	+	−	Coccus
31	6	+	3	−	±	±	−	Coccus
32	2	−	4	−	+	+	−	Coccus
33	−	±	3	−	±	±	−	Coccus
34	2	+	3	−	+	+	−	Coccus
35	4	−	−	−	+	+	−	Coccus
36	−	+	−	−	±	+	−	Fluorescent rod
37	2	+	1	−	+	+	−	Coccus
38	6	+	1	−	±	+	−	Coccus
39	2	+	4	−	+	+	−	Coccus
40	6	+	2	−	+	+	−	Coccus
41	2	+	2	−	+	+	−	Coccus
42	−	±	3	−	±	+	−	Coccus
43	−	±	1	−	±	+	−	Coccus
44	6	−	3	−	+	+	−	Coccus

Table I.—Continued

Number	Hydrogen Sulfid	Ammonia	Glucose Acid	Glucose Gas	Meth. Blue Reduced	Nitrates Reduced	Indol	Morphology
45	−	−	1	1	+	+	−	Proteus-like
46	−	−	−	−	−	±	−	Coccus
47	2	±	−	−	+	±	−	Coccus
48	−	+	4	−	±	±	−	Coccus
49	−	±	3	−	−	+	−	Coccus
50	−	±	1	1	+	+	−	Proteus-like
51	2	+	2	−	±	+	−	Coccus

Hydrogen sulfid, acid and gas production are recorded in the number of days necessary for their production.

Table II.—A study of organisms isolated from hamburger steak in the beginning stages of putrefaction.

Number	Hydrogen Sulfid	Ammonia	Glucose Acid	Glucose Gas	Meth. Blue Reduced	Nitrates Reduced	Indol	Morphology
1A	1	−	1	−	+	+	−	Short rod
2A	1	−	1	−	−	+	−	Short rod
3A	1	±	1	−	−	−	−	Short rod
4A	−	−	2	−	−	±	−	Spore former
5A	1	+	1	−	+	+	±	Short rod
6A	1	±	1	−	+	+	+	Short rod
7A	1	−	1	−	+	+	−	Proteus-like
8A	1	±	1	−	+	+	−	Proteus-like
9A	1	±	2	−	+	+	−	Proteus-like
10A	1	±	1	−	+	+	−	Proteus-like
11A	1	±	1	−	+	−	±	Proteus-like
12A	1	±	1	−	+	+	−	Proteus-like
13A	1	±	2	−	+	±	−	Proteus-like
14A	1	±	6	−	−	−	−	Coccus
15A	1	−	1	−	+	+	−	Proteus-like
16A	−	±	1	−	−	−	−	Coccus
17A	1	±	1	−	+	−	+	Proteus-like
18A	1	±	1	−	+	+	−	Proteus-like
19A	1	+	1	1	+	+	+	Proteus-like
20A	2	−	−	−	−	−	−	Large rod
21A	1	±	1	−	+	+	−	Proteus-like
22A	1	±	1	−	+	+	−	Proteus-like

Table II.—Continued

Number	Hydrogen Sulfid	Ammonia	Glucose Acid	Glucose Gas	Meth. Blue Reduced	Nitrates Reduced	Indol	Morphology
23A	7	±	1	–	–	–	–	Large rod
24A	–	±	1	–	–	–	–	Large rod
25A	1	±	1	1	+	+	±	Proteus-like
26A	1	–	2	–	+	–	±	Proteus-like
27A	1	–	1	–	+	+	–	Proteus-like
28A	1	–	6	–	–	–	–	Short rod
29A	1	–	2	–	–	+	–	Proteus-like
30A	–	±	1	–	–	–	–	Coccus
31A	1	±	1	–	+	+	–	Proteus-like
32A	–	±	4	–	–	–	–	Coccus
33A	1	–	1	–	+	+	–	Proteus-like
34A	1	±	1	–	+	+	±	Proteus-like
35A	–	–	1	–	+	–	–	Coccus
36A	1	–	1	–	+	±	–	Proteus-like
37A	5	–	6	–	–	–	–	Coccus
38A	1	±	1	1	+	+	±	Short rod
39A	2	–	5	–	–	–	–	Streptococcus
40A	1	+	1	–	+	+	±	Proteus-like
41A	1	–	1	–	+	+	±	Proteus-like
42A	1	–	1	–	+	+	–	Proteus-like
43A	1	–	1	1	+	+	±	Proteus-like
44A	1	±	2	–	+	–	–	Short rod

Table II.—Continued

Number	Hydrogen Sulfid	Ammonia	Glucose Acid	Glucose Gas	Meth. Blue Reduced	Nitrates Reduced	Indol	Morphology
45A	−	−	2	−	±	±	−	Short rod
46A	1	−	2	−	+	±	−	Short rod
47A	1	−	2	−	+	−	−	Short rod
48A	1	−	2	−	+	+	−	Short rod
49A	2	±	−	−	−	−	−	Short rod
50A	2	−	1	−	±	−	−	Large coccus
51A	−	−	1	−	−	−	−	Coccus
52A	−	+	1	−	+	+	±	Coccus
53A	1	−	1	−	+	+	−	Proteus-like
54A	1	−	1	−	+	+	±	Proteus-like
55A	−	−	1	−	±	±	−	Large coccus
56A	1	−	1	1	+	+	±	Proteus-like
57A	−	−	2	−	±	−	−	Large coccus
58A	1	−	2	−	+	−	±	Proteus-like
59A	1	−	1	−	+	±	±	Proteus-like
60A	1	+	1	1	+	±	±	Proteus-like
61A	1	+	1	1	+	+	±	Proteus-like
62A	−	−	1	−	−	−	−	Coccus

Hydrogen sulfid, acid and gas production recorded in days necessary for production.

with their physiological characteristics, are recorded in Table I and Table II. Only those characteristics which have been or which might easily be applied as an indication of the presence and activity of the groups concerned were included in the study.

Hydrogen sulfid determinations were made by suspending strips of filter paper soaked in lead acetate solution along side the cotton plugs. Standard infusion broth was used because it was noted that it was more efficient than ordinary peptone solution for this purpose. Difco peptone has been used throughout this work. Nessler's solution was employed to indicate the presence of ammonia in peptone solution cultures. Acid and gas production by the organisms in a one per cent glucose broth was determined. Their ability to reduce nitrates was determined by growing the organisms in a nitrate-peptone solution containing 0.02% potassium nitrate. Ehrlich's method of testing for indol was used. To determine the ability of the organisms to reduce, or more properly to produce products which would reduce, methylen blue solutions, 0.01 cc of a solution of methylen blue, made by diluting 5 cc of a saturated solution to 200 cc with water, was added to each tube of infusion broth. When the methylen blue was added before the organisms were grown, no reduction was noted. The method followed was to add it to a 48 hour culture. Reduction took place immediately; or within an hour, in the case of cultures with weaker reducing properties. These latter were recorded as partial reactions. The number of days necessary for the production of hydrogen sulfid, and of acid and gas from glucose was recorded. This gives an indication of the activity of the organism.

Of the 51 cultures isolated from fresh hamburger steak 45 were coccus forms, 2 were bi-polar rods, 2 were proteus-like rods, 1 produced distinct involution forms, and 1 produced a fluorescent colony. Of the 62 cultures from meat in the beginning stages of putrefaction, only 13 were cocci. This is a percentage of 21 as compared with 88 from the fresh meat. Thirteen were described as short rods, 1 as a spore former, 3 as large rods without spores, and 32 as proteus-like rods. This would indicate that there is an increase in the proportion of the number of rods to the number of cocci during the period of the beginning of putrefaction. A group of organisms described as proteus-like rods, including all short rods with occasional filamentous forms, appears to be the group which increases most rapidly. Only a few of these give the complete reactions of *Bacillus (Proteus) vulgaris.*

Percentages of the organisms from each series which gave positive results with the various physiological tests have been computed and are recorded in Table III.

Two groups of organisms which appear in the series from putrefying meat do not appear, at least to any noticeable extent in the series obtained from fresh meat. These are the groups described as short rods, of which there were 13, and as proteus-like organisms, of which there were 32, in a total of 62 cultures. The characteristics of these groups are recorded in Table IV.

It is of particular note that a larger percentage of the organisms isolated from fresh hamburger steak than of the organisms isolated from the hamburger steak which was in the process of putrefaction reduced methylen blue, nitrates, and produced ammonia. Action on

Table III.—Physiological reactions of the organisms isolated from fresh and from putrefying meat. A summary of tables I and II.

	Organisms from fresh Hamburger Steak % positive	Organisms from putrefying Hamburger Steak % positive
Hydrogen sulfid	51	81
Ammonia	84	50
Acid from Glucose	80	98
Gas from Glucose	14	11
Methylen Blue Reduced	84	73
Nitrates Reduced	92	63
Indol	0	28

Table IV.

	Short rods % positive	Proteus-like organisms % positive
Hydrogen sulfid	92	100
Ammonia	46	53
Acid from Glucose	92	100
Gas from Glucose	8	19
Methylen Blue Reduced	69	97
Nitrates Reduced	62	87
Indol	15	44

glucose seemed to be common to both groups. If these facts be accepted as conclusive, then any test based on these characteristics depends entirely upon the increase in numbers of organisms found in the meat and not upon specificity. This means that any such test would be liable to most of the criticisms of the bacterial count and would probably be of no more value.

No organisms in the first series produced indol. In the second series 28% of the organisms produced it. Comparable with this is the increase in hydrogen sulfid producers. This increase is only partially shown by the percentage of 81 as compared to 51. Most of the hydrogen sulfid producing bacteria found in the first series were coccus forms, producing a sufficient amount to be first observed at the end of two to six days. In the second series, most of the hydrogen sulfid producers were rods which produced the gas abundantly in one day. This fact would indicate that any test based on hydrogen sulfid production should show a much more abundant evolution of this gas during the stages where putrefaction is commencing. It is worthy of note that 92% of the short rods and 100% of the proteus-like organisms produced hydrogen sulfid.

Summary

Of 51 organisms isolated from fresh hamburger steak, 88% were coccus forms. Of 62 isolated from similar meat in the stages of beginning putrefaction, only 21% were cocci. This increase in rod forms was due to the appearance of two groups of bacteria not found in the fresh meat. Thirteen forms were described as short rods and 32 as proteus-like organisms.

When the two series of cultures were compared, it was noted that a larger percentage of the organisms from fresh meat produced ammonia and reduced methylen blue and nitrates, than of the organisms from putrefying meat. There was no appreciable difference in the action of the two groups on glucose.

Judging from these results there is a decided increase in the number of indol and hydrogen sulfid producers during the stage of incipient putrefaction of meat. It was not determined whether this would hold true with meat stored at a different temperature than 20° Centigrade.

Part II.

Hydrogen Sulfid Tests for the Detection of Incipient Putrefaction of Meat

In Part I, it was noted that indol producing organisms made their appearance during the stages in which putrefaction was commencing. Along with this was a very marked increase in the numbers of organisms which actively evolve hydrogen sulfid. An attempt has been made to develop a test or tests for the stage of incipient putrefaction and for the amount of effective contamination, based on the presence of these groups of organisms. The amount of effective contamination is designated as the amount of contamination with those organisms which will be active in decomposing or, more properly, putrefying the meat. In other words, it seemed desirable to have a test which would predict the length of time during which a given meat product would be in condition for consumption. Hamburger steak was again chosen for the investigations.

For the detection of the presence and activity of indol producing organisms, the cotton wool plug test for indol as developed by Gore (41) was used. This is a modification of Ehrlich's method in which the reagents are placed on the cotton plug and the indol volatilized by heating the culture in a water bath. It is a very satisfactory method where the color of the medium would mask the color of the reaction. One gram samples were weighed into tubes containing 10 cc of meat infusion broth and the tubes incubated at 37°. These tubes were removed from the incubator and tested for indol at hourly intervals. It was found that, regardless of whether the hamburger steak was putrid or fresh, the time of the first appearance of indol was between 12 and 24 hours. The amount of indol formed with putrid meat did not appear to be greatly in excess of the amount formed with com-

paratively fresh meat. As these preliminary tests did not appear to give favorable results, the method was not investigated further.

A similar method has been developed to test for the presence and activity of hydrogen sulfid producing organisms. One gram portions of hamburger steak are weighed into tubes containing exactly 10 cc of standard infusion broth, adjusted to pH 7.0. Strips of filter paper soaked in lead acetate solution are suspended beside the plug as indicators of the production of hydrogen sulfid. The tubes are incubated at 37° Centigrade and hourly observations made for blackening of the strips of lead acetate paper. The time of the first appearance of blackening is used as the index.

Samples were collected from various meat markets and tested by this method. Duplicate tubes were run aerobically and also in the partial vacuum induced in a Novy jar by a water pump. Before the completion of the experiments, a vacuum pump was purchased. The pump was found to produce almost identically the same degree of vacuum as the water pump used with the first samples. In the first experiments, the samples were divided and stored in deep culture dishes at 20° Centigrade and at ice box temperature which varied around 5° Centigrade. They were tested at intervals of from one to three days and particular attention was paid to the results at the time when the odor of putrefaction was first noticeable.

Tables V and VI give the results obtained from the examination of two samples. The sample recorded in Table V was purchased from market A. The meat from which it was obtained had been ground and stored in a pan in the refrigerator at the market. It was frozen solid at the time it was bought. It was not tested until the day following the one on which it was collected. It appeared to be in good condition and free from excessive contamination. Table VI gives the results of a sample from market B. All hamburger steak from this market was ground at the time of selling. Anaerobic spore tests were made on both samples according to the method of Weinzirl (40). Positive results were obtained.

Sample B (Table V) after 24 hours incubation at 5° Centigrade contained enough active hydrogen sulfid producers to give a positive test in 8 hours both aerobically and anaerobically. At the end of nine days, when a putrid odor was first noticed, a positive test was obtained in 10 hours aerobically and in five hours anaerobically. After storing at 20° for one day, the odor was described as very sour and there was a very slight indication of beginning putrefaction. A positive test was obtained in six hours aerobically and in five hours anaerobically. At the end of two days, when the meat was decidedly putrid, a positive test was obtained in three hours aerobically and in two hours anaerobically.

Sample C (Table VI), on immediate testing gave a positive result aerobically in eight hours and anaerobically in 10 hours. After one day at 20° Centigrade, it gave off a slightly putrid odor and the hydrogen sulfid test was positive in six hours aerobically and in five hours anaerobically. After five days at 5° Centigrade, the meat had reached a stage comparable to that reached in one day at 20°. A positive test was obtained aerobically in eight hours and anaerobically in five hours.

Aerobic hydrogen sulfid producing organisms do not appear to develop at 5° Centigrade. The test completed under aerobic conditions

Table V.—Hydrogen sulfid production by organisms from hamburger steak. Sample B. Recorded in hours necessary to obtain a positive test. Incubated 37°.

Age in Days	Stored at 5°			Stored at 20°		
	aerobic	anaer.	condition	aerobic	anaer.	condition
0	not	run	good	not	run	good
1	8	8	good	6	5	sour
2	7	7	good	3	2	putrid
5	8	8	fair	.....	.....	.....
7	8	6	sour	.....	.....	.....
9	10	5	put. odor	.....	.....	.....

Table VI.—Hydrogen sulfid production by organisms from hamburger steak. Sample C. Recorded in hours necessary to obtain a positive test. Incubated 37°.

Age in Days	Stored at 5°			Stored at 20°		
	aerobic	anaer.	condition	aerobic	anaer.	condition
0	8	10	good	8	10	good
1	.....	.....	good	6	5	put. odor
5	8	5	put. odor	.....	.....	.....

seems to give comparable results with that run under anaerobic conditions where the meat has been stored at the higher temperature. One sample gave a positive test in 10 hours anaerobically and the other in eight hours, at the time of first testing. It is worthy of note that all examinations made at the time of the first organoleptic indications of putrefaction showed a production of hydrogen sulfid in five hours anaerobically. This was independent of the temperature of storage and seemed to indicate that the anaerobes which produce hydrogen sulfid were the organisms chiefly concerned in active putrefaction. Later experiments showed similar results. This agrees with the findings of recent investigators concerning the causal organisms found in true putrefaction. Table VII gives the results obtained by examining 36 other samples of hamburger steak, using the same test. All of these samples were tested immediately after collection and again at intervals of from one to three days until the meat was shown to be putrid according to organoleptic tests. In all cases, the meat was stored in deep culture dishes at approximately 5° Centigrade. No covers were placed on the dishes and an attempt was made to approximate the conditions under which it would be stored in the average market. For the sake of brevity only the results obtained by testing immediately after collection and those obtained by testing when the first faint odor of putrefaction became evident are given. The meat was graded into four classes according to the appearance, odor, and condition of the shop. These classes were good, fair, poor, and bad. Only meat which gave a slight odor of putrefaction at the time at which it was collected was recorded as bad. These ratings do not give a good method for determining the quality of the meat or the stage of incipient putrefac-

Table VII.—Hydrogen sulfid production by organisms from hamburger steak. Recorded in hours necessary to obtain a positive test. Incubated 37°.

Market	At time of buying		After storing at 5°C until odor appears		Condition
	aerobic	anaer.	aerobic	anaer.	
A	8	8	10	5	good
B	8	10	8	5	good
B	7	5	7	4	fair
A	7	4	—	—	bad odor as bought
B	12	9	8	5	good
A	5	4	—	—	bad odor as bought
B	8	8	7	6	good
D	5	4	5	2	poor
E	8	8	7	4	fair
F	7	7	7	2	poor
B	13	13	7	6	good
A	5	4	5	3	poor
B	8	7	7	5	good
A	3	2	—	—	bad odor as bought
A	.....	4	.....	2	poor
B	.....	7	.....	4	good
B	.....	5	.....	2	good
A	.....	2	.....	—	bad odor as bought
B	.....	6	.....	not run	good
A	.....	5	.....	not run	poor
B	.....	6	.....	3	good
A	.....	6	.....	3	good
B	.....	5	.....	3	fair
A	.....	4	.....	—	bad odor as bought
B	.....	5	.....	—	bad odor as bought
A	.....	2	.....	—	bad odor as bought
B	.....	7	.....	4	good
A	.....	3	.....	2	poor
B	.....	7	.....	5	good
A	.....	8	.....	6	good
B	.....	3	.....	—	bad odor as bought
B	.....	2	.....	—	bad odor as bought
A	.....	4	.....	—	good
B	.....	7	.....	not run	good
A	.....	7	.....	not run	good
B	.....	4	.....	—	bad odor as bought

tion but they do furnish as good a method as was obtainable with which to compare the results of the hydrogen sulfid test. The test was run aerobically only on the first fifteen samples, as it did not appear to give good results on meat stored at 5° Centigrade.

The results agree very well with those given in Tables V and VI. They show conclusively that the aerobic hydrogen sulfid producing bacteria do not increase in activity appreciably during the first stages of putrefaction at 5° Centigrade. On the other hand the anaerobic hydrogen sulfid producing group appears to increase in activity decidedly. A summary is given in Table VIII showing the number of samples rated as good, fair, poor, and bad according to the appearance of the meat and the average number of hours required for the production of hydrogen sulfid under the conditions of the test as applied anaerobically. These averages are computed for the immediate test and also for the test applied when the meat began to evolve odors of putrefaction.

Table VIII.—Hydrogen sulfid production by organisms from hamburger steak. Recorded in hours necessary to obtain a positive test. A summary of Table VII. Incubation at 37° under anaerobic conditions.

Organoleptic Rating	No. of samples	Tested when:	
		Purchased	Putrescent*
Good	16	7.5	4.5
Fair	3	6.0	3.7
Poor	6	4.5	2.2
Bad-putrid odor	11	3.2	—

*When putrid odor first became evident.

According to Table VIII, 16 samples rated as good gave a positive test in an average of 7.5 hours. At the time when noticeable putrefaction appeared these same samples gave positive test at the end of 4.5 hours. Three samples rated as fair gave positive tests at the end of six hours and at the end of 3.7 hours after active putrefaction became apparent. Six samples rated as poor gave positive tests at the end of 4.5 hours and at the end of 2.2 hours at the time of the first appearance of a foul odor. This would indicate that the test shows two conditions: first, the stage of incipient putrefaction; second, the amount of effective contamination. The shortening of the length of time required to obtain a positive test, as the meat ages, shows that the test is indicative of the activity of a group of organisms which increases in numbers or activity or both during the first stages of putrefaction and before the meat would be condemned by organoleptic tests. It is highly probable that the samples rated as poor by their appearance were more highly contaminated than those recorded as good. The "good" samples gave positive tests at the end of 4.5 hours and the "poor" samples at the end of 2.2 hours, when tested at that stage at which they began to evolve odors of putrefaction. This difference is probably due to the increased amount of effective contamination in the poor samples.

Five to seven days was the average length of time that elapsed before the meat which was rated as good appeared to be putrid. The meat rated as fair remained in good condition for approximately three days. That rated as poor usually commenced to evolve foul odors after one day. In general, the length of time which elapsed before noticeable putrefaction became evident agreed well with the condition of the meat as indicated by the hydrogen sulfid test.

Four samples which were collected from a market, later discovered to be using sulfites as preservatives, were tested. These samples gave positive tests in from eight to 12 hours at the time of collection. On storage at 5° Centigrade the time required to obtain a positive test increased until at the time at which the meat became noticeably bad only a slight test was obtained in 24 hours. If the definition of "putrefaction" given by Rettger (35) and other investigators be accepted, for example, that it is marked by the evolution of a decidedly foul odor, then no true "putrefaction" took place in these samples of meat. The spoilage appeared to be more closely comparable to the "decomposition" described by Hunter (36-38) and by others, and which has been ascribed to aerobic bacteria or molds. A species of mold found growing on these samples was isolated and identified as *Chaetostylum fresenii*. This meat remained in apparently good condition for from 10 to 20 days. The fact that the preservative prevents the development of the organisms responsible for a positive anaerobic hydrogen sulfid test and at the same time prevents the development of the organisms responsible for the putrefaction of the meat would serve as evidence that both types of organisms may be the same.

Whether the test as developed for hamburger steak would be applicable for any other class of meat cannot be stated without further investigation. The condition of hamburger steak as sold in the average market is such that some method for determining the amount of effective contamination and the length of time during which it will remain useable after selling, should certainly be applied. From the results obtained on the samples tested and recorded in Tables V, VI, and VII, it appears that this method would be preferable at least to plate counts and to chemical methods in that it is quicker and easier to perform, that it is more specific for the organisms developing during incipient putrefaction, and that it will show incipient rather than advanced putrefaction as well as the amount of effective contamination.

Any definite standard set for the condemnation of unfit hamburger steak must depend upon the quality of meat which is considered salable. Hamburger steak which will not give a positive hydrogen sulfid test, using the method employed in this investigation, in six hours may safely be expected to keep, in a good refrigerator, at least three days before putrefaction becomes evident. This does not appear to be too high a standard to set although it would condemn at least half of the hamburger steak which is now being sold in the markets.

Some samples were tested by the hydrogen sulfid production test and also by the methylen blue reduction method of Tillmans, Strohecker, and Schutze (31). By this method five gms of well mixed meat is placed in a 60 cc flask, which is filled with water heated to about 40° Centigrade. One cc of a methylen blue solution is added. The flask is tightly sealed and placed in a water bath at 45° Centigrade.

The value of the meat is judged by the length of time required for the reduction of the methylen blue. Tillmans and his associates concluded that meat was decomposed and unfit for use as human food when the methylen blue was reduced in less than one hour.

Table IX gives the results obtained by the testing of eight samples by both methods. None of these were of very good quality. All of them gave positive tests for hydrogen sulfid producing organisms in less than seven hours. A period of from four to eight hours was required for the reduction of the methylen blue. All of the samples were tested again at the time when putrefaction became evident by the odor. Portions were taken from the surface, from the center, and from a mixture of the two, for the final test on the last six samples. The results show that the methylen blue was reduced, within the period of one hour set by the German investigators, after the meat became noticeably putrid. There was a wide variation in the length of time which the reduction required depending upon the spot of sampling.

Table IX.—A comparison of the hydrogen sulfid production and methylen blue reduction tests. Recorded in hours necessary to obtain a positive test.

SAMPLE I.

Age in days	Hydrogen Sulfid	Meth. Blue Red.	Condition meat
0	6	6	fair
3	3	5	poor
4	2	½	putrid odor

SAMPLE II.

Age in days	Hydrogen Sulfid	Meth. Blue Red.	Condition meat
0	2	4	poor
3	1	1	putrid odor

SAMPLE III.

Age in days	Hydrogen Sulfid Test			Meth. Blue Reduction		
0	6			8		
	Surface	Center	Mixed	Surface	Center	Mixed
4	6	6	6	½	2	1½

SAMPLE IV.

Age in days	Hydrogen Sulfid Test			Meth. Blue Reduction		
0	5			4		
	Surface	Center	Mixed	Surface	Center	Mixed
4	1	1	1	5 min.	5 min.	5 min.

Table IX.—Continued

SAMPLE V.

Age in days	Hydrogen Sulfid Test			Meth. Blue Reduction		
0	6			6		
	Surface	Center	Mixed	Surface	Center	Mixed
3	4	3	3	1	½	½

SAMPLE VI.

Age in days	Hydrogen Sulfid Test			Meth. Blue Reduction		
0	6			8		
	Surface	Center	Mixed	Surface	Center	Mixed
3	4	4	4	2¾	3¼	3

SAMPLE VII.

Age in days	Hydrogen Sulfid Test			Meth. Blue Reduction		
0	5			8		
	Surface	Center	Mixed	Surface	Center	Mixed
4	3	3	3	7 min.	5 min.	5 min.

SAMPLE VIII.

Age in days	Hydrogen Sulfid Test			Meth. Blue Reduction		
0	4			6		
	Surface	Center	Mixed	Surface	Center	Mixed
4	1	1	1	12 min.	10 min.	11 min.

For example, in the testing of Sample III, when meat from the surface was used the methylen blue was reduced in one-half hour; while, if samples taken from the center required two hours for the reduction to be complete. The place of sampling appeared to have little effect on the hydrogen sulfid test.

The hydrogen sulfid production test appears to be superior to the methylen blue reduction test as it furnishes a method by which the condition of the meat may be indicated before it reaches the stage of evident putrefaction, and, furthermore, the location of the sample in the meat does not seem to influence the result to as great an extent. The methylen blue reduction test would be superior if a test for advanced putrefaction was desired because it is more rapid and is slightly easier to run.

Further investigations were made to see how much the results obtained in the use of the test for a routine method might be influenced by slight errors in technic and to see if the technic employed in the test might be improved so as to make the test more delicate or more

accurate. Eight samples were tested using different amounts of meat. Along with the tubes containing one gram other tubes were used containing one-half and two gram portions. Results showed that within this range the difference in the length of time required to obtain a positive test was insignificant. At no time was there a difference in results between the tubes containing one gram of meat and those containing two grams. Occasionally, one hour longer was required to obtain a positive test in the tube where only one-half gram was used. From these investigations, it was concluded that in the routine use of the test extremely accurate weighings would not be necessary. One gram appears to be the logical amount of meat to use.

The possibility of substituting peptone solution for infusion broth was investigated. The results were very unsatisfactory. The length of time required to obtain a positive test was increased and the reaction was not as well defined. It was found that meat extract broth gave identical results with infusion broth. Broth which contained different amounts of peptone, standard broth, one-half strength broth, and double strength broth was tried. The reaction given in half strength broth was ordinarily as quick but not as strong. No difference was noted between the results obtained with standard and with double strength.

To investigate the effect of the reaction of the medium used, standard broth was adjusted to the following pH's: 6.5; 6.75; 7.0; 7.25; and 7.5. Between 6.75 and 7.25, there was little difference in the results obtained with the six samples tested. Beyond this range a longer period of time was required to obtain a positive test and the reaction was not as well defined. Broth adjusted to pH 6.8 or 7.0 seems to be at the optimum reaction for the production of hydrogen sulfid through the action of the anaerobic organisms concerned in the putrefaction of meat.

Summary

An attempt to apply a test for incipient putrefaction of meat based on the appearance of indol forming organisms was unsuccessful.

Thirty-six samples of hamburger steak were investigated by a test designed to show the increase in activity of hydrogen sulfid producing organisms during the period before putrefaction can be demonstrated by organoleptic methods. The method of testing was as follows: Place one gram of meat in a tube containing exactly 10 cc of standard infusion or extract broth. Suspend a strip of lead acetate paper beside the cotton plug. Incubate at 37° Centigrade in an anaerobic jar under partial vacuum. Examine and record the number of hours necessary to obtain blackening of the acetate paper due to the production of hydrogen sulfid.

Positive tests were obtained from comparatively good hamburger steak in seven to 10 hours. At the time when putrefaction became evident by the production of a foul odor, positive tests were obtained in two to five hours. Hamburger steak which does not give a positive test in six hours will keep at least three days in a good refrigerator. This is suggested as a tentative standard.

Based on results obtained the test is recommended as being efficient

in showing not only the first steps in putrefaction but also the amount of effective contamination. The test is not extremely sensitive to errors in the preparation of the sample of meat used, method of sampling, or in the reaction of the broth employed as a medium.

The hydrogen sulfid test was found to be of more value than the methylen blue reduction test because it shows incipient rather than advanced putrefaction.

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