2. An equimolecular liquid mixture of hexamethyl and hexa-ethyl stannoethane is in equilibrium with solid hexamethyl stannoethane at —3.5°. A mixture of the two compounds in boiling benzene solution gives evidence of interaction between the trimethyl- and triethylstannyl groups.

3. The mixed stannoethanes, $(CH_3)_3Sn{\cdot}Sn(C_2H_5)_3$ and $(CH_3)_3Sn{\cdot}Sn(C_6H_5)_3$, have been prepared and some of their properties studied. The compounds were prepared by two reactions in which the charges on the interacting groups were, respectively, reversed. The same compounds were formed in the two cases, but one reaction in the case of the phenyl derivative led to the formation of the pure compound and the other to a mixture of this compound with hexamethyl and hexaphenyl stannoethanes.

4. Trimethylbenzyl tin has been prepared. On brominating this compound in ether solution at liquid ammonia temperatures, the benzyl group is substituted.

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[CONTRIBUTION FROM THE PROTEIN INVESTIGATION LABORATORY, BUREAU OF CHEMISTRY, UNITED STATES DEPARTMENT OF AGRICULTURE]

THE ROLE OF THE PROTEOLYTIC ENZYMES IN THE DECOMPOSITION OF THE HERRING[1]

BY L. H. ALMY

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The immature herring, *Clupea harengus*, is canned as the sardine in Maine. According to the usual procedure, the fish are caught in weirs, transferred to the hold of the sardine boat, treated there with salt, and transported to the factory, where they are washed, held in brine for one to two hours, removed therefrom and steamed for ten or fifteen minutes, dried somewhat in a current of warm air, and packed in cans. Mustard or oil is added, and the cans are capped, sealed and processed.[2]

Ordinarily the fish decompose very little before they are canned, as the interval between their removal from the water and their steaming is comparatively short. When caught, the fish are usually in search of food; occasionally a large proportion of the herring taken from a weir have feed in their digestive tracts. Such "feedy" fish decompose rapidly, and

[1] The author wishes to acknowledge his indebtedness to the Biological Board of Canada for permission to use the facilities of the Atlantic Biological Station, St. Andrews, N. B., to Professor A. G. Huntsman, Director of the Station, and his Staff, for valuable assistance rendered in the course of the study, and to R. M. Hann of the Bureau of Chemistry, U. S. Department of Agriculture, for electrometric measurements of the hydrogen-ion concentration of buffered solutions.

[2] For details of the canning process see Weber, "The Maine Sardine Industry," *U. S. Dept. of Agriculture Bull.*, No. 908 (1921).

yield a canned product of poor quality. The ventral tissues soften quickly, and, if not already torn, the abdominal wall is ruptured during the steaming process. The appearance of such fish suggests an explosive bursting. These breaks are probably caused to a certain degree by the rapid expansion of gas which is often found in the stomach of feedy fish.

According to Obst,[3] bacteria are responsible for the decomposition of the herring which contain undigested food residues in the alimentary tract. On the other hand, the digestive tract of non-feedy fish was usually sterile. Obst isolated two organisms from the natural feed of the herring (crustacean forms, that is, copepods and schizopods) and from the stomach contents of the fish. Both of the organisms isolated were anaërobes and produced gas when grown in suitable culture media at 37°. The gas in the stomachs of feedy fish was believed to have resulted from the action of these organisms on the feed. Weber and Wilson[4] concur with Obst in the opinion that the decomposition of feedy fish is due to the action of these bacteria. They base this conclusion on their observation that ammonia and amines are formed in abundance during the decomposition of the feed and also by the action of the isolated bacteria on a dextrose and fish-flesh medium. It is apparent that the evidence submitted in favor of this view is largely circumstantial.

Feedy herring decompose much more rapidly than would be expected from the action of the relatively small numbers of bacteria present. Linden[5] believed that bacteria take only a secondary part in spoilage of this type. The present paper reports a study of the proteolytic enzymes of the herring from the standpoint of the part they may play in the deterioration of feedy fish.

Numerous studies on the enzymes of fish reported in the literature[6] show that the proteolytic enzymes of fish are more active at temperatures of 37° to 40° than at lower temperatures and that the optimum hydrogen-ion concentrations for their action appear to be about the same as those for the corresponding mammalian enzymes. Very little exact work on this phase of the subject, however, has come to the attention of the author.

The stomach of the herring consists of two sacs, the larger, the cardiac sac, extending straight back from the esophagus, and the smaller, the pyloric sac, branching off from the junction of the esophagus and cardiac sac. The posterior end of the cardiac sac communicates with the air

[3] Obst, *J. Infectious Diseases*, **24**, 158 (1919).

[4] Weber and Wilson, THIS JOURNAL, **42**, 841 (1920).

[5] Linden, unpublished report, Bureau of Chemistry, U. S. Department of Agriculture.

[6] For reviews of the literature on the enzymes of fish see Sullivan, *U. S. Bur. Fisheries Bull.*, **1908**, XXVII, 3, and Biedermann, in Winterstein's "Handbuch der vergleichenden Physiologie," Jena, **1911**, ii, p. 1049. Recent work is discussed by Kenyon, *U. S. Bur. Fisheries Bull.*, **1925**, XLI, 181.

bladder through the pneumatic duct. The lower end of the pyloric sac joins the intestine, at the head of which are attached tubular organs called pyloric ceca. The peptic enzyme is found in both the cardiac and pyloric sacs. The tryptic enzyme is found in the pyloric ceca.[7]

The investigation here reported deals in the main with the relative strengths of the proteolytic enzymes from feedy and non-feedy fish, together with the determination of the temperature coefficient of the tryptic activity, and the autolysis of the flesh of the fish under different conditions.

The digestive organs were readily comminuted by grinding in a mortar with sea sand. The extractions were made at 0° to 5° by intermittent mechanical shaking for four hours, using 10 parts of extracting liquid to one part of stomach or cecal tissue. The extracts were filtered in the cold. When water was used as the extracting fluid the filtrate was diluted with an equal volume of glycerol to act as an enzyme preservative.

Pepsin

Tests of the action of the enzyme on fibrin stained with Congo red in buffered solutions of known Sörensen value (P_H) showed that (a) water is slightly more efficacious than 30% alcohol or 85% glycerol as an extracting medium for the enzyme of the stomach, (b) digestion by the aqueous extract is greater at 37° than at 25° and greater at 25° than at 15° and (c) the aqueous extract is more potent at P_H 2.5 to 2.85 than at higher or lower hydrogen-ion concentrations.

In comparing the relative strengths of the enzymes from feedy and non-feedy fish, the organs from five fish of uniform size were combined to obtain sufficient material to work with conveniently. For the determination of the enzymic strength of the extracts gelatin was used as the substrate and the rate of hydrolysis was followed by periodic determinations of the viscosity of the solution, using the method devised by Northrop and Hussey.[8] The gelatin employed was electrolyte-free, having been prepared by the procedure recommended by Smith.[9] A 3% gelatin solution was made by soaking the air-dry gelatin in a small quantity of Sörensen's citrate buffer of P_H 1.95, melting the swollen gelatin mixture by heating for 10 minutes at 60°, and then diluting to the appropriate volume with the buffer solution. The resulting solution had a P_H of 2.65, which is in the optimum range for the action of the enzyme on fibrin. The viscosity determinations were made in ordinary 2cc. Ostwald viscosity pipets submerged in a water-bath held constant at 37.5 ± 0.05°. Five-tenths cc. of the enzyme solution was added to 25 cc. of gelatin solution, which had previously been held at 37.5° for one hour. After thorough mixing,

[7] Stirling, *2nd Annual Rept. Fish. Bd. Scotland*, **1884**, Appendix F, No. 1, 31.

[8] Northrop and Hussey, *J. Gen. Physiol.*, **5**, 353 (1923).

[9] Smith, THIS JOURNAL, **43**, 1350 (1921).

5 cc. of the mixture was transferred to the pipet and the viscosity determined immediately and at convenient intervals thereafter during one hour's digestion. Two pipets were used, with each of which the enzymic strengths of the extracts from both feedy and non-feedy fish were tested.

The stomach enzyme in the preliminary tests with fibrin was found to be only very slightly active at P_H 4.0 and practically inactive at higher P_H values. The P_H of the tissue of the abdominal wall of the herring was found by the colorimetric method, using Clark's indicators, to be between P_H 6.2 and P_H 6.6, a range outside of the effective range for the activity of the peptic enzyme. The curves in Fig. 1 show that less enzyme is extractable from the stomach of feedy fish than from the stomach of non-feedy fish, whether the stomach and contents or only the stomach tissue is extracted. The evidence is, therefore, against the supposition that the stomach enzymes are concerned in the rapid decomposition of feedy fish.

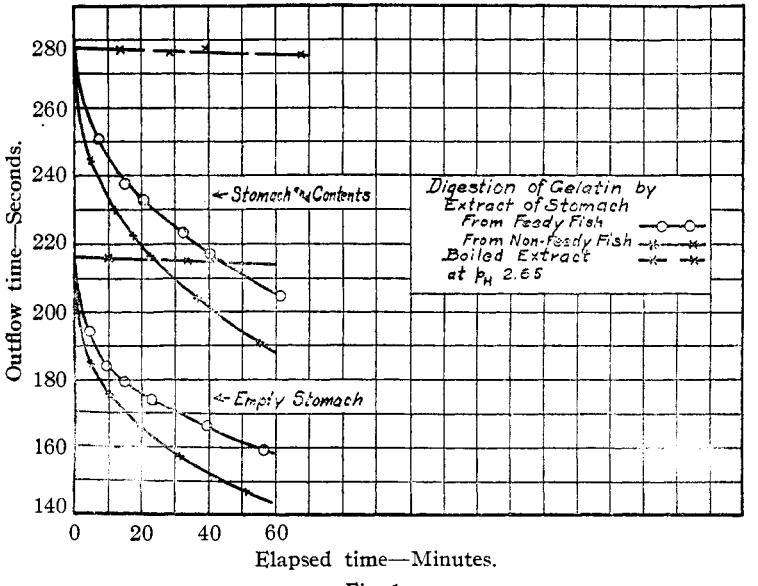

Fig. 1.

Trypsin

Again using fibrin, the aqueous extract of the pyloric ceca was found to be most active at blood heat and at approximately P_H 9.0 ± 0.5. The enzymic strength was then determined as before with gelatin as the substrate. A 3% gelatin solution was prepared as previously described, except that solution was effected with Sörensen's borate buffer to yield a final value of P_H 8.76. As the trypsin solution was much more active than the corresponding pepsin solution, it was necessary to dilute the cecal extract with four volumes of 1:1 glycerol solution before testing in

order to procure a rate curve comparable with that obtained with the stomach extract.

The tryptic enzyme in the tests with fibrin was found to be active at P_H 6.85, that is, near the range of the Sörensen value of the flesh of the abdominal wall. The action, however, was slight compared with that at the optimum hydrogen-ion concentration. As the ceca are adjacent to the ventral wall, it is reasonable to suppose that the enzyme of the pyloric ceca may play some part in the decomposition of feedy fish. Furthermore, the ceca of the feedy fish appeared to be congested and easily ruptured compared with those of the non-feedy fish. The curves in Fig. 2 show that

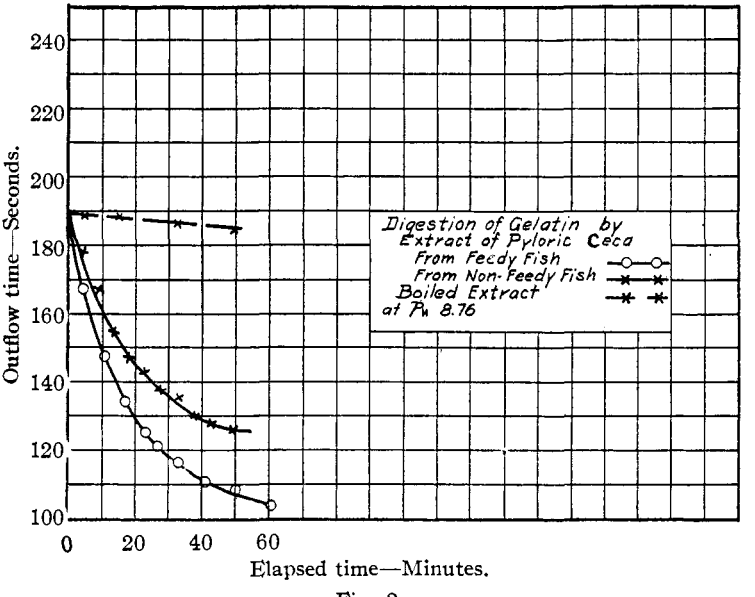

Fig. 2.

the extract of the ceca of feedy fish is more active or stronger than that of the ceca of non-feedy fish. These results were confirmed by tests on three lots of fish of both types. Considered in connection with the condition and location of the ceca, they seem to point to the enzymes of this organ as the cause for the rapid decomposition of the body wall of feedy herring.

Temperature Coefficient of Trypsin.—As the temperature of the herring seldom rises above 20° from the time they are removed from the water until they are placed in the steam chest of the cannery, it is apparent that the proteolytic enzyme of the ceca must be active at comparatively low temperatures if it is to produce any appreciable change. Working with mammalian trypsin, Ramsey[10] found that for "the tempera-

[10] Ramsey, "Temperature Coefficients of Enzyme Activity and the Heat Destruction of Trypsin," *Dissertation*, Columbia University, **1925**.

ture range where the destruction of the enzyme does not play an important part the rate of hydrolysis is about doubled for a rise of ten degrees in temperature." Using casein as the substrate and following the method essentially as outlined by Sherman and Neun,[11] the effect of temperatures on the activity of the tryptic enzyme from the herring was studied. The amino-acid nitrogen of the digested casein was determined by the Sörensen titration method, the results being expressed in terms of 0.2 *N* sodium hydroxide solution. The results (Fig. 3) indicate that for every 10°

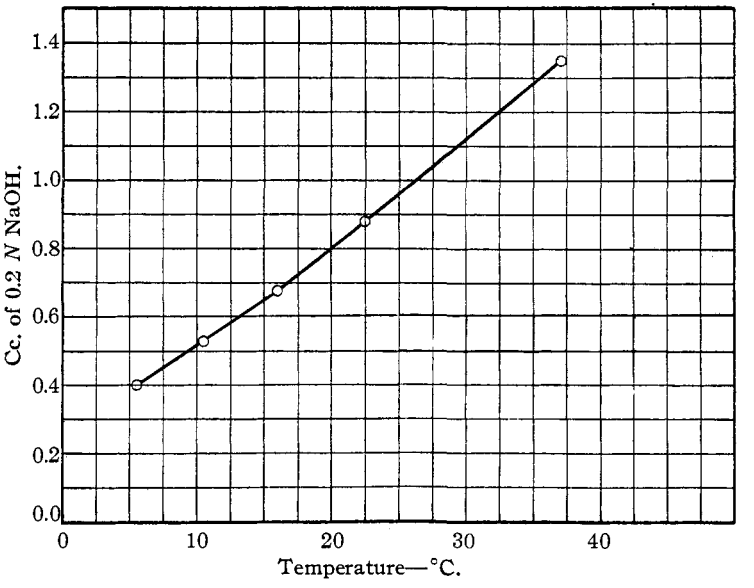

Fig. 3.—Digestion of casein by extract of pyloric ceca at different temperatures; 14 hours' incubation.

rise the activity of the enzyme is increased not more than 50%, corresponding to a temperature coefficient of 1.5. This fish enzyme, therefore, is relatively more active at the lower temperatures than the corresponding mammalian enzyme, a fact which is in harmony with the idea that the enzyme is responsible for the breaking down of the tissues of feedy fish at the comparatively low temperature range of 10° to 20°.

Autolysis[12]

According to Chen and Bradley,[13] the muscle tissue of vigorous swimming fish appears to autolyze more extensively than that of sluggish swimming

[11] Sherman and Neun, THIS JOURNAL, **38**, 2199 (1916).

[12] As ordinarily used, the term "autolysis" means digestion of a tissue by the enzymes in the cells themselves. It is here used to cover digestion of a mixture by any enzymes that may be present in one or more constituents.

[13] Chen and Bradley, *J. Biol. Chem.*, **59**, 151 (1924).

fish. If this conclusion is correct, the rate of autolysis of the flesh of the herring, which may be considered an active fish, should be comparatively high. A number of experiments were performed to determine the part played by muscle autolysis in the decomposition of feedy herring and the influence of different organs on the enzymic digestion of the muscle proteins. The method of Bradley and Taylor,[14] with slight modification, was used. The mid-section of the fish was obtained by cutting off the head just back of the gill cover and the tail at the anus. The entrails were removed, and the remaining tissues consisting of the muscle tissue, bones, skin and dorsal fin, were ground twice in a meat chopper, with and without the addition of various internal organs. Two parts of this material, together with 7 parts of water and 1 part of toluene, were placed in a pint (500 cc.) fruit jar. The mixture was thoroughly shaken and held for a few days at 37° or at room temperature. In the beginning and at intervals, 25cc. portions measured in a graduated cylinder were transferred to 100cc. volumetric flasks and treated with 50 cc. of 5% trichloro-acetic acid solution. The mixture after being shaken was diluted to the mark with water. Twenty-five cc. of the filtrate therefrom was titrated by the formol method, the results being expressed in the tables in terms of cubic centimeters of 0.2 *N* sodium hydroxide solution per 25 cc. of filtrate.

TABLE I

AUTOLYSIS OF HERRING AT 37°

Expt.	Mixture	0.2 *N* amino acids, cc. Days 0	1	2	3	7	14
I	Flesh of feedy fish	0.45	0.65	0.70	..	...	1.00
	Flesh of non-feedy fish	.45	.65	.60	..	...	1.00
II	Flesh and entrails of feedy fish	.45	5.40	6.90	7.90	...	..
	Flesh and entrails of non-feedy fish	.45	1.70	2.55	3.80	...	..
III	Flesh and entrails (except stomach) of feedy fish	.60	4.15	5.40	7.10	...	..
	Flesh and entrails (except stomach) of non-feedy fish	.50	2.05	3.40	5.25	...	..
IV	Flesh and entrails (except ceca and intestines) of feedy fish	.50	1.10	1.40	1.70	...	..
	Flesh and entrails (except ceca and intestines) of non-feedy fish	.50	0.90	1.05	1.20	...	..
V	Flesh and stomach of feedy fish	.60	.90	1.20	..	...	..
	Flesh and stomach of non-feedy fish	.50	.85	0.95	..	...	..
VI	Flesh and ceca of feedy fish	.50	3.90	5.70	..	7.4	..
	Flesh and ceca of non-feedy fish	.45	1.60	3.40	..	6.1	..
VII	Flesh and intestines of feedy fish	.55	0.75	1.00	..	...	..
	Flesh and intestines of non-feedy fish	.50	.80	1.10	..	...	..

Table I shows that marked decomposition occurred only in the mixtures containing the pyloric ceca (Expts. II, III and VI). Similar results were obtained when the mixtures were incubated at 17° to 18°. The autolysis

[14] Bradley and Taylor, *J. Biol. Chem.*, **25**, 261 (1916).

of the muscle tissue (Expt. I) plays a comparatively insignificant part in the breaking down of the muscle proteins, and the stomach and intestines (Expts. V and VII) are likewise relatively inactive. It is apparent that the ceca of feedy fish produce a much greater change than those of the non-feedy fish.

When the crustacea are plentiful the herring gorges itself with food and the cardiac sac of the stomach of feedy fish is usually distended to several times its size when empty. The resultant crowding of the other organs causes a pressure of the ceca against the abdominal wall. The fish in the lower layers in the holds of the sardine boats and in the pickling vats in the cannery are subjected to the pressure of the upper layers. The external and internal pressure and numerous handlings, coupled with the fact that the ceca of feedy fish are congested and readily ruptured, offer a condition favorable to the diffusion of the tryptic enzyme from the ceca into the tissues of the body wall. That diffusion actually occurs was demonstrated by experiment. Feedy and non-feedy fish were held for eight hours at approximately 20°. The abdominal wall was then excised and carefully cleaned to remove any adhering organ tissue. When allowed to autolyze as in the previous experiments, the results, similarly expressed, for 0, 1 and 3-day incubation at 37°, were as follows: feedy fish, 0.6 cc., 1.60 cc. and 3.90 cc.; non-feedy fish, 0.50 cc., 0.60 cc. and 0.90 cc. Enough of the enzyme had penetrated the tissues of the feedy fish to cause a rapid hydrolysis of the proteins.

As the alimentary tract of feedy herring contains bacteria and that of the non-feedy fish is usually sterile,[3] it might be conjectured that bacteria as well as enzymes would play a part in the decomposition of the feedy fish. The experiments reported in Table II show that during incubation for eight hours at 37° bacteria are apparently not concerned in the decomposition of non-feedy fish, but do assist in the decomposition of feedy fish. That putrefactive bacteria are present in the feed of the herring is shown

TABLE II

DECOMPOSITION OF HERRING AT 37° WITH AND WITHOUT TOLUENE AS PRESERVATIVE

Expt.	Mixture	0.2 *N* amino acids, cc. Hours 0	5	8	24	48
VIII	Flesh and entrails of feedy fish, no preservative	0.50	3.5	5.0	...	..
	Flesh and entrails of feedy fish, with toluene	.50	2.75	3.75	...	..
IX	Flesh and entrails of non-feedy fish, no preservative	.40	0.80	1.00	...	..
	Flesh and entrails of non-feedy fish, with toluene	.40	.70	0.90	...	..
X	Flesh of non-feedy fish and copepods, no preservative	.55	.80	..	3.2	9.80
	Flesh of non-feedy fish and copepods, with toluene	.55	.65	..	1.05	1.50

in Expt. X. For this test live copepods amounting to a volume of 4 cc. were killed by grinding in a mortar with sand, and the finely divided material was added to the usual autolysis mixture containing 50 g. of flesh.

Discussion

It has been shown that the proteolytic enzyme of the stomach is weaker and that of the pyloric ceca is stronger in feedy than in non-feedy fish. A plausible explanation of the former condition is that part of the peptic enzyme has been fixed by the food present and part has been eliminated through the intestine, leaving only a fractional part of that which was originally stored. Apparently the enzyme is formed more slowly than it is eliminated from the field of action.

In view of the large quantity of trypsin demonstrable in the pyloric ceca, it may reasonably be assumed that this organ secretes the enzyme. Massed copepods or schizopods appear to be practically white except for the black eyes of the schizopods. During digestion in the alimentary tract of the herring, however, the mass becomes pink or red, a change probably related to the presence of chitinous tissue. It was noted that the ceca of non-feedy fish were pale and contracted, whereas those of feedy fish were of the same color as the partially digested crustacea and somewhat swollen. It is judged from this that some at least of the liquid food residues leaving the stomach enter the ceca, undergo further digestion and are probably absorbed there. The appearance of the pyloric ceca of feedy king salmon as described by Greene[15] agrees with that observed by the author in the herring. Greene believed that the ceca are the principal organs of absorption of fats in the king salmon.

The tryptic enzyme is probably being formed and actively secreted as long as food residues are present, and the rate of formation would have to be greater than the destruction or elimination to account for the difference in proteolytic activity of the cecal extract of feedy and non-feedy fish. Other factors may enter into this, however. The presence of food in the stomach, before and after it has reached the ceca, may induce the formation and secretion of the enzyme in the ceca, thus building up a supply which, during subsequent digestion, is never depleted to the predigestion level. Furthermore, the mineral salts in the food, particularly the calcium salts, may increase the activity of the trypsin. The catalytic effect of calcium salts on the activity of the cecal extract of non-feedy fish was not studied.

The rapid decomposition of feedy herring has been shown to be largely enzymic. The data also show, however, that bacteria may play a part. Micro-organisms are present not only in the digestive tract of feedy fish, but also on the gills. The fecal material eliminated by the fish voluntarily or mechanically contains large numbers of bacteria. It can readily

[15] Greene, *Bur. Fisheries Bull.*, **33**, 153 (1913).

be seen that there is the possibility that practically all of a lot of mixed feedy and non-feedy fish piled in the hold of a sardine boat would become infected through the exterior surface.

In reference to the possibility of the penetration of bacteria into the muscular tissue of the abdominal wall from the intestine, perhaps one could not do better than to quote Anderson:[16] "One frequently finds, especially in dealing with herring or cod, some whose stomachs were evidently packed with crustaceans or small fish at time of capture, and in these cases digestion and solution of the wall of gut may take place in a few hours, whereas if the gut is comparatively empty the digestion may be considerably delayed. This certainly takes place very rapidly in herring in the above condition..... But when one has due regard to the rapidity in many cases with which solution of the gut wall takes place, it appears to be at least initiated by *post-mortem* digestion, although this process may be accompanied by, and is certainly soon superseded by, the action of bacteria of putrefaction which abounds in the gut..... I have frequently found *bacillus coli* (in the peritoneal fluid[17]) in about 45 to 60 minutes after death, and in a very few cases even 30 minutes after death. After one hour they will be found readily in greatly increasing numbers." The rapid disintegration of the tissues of the ventral wall of the immature feedy herring resulting from the action of the enzyme of the pyloric ceca makes possible the ready penetration of the bacteria from the peritoneal cavity. It is apparent, therefore, that under the conditions obtaining in the sardine industry when feedy fish are used the flesh is subject to infection by bacteria which may gain entrance through both the outer integument and the peritoneum. The visible evidence of decomposition—the softening and bursting of the abdominal wall—is, however, practically wholly an enzymic manifestation.

The gas often found in the posterior end of the cardiac stomach of feedy fish, the rapid expansion of which during the steaming process appears to be the cause of the bursting of the stomach and abdominal walls, is believed by Obst[3] (but not proved) to have its origin in the action of gas-producing anaërobic bacteria present in the feed. Attention should be called, however, to the fact that this sac communicates with the air bladder through the pneumatic duct and it is not impossible that, owing to the paroxysms accompanying death by asphyxiation and also perhaps to pressure on the air bladder during subsequent handling, gas from the bladder gains entrance to the stomach and becomes locked therein. Consideration of this possibility raises a reasonable doubt as to the correctness of the theory that the gas results wholly from the action of bacteria.

[16] Anderson, *26th Annual Rept. Fish. Bd. Scotland*, **1907**, Part III, Sci. Investigations, 13.

[17] Inserted by the author.

Summary

A biochemical investigation on the immature herring (used in sardine packing) to determine the cause of the decomposition of feedy fish, which makes them unacceptable for food purposes within a few hours, showed the following points.

1. The pepsin extracted with water from the stomach of the fish is more active at 37° than at lower temperatures and at hydrogen-ion concentrations between P_H 2.5 and P_H 2.85 than at higher or lower values. It is comparatively inactive above P_H 4.0. Less pepsin is extractable from the stomach of feedy fish than from the stomach of non-feedy fish.

2. The trypsin extracted with water from the pyloric ceca is more active at blood heat than at lower temperatures and between P_H 8.5 and 9.5 than at higher or lower values. It acts slowly, however, at P_H 6.85. Decidedly more trypsin is extractable from the ceca of feedy fish than from the ceca of non-feedy fish.

3. In mixtures of ground flesh and digestive organs, enzymic digestion is rapid and is attributable mainly to the presence of the pyloric ceca. The stomach and intestines, as well as muscle autolysis, play only an insignificant part in the breaking down of the proteins.

4. The flesh of feedy fish is invaded by bacteria and by the trypsin of the pyloric ceca, but the visible evidence of decomposition—the softening of the abdominal wall—is due almost solely to the action of the trypsin, which is greater in amount or more active than that in the ceca of non-feedy fish, and which readily escapes from the delicate and highly congested tubules, quickly penetrating to the adjacent tissues of the ventral wall.

WASHINGTON, D. C.

[CONTRIBUTION FROM THE HAVEMEYER CHEMICAL LABORATORY, NEW YORK UNIVERSITY]

THE BASIS FOR THE PHYSIOLOGICAL ACTIVITY OF CERTAIN -ONIUM COMPOUNDS. V. THE MOBILITIES OF THE -ONIUM IONS. II[1]

BY ISAAC BENCOWITZ[2] WITH R. R. RENSHAW

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The problem of determining which properties of the -onium compounds are responsible for their physiological effects has been discussed in some detail elsewhere. It was there pointed out that evidence exists for believing that some sort of an electrical effect[3] is involved. It seems de-

[1] This problem is being carried on in coöperation with Dr. Reid Hunt of the Harvard Medical School. The physiological data are the basis of a series of papers published elsewhere by him.

[2] National Research Fellow in Chemistry.

[3] Renshaw, *Science*, **62**, 384 (1925).