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DECOMPOSITION OF FREE AND COMBINED CYSTINE WITH SPECIAL REFERENCE TO CERTAIN EFFECTS PRODUCED BY HEATING FISH FLESH

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Other investigators have studied the effect of acid and alkali hydrolysis of proteins upon the combined cystine, as shown in a review of the subject by Hoffman and Gortner.[1] The experimental evidence on the extent of the breaking down of cystine during acid hydrolysis is somewhat divided, but the destructive effect of alkali hydrolysis is generally accepted. The variability of the results obtained by different investigators is due to the lack of uniformity in experimental conditions. The method employed in the study here reported differs decidedly from those generally used. The results, therefore, may not be compared strictly with those of others. Briefly, the procedure consists in heating flesh in a sealed tube with the addition of little or no water or aqueous solutions. This method of heating was decided upon since it was desired to use a procedure which would imitate to a degree the conditions existing in a can of food material during the sterilization process. The purpose of the study was to gain sufficient information on the changes which take place during the heating of proteinaceous food products to afford a basis for the interpretation of the hydrogen sulfide content of the canned product.

Experimental Procedure

The flesh from fresh marine fish of various species was ground thrice to make a homogeneous sample. Twenty grams of the flesh, with or without the addition of other substances, was placed compactly in a 20 × 150 mm. test-tube of soft glass. The tube was sealed in the blast lamp and heated in a constant-temperature bath for 45 minutes. It was then opened and the heated product examined. Because of the nature of the tests, but one determination could be made on the contents of each tube.

For the determination of hydrogen sulfide, the tube was inverted in a beaker containing about 25 cc. of 0.6% zinc acetate solution and the tip of the tube was broken off beneath the surface of the liquid. The beaker, with the tube, was placed in a vacuum desiccator, the evacuation of which drew the gas in the tube through the zinc acetate solution, thus trapping any hydrogen sulfide contained in the air space above the flesh and rendering possible the quantitative determination of the hydrogen sulfide remaining after the heating. The tube was then carefully broken in the center. The contents were ground thoroughly in a mortar and finally washed into a cylinder for the determination of hydrogen sulfide by the methylene blue method.[2] This method estimates both the gas which is combined as sulfide and that which exists free in the flesh. The term "hydrogen sulfide" as used throughout this paper includes both forms.

For the estimation of cystine, the tube was opened, the flesh ground in a mortar

[1] Hoffman and Gortner, THIS JOURNAL, **44**, 341–360 (1922).

[2] Almy, *ibid.*, **47**, 1381–1390 (1925).

with about 50 cc. of water, the protein precipitated by the tungstic acid method of Folin and Wu,[3] and the mixture diluted to 100 cc. with distilled water. The protein-free filtrate therefrom was then analyzed for cystine by Sullivan's method.[4]

Hydrogen-ion determinations were made either electrometrically[5] by means of the hydrogen electrode, or colorimetrically, and sometimes by both methods, on the filtrate from 25% aqueous suspensions of the finely divided material.

For certain of the tests cystine dissolved in *N* hydrochloric acid solution was added to the flesh before the heating. The cystine solution was varied in concentration, but was always used in the proportion of 2 cc. to 50 grams of flesh. Two cc. of *N* sodium hydroxide solution was then added to neutralize the hydrochloric acid. Cysteine was likewise added in *N* hydrochloric acid solution, the hydrochloric acid of the cysteine hydrochloride having first been neutralized with dilute sodium hydroxide solution. Control samples were similarly treated with equivalent quantities of *N* hydrochloric acid and *N* sodium hydroxide solution.

Experimental Results

Effect of Heating on the P_H of the Product.—As variations in the H-ion concentration may cause differences in the results, it was necessary to determine the extent of the change in reaction produced by the heating in soft glass. It was found that the P_H of the material before and after heating seldom differed by more than 0.2 of a Sørensen unit. This observation is in general harmony with that of Esty and Cathcart,[6] who found that soft glass tubes affected the H-ion concentration of vegetable juices heated therein less than the hard glass tubes.

Effect of Heat on Fresh and Stale Flesh

The flesh of strictly fresh fish seldom contains hydrogen sulfide. Of twelve species examined by the methylene blue method, only one gave a measurable quantity of the gas. As the flesh becomes stale, it may or may not give a positive test. The amount of hydrogen sulfide obtained on heating the flesh in a sealed tube, however, increased with the length of time the product was held prior to the heating (Table I). Although the P_H shifts from the acid toward the alkaline side, it does not seem to be a requisite that the flesh be neutral or alkaline to yield a considerable quan-

TABLE I

HYDROGEN SULFIDE LIBERATED FROM FRESH AND STALE FLESH ON HEATING FOR 45 MINUTES AT 120 °C.

			After holding at room temperature					
	Fresh		24 hours		48 hours		72 hours	
Fish	H_2S, mmg. per 100 g.	P_H	H_2S, mmg. per 100 g.	P_H	H_2S, mmg. per 100 g.	P_H	H_2S, mmg. per 100 g.	P_H
Weakfish, *Cyanoscion regalis*	33	6.5	3296	7.0	3859	7.1	..	7.3
Rockfish, *Roccus lineatus*	34	6.0	59	6.3	1809	6.5	2922	6.6

[3] Folin and Wu, *J. Biol. Chem.*, **38**, 81 (1919).

[4] Sullivan, *Public Health Reports*, **41**, 1030–1056 (1926).

[5] Thanks are due to Mr. H. C. Waterman of the Food Control Laboratory of the Bureau of Chemistry for the H-ion measurements made in connection with this study.

[6] Esty and Cathcart, *J. Infectious Diseases*, **29**, 29–39 (1921).

tity of the gas. None of these samples of flesh before heating gave a test for hydrogen sulfide except the rockfish sample at the 72-hour period, the amount being only 13.4 mmg. (micromilligrams) per 100 g.

Effect of Added Cystine and Cysteine on Hydrogen Sulfide Produced by Heating Flesh

In an effort to determine what part cystine or cysteine plays in the production of hydrogen sulfide on heating the flesh, the following experiment was made.

Twelve 40 g. portions of rockfish flesh were thoroughly mixed with varying quantities of *N* hydrochloric acid and *N* sodium hydroxide solution to give samples having Sørensen values ranging from *P*H 5.0 to *P*H 8.4. There were thus obtained four groups of samples of three each, each group having a different Sørensen value. To one of the samples of each group was added 5 mg. of cystine and to another 5 mg. of cysteine, the remaining sample being used as a control. The dilution of each sample with liquid was made the same by appropriate additions of water. The total dilution amounted to but 3 cc. for each 20g. sample. The samples were heated in sealed tubes for 45 minutes at 120° and then analyzed for hydrogen sulfide. The test was made on fresh flesh and on flesh which had decomposed to some extent during 24 hours' standing at room temperature. The results are shown in Table II.

TABLE II

ROCKFISH FLESH (20 G.) HEATED WITH AND WITHOUT ADDED CYSTINE OR CYSTEINE

Material	Fresh		After standing at room temperature for 24 hrs.	
	*P*H before heating	H_2S after heating, mmg.	*P*H before heating	H_2S after heating, mmg.
Flesh alone	5.0	5	5.6	106
Flesh + 5 mg. cystine	5.0	0	5.6	204
Flesh + 5 mg. cysteine	5.0	0	5.6	231
Flesh alone	5.6	10	6.6	342
Flesh + 5 mg. cystine	5.6	0	6.6	544
Flesh + 5 mg. cysteine	5.6	0	6.6	560
Flesh alone	7.0	0	7.6	751
Flesh + 5 mg. cystine	6.8	0	7.6	1064
Flesh + 5 mg. cysteine	6.8	0	7.6	829
Flesh alone	7.8	0	8.4	1728
Flesh + 5 mg. cystine	7.8	0	8.4	2293
Flesh + 5 mg. cysteine	7.8	5	8.4	2137

Little, if any, hydrogen sulfide could be detected in the fresh samples after heating, regardless of the H-ion concentration. In the stale samples the amount of gas produced was greater the higher the *P*H and also greater in the samples containing cystine or cysteine than in the flesh alone. In endeavoring to reach a satisfactory explanation of these results, the

author considered two possibilities. One is that neither cystine nor cysteine under these conditions decomposes to give hydrogen sulfide, the gas obtained having been derived from a thermolabile decomposition product of cystine and cysteine. The other is that these amino acids are decomposed by the heat with the direct production of hydrogen sulfide, the gas, however, being partially or wholly destroyed only in the case of the fresh flesh.

The following experiments were performed for the purpose of testing the validity of these explanations.

Decomposition of Cystine

If cystine, during its contact with the stale flesh, is changed to a thermolabile product, the longer the contact the greater the change. Accordingly, cystine was allowed to remain admixed with stale rockfish flesh for different periods before heating. The shortest period used was the minimum time required to insert the flesh in a tube, seal the tube, and place it in a hot calcium chloride brine bath. All samples were heated at 120° for 45 minutes. The results (Table III) show that the duration of contact of the cystine with the stale flesh had little influence, if any, on the amount of hydrogen sulfide produced. This tends to disprove the first hypothesis.

TABLE III

INFLUENCE OF DURATION OF CONTACT OF CYSTINE WITH SPOILED ROCKFISH FLESH (20 G.) ON THE AMOUNT OF HYDROGEN SULFIDE PRODUCED DURING HEATING OF THE MIXTURE FOR 45 MINUTES AT 120°

Material	Duration of contact, min.	Hydrogen sulfide, mmg.
Flesh alone	..	654
Flesh + 4.8 mg. cystine	3	825
Flesh + 4.8 mg. cystine	20	801
Flesh + 4.8 mg. cystine	40	836

Several experiments, the details of which it will not be necessary to describe, showed that when fresh flesh was heated, any hydrogen sulfide which may have been present was practically wholly destroyed, probably by oxidation. When stale flesh, which alone yielded large quantities of hydrogen sulfide when heated, was mixed with fresh flesh and then heated for 45 minutes at 120°, analysis revealed little or no hydrogen sulfide. Likewise, when hydrogen sulfide water was added to fresh flesh, none of the gas could be detected after heating the mixture. The gas was not lost in this way, however, when added to stale flesh.

Tillmans and Otto[7] suggest three methods of detecting incipient decomposition of fish, all of which are based upon the reducing power of the flesh. The most delicate is an adaptation of Winkler's method[8] for the

[7] Tillmans and Otto, *Unters. Nahr. u. Genussm.*, **47**, 25–37 (1924).

[8] Winkler, *Ber.*, **21**, 2843 (1888); **22**, 1764 (1889); **24**, 3602 (1891).

determination of dissolved oxygen in water. Five grams of ground flesh is placed in a Winkler bottle, distilled water is added to overflowing, the stopper is inserted, and the bottle is agitated to give a fine suspension. After incubation for one or two hours at 22°, the Winkler reagents are added and the determination of dissolved oxygen is completed. If no oxygen is present under these conditions, according to Tillmans and Otto, the product is unfit for human consumption.

That the reducing power of the flesh is quickly built up while the product is held at room temperature was shown in tests conducted by the author. The titration figures for fish flesh so held changed from about 1.0 cc. of 0.1 *N* sodium thiosulfate solution to practically nil in 18 hours. The possibility of the destruction of hydrogen sulfide by oxidation by the flesh before it has reached the reducing stage is thus explained.

The data presented thus far seem to point to the destruction of part at least of the cystine during the heating of either the fresh or the stale flesh. If heat will destroy the cystine, the destruction should be a function of the temperature. Using Sullivan's method,[4] cystine was determined in flesh which had been heated at 100 and at 120° and to which cystine had been added prior to heating. The results showed an apparent destruction of the cystine, for the mixture (20 g. of flesh + 15 mg. of cystine) at 100° gave 18 mg. of cystine, whereas that at 120° gave only 5 mg. The flesh without cystine gave about 2 mg. of cystine after heating at 120°.

Such results, however, might be explained as being due to a combination of the cystine with other constituents of the flesh, the combining power of cystine being generally recognized. To settle this point, the cystine-flesh mixture, after heating, was hydrolyzed with 20% hydrochloric acid for six hours.[9] Following the procedure recommended by Sullivan[10] for the determination of cystine in casein, the hydrolysate was filtered, decolorized with Norit, neutralized and tested for cystine. The amount of cystine in 20 g. of the mixture before heating was 128 mg. After heating there were but 61 mg. Since only 15 mg. of cystine had been added, it is evident that some of the original combined cystine of the flesh was also destroyed by the heating. This was checked by heating flesh to which no cystine had been added. The cystine content of the hydrolysate decreased as a result of the heating from 107 mg. to 82 mg. There seems to be little doubt, therefore, that some of the cystine is destroyed during the heating of the flesh.

Summary and Conclusions

When fresh fish flesh was heated in a sealed tube at 120° for 45 minutes, either no hydrogen sulfide or only a comparatively small amount could

[9] Sullivan, unpublished data.

[10] The author acknowledges his indebtedness to Dr. M. X. Sullivan for his helpful suggestions in connection with this study.

be detected in the heated product. When the flesh had become somewhat stale, the same heat treatment yielded the gas in relatively large amounts. Added cystine increased the amount of residual hydrogen sulfide only in the case of the stale flesh. The cause of this difference in results with fresh and stale flesh was traced to the ability of the fresh flesh, and, conversely, the inability of the stale flesh, to destroy hydrogen sulfide formed by the heating. The gas is destroyed apparently by oxidation. Cystine added to fresh flesh, as well as that present in combined form in the flesh, is partially destroyed by this heating.

On the plausible assumption that the effect of processing a can of fish is the same as the effect of the above-mentioned heat treatment in a sealed tube, it may be concluded that the presence of considerable hydrogen sulfide in a canned product of this nature indicates that the raw material at the time of canning was in poor condition.

It is recognized that sulfur compounds other than cystine may exist in the protein or other constituents of the flesh examined, but the possibility of their existence therein need not be considered in connection with this study. While these results were obtained with fish flesh, which procedure had the advantage that the raw material could be obtained in fresh condition at any time desired with little inconvenience, it is probable that similar results would be obtained with other flesh products. Therefore, the conclusion seems justified that when flesh products in general are heated at temperatures above 100° some of the cystine is destroyed and hydrogen sulfide derived therefrom may be detected in the product, providing the gas is not oxidized by the material during the heating process.

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THE BECKMANN REARRANGEMENT OF SALICYLHYDROXAMIC ACID DERIVATIVES[1]

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Several benzene derivatives containing an hydroxyl group and a nitrogenous group in the ortho position to each other can be converted into oxycarbanil by suitable treatment.[2] Especially in the cases of urea and urethan derivatives is there the possibility of the intermediate formation

[1] This paper is based upon a thesis presented by Mr. J. H. Mote to the Graduate School of the University of Georgia, in partial fulfilment of the requirements for the degree of Master of Science.

[2] (a) Groenvik, *Bull. soc. chim.*, **25**, 177 (1876); (b) Kalckhoff, *Ber.*, **16**, 1828 (1883); (c) Leuckart, *J. prakt. Chem.*, [2] **41**, 327 (1890); (d) Bender, *Ber.*, **19**, 2269, 2951 (1886); (e) Sandmeyer, *Ber.*, **19**, 2656 (1886); (f) Chetmicki, *Ber.*, **20**, 177 (1887); (g) Jacoby, *J. prakt. Chem.*, [2] **37**, 29 (1888); (h) Lellmann and Bonhöffer, *Ber.*, **20**, 2126 (1888).