Chemical Studies on Freshness of Whale Meat. IV.

Some Informations of *Achromobacter ubiquitum* isolated from Whale Carcass

By
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(This was reported in Japanese on pp. 152–156, Vol. 71, Journal of the Pharmaceutical Society of Japan (1951))

Prof. Dr. T. Akiba and his co-operators[1] isolated various kinds of bacteria from sperm whale carcass low in freshness, among which many strains of *Achromobacter ubiquitum* were isolated from any of muscle, blood and intestines. In order to study what part this bacterium played in the decrease of freshness in the whale carcass, I took over a strain of this bacterium from him and investigated the decomposing state of the extract of whale meat by it. Then I searched the acids produced by the decomposition of glucose by this bacterium. Some informations obtained are reported hereon.

Experiment and Study

I. Decomposition of the extract of whale meat

Achromobacter ubiquitum (abbreviated to A. u. bacterium) was inoculated on the extract of whale meat and the decomposing process of the extract was traced by determination of volatile basic nitrogen with the lapse of time.

Preparation of the extract of whale meat:—The refrigerated meat of a whale (*Balaenoptera*) was minced, to 500 g of which 1 *l* of physiological salt solution was added and after 3 hours' standing under occasional stirrings, it was heated in the steam-kettle for 1 hour and filtered. 1 *l* of the filtrate was sterilized for 1 hour. (Per 100 cc of the extract, total nitrogen was 237.4 mg, non-protein nitrogen 226 mg and volatile basic nitrogen 10.2 mg.)

To the extract obtained in such a way, 0.1 cc of suspension of one platin loopful of A. u. bacterium for 5 cc of physiological salt solution was added and incubated at 26~28°C.

Determination of volatile basic nitrogen:—It was determined in aeration method. To 5 cc of cultural fluid 0.5 cc of saturated K_2CO_3 solution, 0.1 g of NaF and 5 drops of octyl alcohol were added, where to

air washed through dilute sulfuric acid was sent so violently that the volatile base was expelled into 0.02 n H_2SO_4. Then the surplus acid was titrated back with 0.02 n NaOH.

The result is shown in Fig. 1. For the first 250 hours, the amount of volatile basic nitrongen (abbreviated to V-N) showed little increase. After that, about till the 750th hour, it kept the steady increase and then decreased suddenly. After the decrease from 5.9 to 5.5 in pH, increasing again, it was 6.5 instantly before V-N showed the clear increase. Perhaps the first decrease in pH is due to the acid brought from the small amount of saccharide contained in the extract by the acid productivity of A. u. bacterium. Inspite of no increase of V-N amount, the value of pH increased in the period about from 60th to 200th hour. This probably shows the formation of non-volatile amine. Clearly the generation of gas was recognized after 24 hours of inoculation. The cultural fluid smelled rather sourish and of its own, not bad.

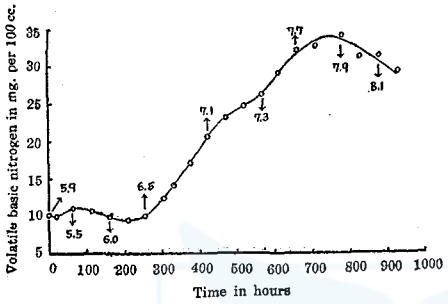


Fig. 1. Decomposition of whale meat extract. Figures marked with ↑ mean the value of pH of the solution at that time.

II. Comparison of decomposition between suspension and extract of whale meat

The state that whale meat was being eroded by A. u. bacterium was inferred from the decomposing state of whale meat suspension by this bacterium, and at the same time it was compared with that of the extract. The whale meat used was of the same kind as I.

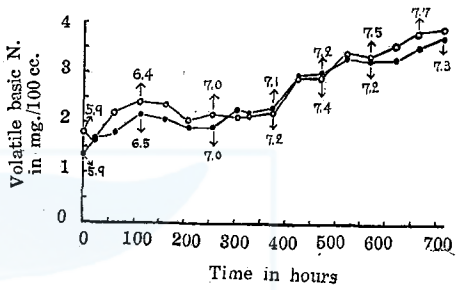
Preparation of whale meat suspension:—50 g of whale meat minced with the meat-chopper was heated in the steam-kettle for 30 minutes and brayed in the mortar. To it, 1 *l* of physiological salt solution was added and after 1 hour's heating in the steam-kettle, its supernatant was put in another vessel. Its residue was fully brayed again and sterilized for 1 hour after dispersing into the supernatant. Total nitrogen per 100 cc was 171.1 mg.

Preparation of the extract:—60 g of the above-mentioned whale meat was heated in the steam-kettle for 30 minutes, and brayed in the mortar. To it, 1.2 *l* of physiological salt solution was added. After

2 hours' heating in the steam-kettle and filtering, 1050 cc of the filtrate was sterilized for 1 hour. Total nitrogen per 100 cc was 36.3 mg.

5 drops of the above A. u. bacterium suspension were added to thus made suspension and extract respectively, which were kept at 27°C. The amount of V-N was measured by the aeration method, with 20 cc of each liquid.

The result is shown in Fig. 2. Fluctuation of V-N amount of the suspension agrees nearly quite with that of the extract. This is, it is not influenced by the existing amount of muscle protein. This fact can be easily understood from the known fact that A. u. bacterium has no gelatine decomposing power. Therefore, on whale meat eroded by A. u. bacterium, not muscle protein but mainly non-protein matters will be decomposed. The change of pH in both liquors agreed about till 300th hour (pH 7.1) and after that with an increase of the amount of V-N, these liquors showed the different value in pH and its difference became larger and larger. And the then value of pH was smaller in the suspension than in the extract. Probably this is due to protein's buffer action.



o—Extract •—Suspension of whale meat

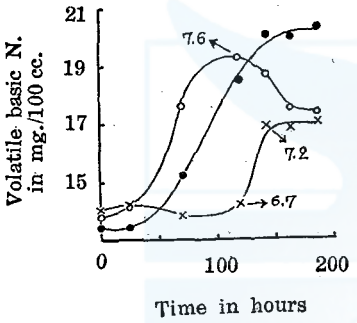
Fig. 2.

III. Effect of the initial value of pH upon volatile base formation

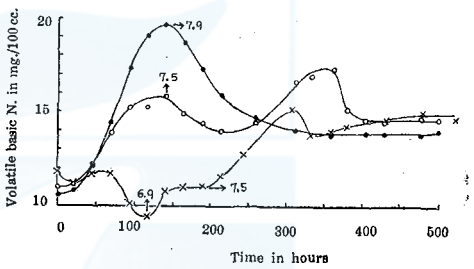
From the above experiments and the general fact that optimum pH for deaminase of bacteria is alkaline, I imagined this bacterium deaminated substrate in neutral and alkaline, but scarcely about pH 6. Therefore, if pH of the extract in the beginning of the experiment was neutral or alkaline, this bacterium would deaminate the substrate at once and the amount of V-N would show a clear increase from the beginning, I imagined. So inoculating A. u. bacterium on the different extracts in pH, I examined the change of the amount of V-N. The whale meat used for the experiment was of the same species as in the above one.

1.6 *l* of physiological salt solution was added to 800 g of minced whale meat and after 3 hours' standing under occasional stirring, boiled

for 10 minutes and filtered. After steam-sterilizing the filtrate for 1 hour, there was inoculated a platin-loopful of A. u. bacterium and kept standing for one night at 27°C. Thus, fermentable matter (carbohydrate) in the extract was consumed, to preventing as much as possible from decreasing pH instantly after the commencement of the experiment as in experiment I and II. And through the layer of kieselguhr, bacteria were sucked off. Each 480 cc of the filtrate was made at 6, 7 and 8 in pH and after 30 minutes' heating in the steam-kettle, changed pH was adjusted with NaOH and added physiological salt solution made its amount 500 cc. It was put in a 1 *l* Erlenmeyer's flask and sterilized for 30 minutes every day for the following three



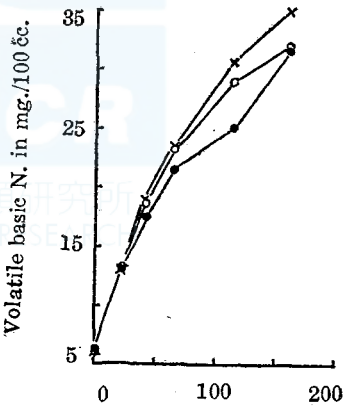
Initial value of pH in the extract:
×—6, ○—7, ·—8
Fig. 3.



Initial vulue of pH in the extract:
×—6.1, ○—6.9 ·—7.9
Fig. 4.

days. To each of them, 0.1 cc of the above mentioned suspension of bacteria was added and kept at 27°~28°C. Often 10 cc of this solution was taken out aseptically to measure the amount of V-N.

The experiment was carried out twice and its result is shown in Figs. 3 and 4. In the extracts of 7, 6.9 and 8, 7.9 in pH at the beginning of the experiment, the amount of V-N increased from the outset but in the extracts of 6, 6.1 in pH it did not increase in the early stage of the experiment with even a decrease until towards 7 in pH. This result seemed to coincide fully to my expectation before the experiment. But in case of peptone



Decomposition of pepton salt solution. Intial pH:
×—4.9 ○—7.1 ·—8.1
Fig. 5.

solution (1% of Polypepton "Takeda", 0.5% of NaCl) used as cultural medium, in any of 5.9, 7.1 and 8.1 in the initial pH, the amount of V-N increased from the outset and the increase of the amount of V-N at 5.9 in pH was the most till 162nd hour (Fig. 5). Therefore, we can consider that peptone contains some substance that can give the volatile base by A. u. bacterium's action even at 6 in pH but the extract of whale meat does not contain it.

In the extract of whale meat, at a certain stage the amount of V-N decreased in sudden with state of equilibrium in conclusion. In this state the amount of V-N was nearly same, with no relation to pH in the beginning of the experiment (Fig. 4).

IV. Decomposition of autolyzate of whale meat

In the decreasing process of freshness of whale carcass, prior to or at the same time with the decomposition by bacteria, autolysis of muscle takes place and intermediary products of protein decomposed increase gradually and the amount of the extractive matter also. To find out the relation between it and the A. u. bacterium's action, the extracts of autolyzed and normal whale meat were made and their decomposing state by A. u. bacterium was compared.

The preparation of the extract of normal whale meat (control solution):—To 500 g of minced sperm whale meat, 1 *l* of physiological salt solution was added and after 3 hours' standing, it was boiled for 10 minutes. The filtrate was sterilized for 1 hour and a platin-loopful of A. u. bacterium was inoculated on it and kept at 28°C for a night. Thus, after consuming the acid-fermentable matters, the solution was sucked through the kieselguhr layer and the filtrate was neutralized with NaOH, of which 500 cc was put in a 1 *l* Erlenmeyer's flask and sterilized for 30 minutes every day for the following 3 days. Total nitrogen was 117 mg per 100 cc.

Preparation of the solution of autolyzate:—To 500 g of minced meat of sperm whale, 1 *l* of physiological salt solution saturated with chloroform and 5 g of chloroform were added and kept at 27°~28°C for 5 days. Then, after 10 minutes' boiling, the filtrate was treated as mentioned above. The total nitrogen was 190.3 mg per 100 cc.

0.1 cc of A. u. bacterium suspension was added to thus obtained two kinds of solutions and kept at 27°~28°C. The result was shown in Fig. 6.

The amount of V-N was always more in the solution of autolyzate than in the control solution but both solutions showed nearly same

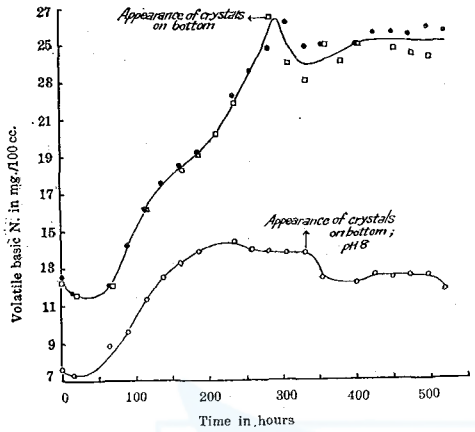


Fig. 6. Comparison of the decomposing process of the extract between autolyzed whale meat and normal whale meat.
•, □—Autolyzed, ○—Normal

changing state till about 200th hour. However, after the 200th hour, the control solution showed little increase of the amount of V-N and then gradual decrease, but the solution of autolyzate showed the remarkable increase again. Can't we interprete this phenomenon as follows? In the solution of autolyzate, kinds of substances decomposed by A. u. bacterium were different between till and after about the 200th hour ("The substance decomposed by A. u. bacterium" is limited to the substance forming the volatile base by the action of A. u. bacterium, in this case). Till about 200th hour, the substance decomposed by A. u. bacterium was of same kind in both solutions and consumed almost all by this time. After this time, the control solution had no substance decomposed by A. u. bacterium, so the amount of V-N did not increase. In the solution of autolyzate, A. u. bacterium found another kind of substance to be newly utilized in the product of autolysis and began to decompose actively and the amount of V-N increased again.

As in the preceding experiments, both solutions showed the sudden decrease of the amount of V-N in a certain stage.

V. Effect of A. u. bacterium upon the decreasing freshness of whale carcass

The results of the above experiments can make us imagine as follows:—No A. u. bacterium decomposes protein and gives out the bad smell. So if this bacterium only acts on the whale carcass, it gives no remarkable effect on freshness. However, generally speaking, in the decreasing process of freshness, only one species of bacterium acts rarely, and simultaneously various kinds of bacteria work. So in such a case, A. u. bacterium decomposes actively various intermediary decomposition products of protein which are produced by other bacteria, especially bacteria with protein decomposing power, and gives abundantly the last products of protein decomposed. Namely it can be safely

said that A. u. bacterium can not become the main cause of decreasing freshness on whale carcass but plays a part by participating in the last process of protein decomposing.

VI. The cause why the sudden decrease in the amount of V-N takes place in a certain stage of decomposing process of extract

In most of the above experiments, the sudden decrease of the amount of V-N was found in a certain stage. The pH was always bigger than 7.5. From the further shape of the curve we can judge that this decrease is not caused by volatilization. After my incessant observation, I found that in this stage fine sand-like or short prismal crystals appeared on the bottom of the vessel without fail and they increased their number with the decrease of the amount of V-N (Fig. 6 and 7). Wondering if this crystal had any relation with the decrease of the amount of V-N, I investigated to find that this crystal contained ammonia as my expectation.

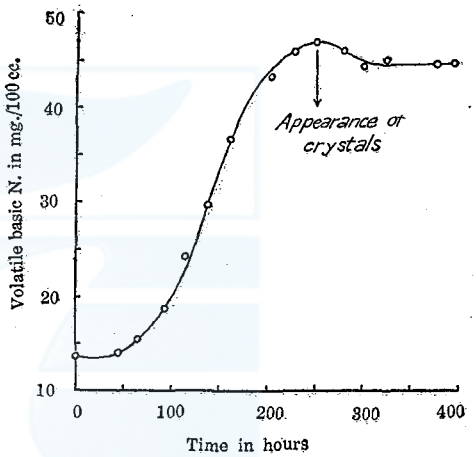


Fig. 7. Appearance of crystal of $NH_4MgPO_4 \cdot 6H_2O$ and decrease of the amount of vatile basic N

This crystal is an inorganic substance, hardly soluble in water and alkali carbonate solution and readily in dilute mineral acids. Its dilute mineral acid solution, if it is alkalified with NaOH, gives the gelatinous precipitate and generates ammonia. This crystal was confirmed to be magnesium ammonium phosphate from the fact of containing $Mg^{\cdot\cdot}$ and PO_4''' as well as ammonia and the content of phosphorus. The determination of phosphorus was done by Bell-Doisy's[2] colorimetry with solution of the crystal in 0.01 n HCl as test solution.

Sample :	1.63 mg,	1.51 mg
P :	0.204 mg,	0.188 mg
$NH_4MgPO_4 \cdot 6H_2O$:	calculated	12.63
	found	12.52, 12.45

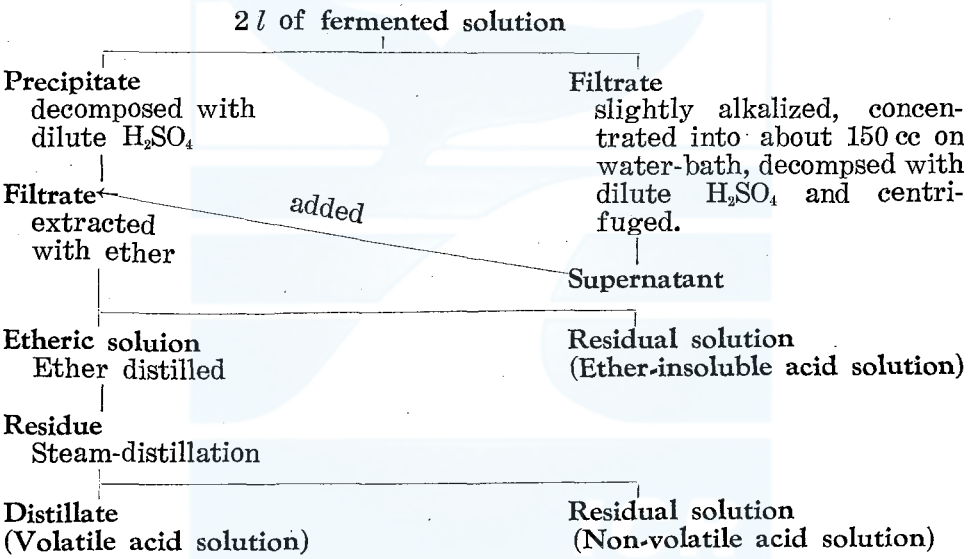
Accordingly, the cause for decrease of the amount of V-N was found on the crystallization of magnesium ammonium phosphate. It was certificated by its crystallization even in the extracts in Fig. 1 and Fig. 7 which were made without the treatment through kieselguhr.

VII. Acids as the metabolic products of Glucose by A. u. bacterium

A. u. bacterium produces acids from saccharide. Acids produced aerobically from Glucose were searched.

Preparation of cultural medium :—The solution of 1 *l* of whale broth, 10 g of Glucose and 5 g of NaCl were neutralized with NaOH and aftr sterilization, added 5 g of $CaCO_3$ separately sterilized. A 2 *l* Erlenmeyer flask was used as vessel.

A platin loopful of A. u. bacterium was inoculated on this cultural medium and incubated at 28°~30°C for 3 days under occasional stirrings. 2 *l* of thus fermented solution was treated as the following table and divided into the volatile acid solution, ether-soluble non-volatile acid solution and ether-insoluble acid solution.



I tried to isolate acids from the above each solution.

Volatile acid :—To volatile acide solution, PbO was mixed in excess ond evaporated to dryness on the water bath and the residue was infused with about 35 cc of tepid water. The filtrate was heated in the violently boiling water-bath but no basic lead propionate was found. Therefore, after removing lead by adding dilute H_2SO_4 to the filtrate, ZnO was added and evaporated to dryness on the water-bath. The residue was infused with absolute alcohol after two hours' desiccation at 150°C. To the residue, phophoric acid was added and distilled with steam. The distillate showed negative reaction against formic acid. Alcohol distilled off the infusion and after adding phosphoric acid to

the residual solution, it was distilled with steam. Though white substance was found in the distillate, its scarcity made the further investigation impossible. Then it was filtered off. The filtrate was neutralized with NaOH and concentrated into about 3 cc on the water-bath and added saturated $AgNO_3$ solution. Instantly after, white substance appeared, which was filtered out and recrystallized from warm water. About 0.2 g of needle crystal of silver acetate was obtained.

Sample 68.7 mg Ag 43.8 mg
CH_3COOAg calculated 64.64 found 63.75

Non-volatile acid:—Non-volatile acid solution was neutralized with saturated $Ba(OH)_2$ solution and concentrated on the water bath. 25 gr of the concentrated viscous substance was dissolved into 250 cc of 80% alcohol under warming and after cooling, it was filtered. The residue was inorganic. After distilling alcohol off, the filtrate was diluted with water and decomposed with dilute H_2SO_4 and filtered. The filtrate was extracted with ether and then it was distilled off. After the volatile substance which somewhat seemed to be contained in the residue was expelled off through steam-distillation, some water and $ZnCO_3$ in excess were added and heated on the water-bath and filtered. The filtrate was treated with active carbon and concentrated on the water bath. About 10 g of crude zinc lactate was obtained. After recrystallizing from hot water and desiccating at the room temperature, the yield was 5.4 g. Some more crystals were yielded from the mother liquor.

Sample 298.2 mg ; 215.5 mg (yielded from the mother liquor)
Loss on heating at 115°C 53.7 mg . 39.0 mg
ZnO 81.7 mg ; 58.7 mg
$Zn(C_3H_5O_3)_2 \cdot 3H_2O$ calculated crystal water 18.16
ZnO 27.35
found crystal water 18.01 ; 18.1
ZnO 27.43 ; 27.24

According to this analysis value, the lactic acid produced by A. u. bacterium is racemic mixture. Therefore, A. u. Bacterium is imagined to contain racemiase.[3]

Ether-insoluble acid:—It was searched with newly made fermented solution. It was concentrated on the water-bath and decomposed with dilute H_2SO_4 and centrifuged. The supenatant was extracted with ether for removing the ether-soluble acids and the residual solution was boiled with excess $CaCO_3$. In the filtrate and the residue, acid

was searched but no organic acid could be found.

Accordingly, organic acids found as metabolic products of glucose were considerable amount of lactic acid and minute amount of acetic acid.

In conclusion, sincere thanks are expressed to Dr. T. Maruyama for his kind advice and to Prof. Dr. T. Akiba for transfering the precious bacterium and to Mr. H. Okuda for revision.

Literatures

1) Sci. Rep. Whales Research Institute No. 3, 63 (1950)
2) J. Biol. Chem. 44, 55 (1920)
3) H. Katagiri, K. Kitahara: Bull. Agr. Chem. Soc. Jap. 12, 127 (1936)