53. Kyosuke Tsuda* and Masaaki Kawamura**: The Constituents of the Ovaries of Globefish. VI.¹⁾ Purification of Globefish Poison by Chromatography.

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The poisonous component of globefish precipitates by lead acetate solution in alkaline state from the hot aqueous extract of globefish ovaries. By the removal of lead from this precipitate, a crude poison is obtained.

In Part I²⁾ and Part IV³⁾ of this series, the authors gave a summarized report of the purification of this crude poison by various precipitation reagents, and also described on the determination of a carbohydrate isolated during the course of this purification.

As a preliminary step in this purification, one-dimensional paper chromatography was carried out with the crude poison. A 90% aqueous phenol solution was used as a partitioning solvent. Several strips of paper was lined and the chromatograms developed. Each strip was then examined for the location of amino acids, reducing sugar and the poison. Spots for amino acids appeared, as shown in Fig. 1, at R_f 0.3~0.8 and those of reducing sugar at 0.3~0.5. The poisonous component was very slow in moving, and the spots were located at R_f 0~0.1. The dyestuff spot moved very rapidly, rising with the partitioning solvent. The spots for amino acids here observed were those of glycine, taurine, alanine, valine and leucine, and those of reducing sugar were those of glucose, as reported in the previous paper.³⁾

Fig. 1.

One-dimensional Chromatogram of the Crude Poison.

Ascending System—at 25°.

Filter paper used-Toyo Roshi No. 2. 3×30 cm.

Spraying reagents: Ninhydrin (amino acids); aniline
hydrogen phthalate (reducing sugar).

The location of the poisonous component was determined by cutting the strip into 10 portions, extracting each portion with water, and noting its effect on mouse when injected.

Strip I—Poison map.

Strip II—Amino acid map.

Strip III—Reducing sugar map.

Spots: 1) Poison; 2) glycine; 3) taurine; 4) alanine;
5) valine plus leucine; 6) glucose.

Fig. 1

Purification of the Poison by Column Partition Chromatography

An aqueous solution of the poison was flowed in from the top of a column composed of a mixture of potato starch and celite. The column was then developed by phenol water which was stopped when the solution reached the tip of the column. The column

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1) Part V: This Journal, 72, 182 (1952).

2) K. Tsuda and M. Kawamura: This Journal, 70, 433 (1950); C. A. 45, 3950 (1951).

3) K. Tsuda and M. Kawamura: This Journal, 71, 282 (1951); C. A. 45, 8151 (1951).

was then divided into 10 equal portions, numbered 1 to 10 from the top to the bottom, and each was washed with ether, then extracted with cold water. The extract was evaporated in vacuo, its residue extracted with 70% ethanol without application of heat, and the alcoholic extract evaporated to dryness in vacuo. The toxic potency of each portion was examined and the results showed the presence of poison in No. 1 (0.8 γ) and No. 2 (1.0 γ) only, portions below No. 3 showing no poisonous action. This shows that the poisonous components in the original material has been condensed in the No. 1 and No. 2 portions in 60~70% and in 20~30%, respectively. Substances that reduce Fehling's solution were contained in Nos. 1, 2, 8, 9 and 10, that in Nos. 1 and 2 being glucose, but those in the others are still unknown.

Amino acids contained in these ten portions of the column were examined and the presence detected of glycine, taurine, alanine, valine, leucine and phenylalanine. Identification of these amino acids was made by direct comparison of their one- or two-dimensional paper chromatography with those of authentic samples. The results are shown in Fig. 2 from which it can be seen that the movement of the poison is very slow compared to amino acids and reducing substances that this method would be highly satisfactory as a method of the purification of globefish poison.

Fig. 2

Fig. 2. Distribution of Poisonous Component and Amino Acids in the Column of Columnar Partition Chromatography.

Column : Starch+celite* (2:1), 1.5×40 cm. Crude poison: 500 mg. Developing reagent : 90% Phenol water.

Column was divided into 10 equal portions, numbered No. 1 to No.10, from top to bottom. Each portion put to one-dimensional paper chromatography, by the ascending system.

Filter paper used : Toyo Roshi No. 2, 3×40 cm. Developing reagent, 90 % Phenol water. Spraying reagent : 0.2% Ninhydrin butanol solution. Numbers on the map designate the following: 1) glycine; 2) taurine; 3) alanine; 4) valine; 5) leucine+phenylalanine. Leucine and phenylalanine can be separated by developing with a mixture of butanol:acetic acid:water in 4:1:1 ratio.

* Celite No. 535, a product of Johns-Manville Co., New York.

Analysis of Amino Acid Mixture obtained from the Sulfuric Acid Hydrolysate of Globefish Ovaries

In order to make a comparison of the paper chromatography of amino acids contained in the crude poison, paper chromatography was carried out with the hydrolysate of the globefish ovaries.

Ovary tissues left after the extraction of poison was washed well with water and 200 mg. of the tissues was refluxed for 20 hours with 40 cc. of 8N sulfuric acid. After cool, the reaction mixture was corrected to pH 11~12 with baryta, filtered, and the filtrate was concentrated in vacuum to remove ammonia. The pH of the solution was then corrected to 6 by 0.1N sulfuric acid, barium sulfate removed by filtration, and the filtrate evaporated. The residue was dissolved in 1 cc. of 10% isopropanol, and this solution was put to two-dimensional paper chromatography. The map of chromatogram here obtained is shown in Fig. 3.

As can be seen from Fig. 3, the presence of glycine, taurine, threonine, lysine, alanine, arginine, proline, valine, phenylalanine and leucine was confirmed. This has shown that the amino acids which accompany the poison in its purification are those contained in the hydrolysate of ovary tissues.

Fig. 3

Fig. 3. Two Dimensional Paper Chromatography of the Hydrolysate of Globefish Ovaries.

Ascending system. Filter Paper used : Toyo Roshi No. 2, 40×40 cm. Solvent : 90%phenol-water (0.1%NH₃) and BuOH:AcOH:H₂O (4:1:1) 28°.

Sample was placed at 0 point in an amount to make a spot 1 cm. in diameter.

Spraying reagent : 0.2% BuOH solution. Each amino acid spot was auxiliarily examined by Sakaguchi and Pauli reactions, etc.

Numbers in the map are : 1) glycine; 2) taurine; 3) threonine; 4) lysine; 5) alanine; 7) arginine; 8) proline; 9) valine; 10) phenyl-alanine; 11) leucine. Spot No. 6 could not be determined.

Purification of the Poison by Adsorption Chromatography

As an adsorbent, active charcoal, "Edokol"™ was used, and retention volume⁵⁾ was first estimated with glucose solution. Its specific retention volume was 13.4 cc./g., which was slightly smaller than the values given in literature for various active charcoal.

A solution containing 200 mg. of crude poison in 25 cc. of water was passed through a column (1×7 cm.) of "Edokol"+celite (1:0.5). The column was washed first with 200 cc. of water, 30 cc. of 5% ethanol, then with 30 cc. of 10% ethanol. The aqueous effluent was fractionated into 25 cc. each as filtrate Nos. 1~9, 5% EtOH effluent as No. 10, and 10% ethanol effluent as No. 11. Each fraction was evaporated in vacuo and their residue was examined for poisonous potency, amino acids and reducing sugar. The results are shown in Table I, from which it can be seen that 60% of the poison is eluted by 5% ethanol and 20% by 10% ethanol, with almost none in the aqueous effluent. Amino acids and reducing sugar appear only in the aqueous effluent and not in hydrous alcoholic effluent. In this manner, poison is separated from amino acids and reducing sugars. Elution of the remaining column by 50% acetone gave a brownish resinous substance which showed no poisonous activity.

Table I. Composition of the Liquid Chromatogram obtained by Active Charcoal**

Fraction	1	2	3	4	5	6	7	8	9	10	11
Effluent	H ₂ O	H ₂ O	H ₂ O	H ₂ O	H ₂ O	H ₂ O	H ₂ O	H ₂ O	H ₂ O	5 % EtOH	10% EtOH
Amino acids	alanine proline leucine	leucine valine alanine	valine	glycine taurine	glycine taurine	glycine taurine	nil	nil	argi- nine	nil	nil
Reducing sugar	±	±	±	±	±	±	±	±	±	—	—
Toxicity MLD/g.	nil	nil	nil	nil	nil	nil	nil	nil	nil	0.6γ	0.6γ

Amino acids detected by two-dimensional paper chromatography, ascending system, at 25°. Solvent : Phenol-water, butanol-acetic acid-water (4:1:1). Spraying reagent : Ninhydrin 0.2 % butanol solution.

Potency tested by parenteral administration in mice.

** Retention volume of active charcoal : One g. of "Edokol" was well washed with 5% NaOH, then with water and dried. To 1 g. of this dried charcoal, 0.5 g. of Celite (*loc. cit.*) was mixed and prepared into a column 1×7 cm. The tip of the percolator was fixed so as to give 0.05 cc. of solution in one drop. The 0.2% aqueous solution of D-glucose was then passed through the column and each drop of the effluent was allowed to make a separate spot on a filter paper. Spots were thus taken of 15~20 cc. of the effluent. Aniline hydrogen phthalate solution was then sprayed on the whole spots and the presence of D-glucose examined. The point at which D-glucose initially appears in the effluent thus examined was at 268 th drop, corresponding to 13.4 cc. of the effluent.

* A product of the Dai-Nippon Active Carbon Co., Ltd., Tokyo.

5) Claesson : Ann. N. Y. Acad. Sci. 49, Part 2, 183 (1948).

Summary

Crude poisons from globefish ovaries was purified by starch column partition chromatography and by adsorption chromatography using active charcoal. In both cases, amino acids and reducing sugars mixed with crude poison were found to flow at a faster rate than the poison by development. The separation of the poison and amino acids was especially complete in the case of active charcoal. Globefish poison thus obtained showed a potency of 0.6 MLD/g. mouse and when dried, became colorless, and brittle, resin-like solid containing some crystals. This is still not completely pure. The amino acids mixed in the poison were identified by paper chromatography by which it was seen that these amino acids are found in the hydrolysate of the ovary tissues of globefish.

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54. Shigehiko Sugasawa, San-ya Akahoshi, Jun'ichi Himizu and Mitsutaka Kawazu: Preparation of Some 2-Quinolones.

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In connection with other investigation now going in this laboratory *N*- β -3',4'-dimethoxyphenylethyl-4-methyl-5-methoxy-2-pyridone (II) was needed, which, we thought, might be obtained from *N*- β -3',4'-dimethoxyphenylethyl-3-methoxy-4-methylpyridinium bromide (I) by ferricyanide oxidation. The reaction was, however, unexpectedly difficult and numbers of fruitless experiments had been made, before we at last abandoned this project. The product was always untractable tarry substance. Some ten years ago one of us with Sakurai and Okayama¹⁾ made known that *N*- β -3',4'-dimethoxyphenylethyl-3-carbalkoxy-pyridinium bromide (III) undergoes smooth oxidation, giving *N*- β -3',4'-dimethoxyphenylethyl-2-pyridone-5-carboxylic acid (IV) in more than 90% yield. Thus it seems probable that the methoxy group at 3-position in (I) would be responsible for the failure of smooth oxidation.

This failure prompted us to investigate the oxidation reaction of various aromatic cyclic ammonium halides with different oxidation agents, when we found that some quinolinium alkyl halides can be oxidized smoothly by means of silver oxide, often with better results than by ferricyanide.

As for the mechanism of this reaction, quinolinium alkyl hydroxide (V), produced from the halide by silver oxide, in its amino-aldehyde form (VI) (via carbinol amine (V')), undergoes oxidation by silver oxide, giving aminocarboxylic acid (VII), which in turn cyclizes to give 2-quinolone (VIII) with loss of water.

If so some isoquinolines should give the corresponding isoquinolones under similar conditions, and this was proved to be the case with 6,7-methylenedioxy-2,3-dimethyl-isoquinolinium iodide (IX), yielding the corresponding isoquinolone (X), although in this case in lower yield than by ferricyanide oxidation.

When applied to pyridinium salt this oxidation method failed to give result of any practical importance.

The influences of the various substituents (e. g. R and R' in compound I) both electron-donating and attracting, and of their positions upon the course of the oxidation are now being investigated.

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1) Sugasawa, Sakurai and Okayama: Ber. **74**, 537 (1941).