synthesis left much to be desired, for the first steps were accompanied by many difficulties. Considerable gum formation during the pyrolytic cyclization was responsible for much of the tediousness involved in the separation of isomers by fractional crystallization. Since there was but slight success in use of the method employed with the related 3-methyl type (*cf.* ref. 4c), fractionation was accomplished from alcohol, or, alternating, alcohol and aqueous acetone. The less soluble of the fractions was the 7-chloro isomer, for the bz-chloro-4-hydroxy-3-propylquinoline-2-carboxylic acid produced by its hydrolysis was oxidized to 4-chloroanthranilic acid by alkaline permanganate.[4c] An inadequate amount of the desired 5- and 7-chloro-4-(4′-diethylamino-1′-methylbutylamino)-3-propylquinolines was obtained for screening as antimalarials, but all pertinent information relative to them and intermediates required is given in Table III.

Acknowledgement.—The authors are pleased to have had the advantage of advice and encouragement from Drs. C. M. Suter and J. S. Buck during the course of these investigations. The analytical staff of the Institute, under the direction of Mr. M. E. Auerbach, has shown great patience and care in carrying out the many determinations required. Mrs. N. P. Gorman and Mrs. E. J. Altier have rendered further valuable technical assistance.

Summary

A group of 4-(4′-diethylamino-1′-methylbutylamino)-quinolines has been prepared, including all possible bz-fluoro-3-methyl derivatives, and also 3,6,5/7-trimethyl, 7-chloro-2-methyl and the 5/7-chloro-3-propyl types.

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[CONTRIBUTION FROM THE GEORGE WILLIAMS HOOPER FOUNDATION, UNIVERSITY OF CALIFORNIA, AND THE CHEMICAL LABORATORY OF NORTHWESTERN UNIVERSITY]

Paralytic Shellfish Poison. I. Occurrence and Concentration by Ion Exchange[1,2,3]

BY HERMANN SOMMER, ROBERT P. MONNIER, BYRON RIEGEL, D. WARREN STANGER, JAMES D. MOLD, DONALD M. WIKHOLM AND ELIZABETH SHANESY KIRALIS

The paralytic form of shellfish poisoning in man has been recognized for over a century as a clinical entity.[4] Shellfish become poisonous when they feed on the marine plankton organism, *Gonyaulax catenella* Whedon and Kofoid. This was established by Sommer and co-workers,[5] who showed: (1) that in the three-year period studied, there was a close correlation between the toxicity of shellfish and the number of *Gonyaulax catenella* per liter present in sea water; (2) that non-toxic bivalves kept in the laboratory became toxic when supplied with fresh sea water rich in this dinoflagellate; and (3) that the poison could be obtained directly from this plankton organism.

The California mussel, *Mytilus californianus* Conrad, has proved to be a better source of the poison, on a scale sufficient for chemical study, than the dinoflagellate. It has been estimated that the average mussel filters 38 liters of sea water a day to obtain its food supply of plankton.[5] Extensive beds of these mussels are found along the rocky Pacific coast of North America. From April to November selected beds along the coast 150 miles north and south of San Francisco were sampled semimonthly and the poison titer of the mussels determined. A large-scale collection was made when the poison content reached or exceeded 4000 MU. per 100-g. mussel.[6]

Daily collections can be made only during the last four or five days of the minus tide period, which occurs every two weeks with the new or full moon. Usually there is only one such period in the entire season during which the mussels are sufficiently toxic to warrant collection. In summer the lower low tide is at daybreak or shortly afterward. In the two-hour period when the beds were accessible, the mussels were pried loose from the rocks and carried up on the beach above tidewater. There they were sorted, washed and opened.[7] The "livers" or digestive glands were dissected out and preserved in acidified ethanol.

In the three-year period, 1944–46, a total of 4360 kg. of mussels containing 160×10^6 MU of poison was collected. The extraction of one of the many collections is shown in Table I. This collection was made south of Pedro Point, San Mateo County, California, on July 18, 1946.

(1) The work described in this paper was initiated under a contract between the Federal Security Agency and the University of California and Northwestern University. It was continued under a contract with the Chemical Corps, Camp Detrick, Frederick, Maryland.

(2) Since the mass poisoning in the San Francisco area in 1927, Dr. Karl F. Meyer, Director of the George Williams Hooper Foundation for Medical Research, University of California, has sponsored research on shellfish poison. He was responsible for renewed interest in the problem in 1944, when the contracts for further research in this field were made. The members of the Northwestern group are greatly indebted to Dr. Meyer for making the facilities of the Foundation available to them each summer during the collection period.

(3) The authors wish to thank Dorothy Butler, Ardath Clark Van Tuyl, Patricia Garbutt, Esther Kline and Ruth Nell for their technical assistance.

(4) (a) K. F. Meyer, H. Sommer and P. Schoenholz, *J. Preventive Med.*, **2**, 365 (1928); (b) H. Sommer and K. F. Meyer, *Arch. Path.*, **24**, 560 (1937).

(5) H. Sommer, W. F. Whedon, C. A. Kofoid and R. Stohler, *Arch. Path.*, **24**, 537 (1937).

(6) The mouse unit (MU.). or average lethal dose, is defined as the amount of mussel poison contained in 1.0 ml. of aqueous solution that, injected intraperitoneally into a 20-g. white mouse, will cause death in fifteen minutes. Directions for carrying out this bioassay, together with the tables for calculating the number of MU in the test solution from the weight of the mouse and the dying time, were furnished by H. Sommer to the other workers in this field. These tables are based on graphs[4b] recorded in the literature.

(7) Many of the people in the collecting party were members of the Hooper Foundation who volunteered their services. The authors are especially indebted to Lucile Foster, Florence Hockin, Vera Kreekis, Adelien Larson, Alma McDole, Ethel Meyer, Edward Sherry, Susanne Sommer and Richard Sommer.

TABLE I

EXTRACTION OF POISON FROM A COLLECTION OF TOXIC MUSSEL LIVERS

Collection

		Assay, MU./100-g. mussel,	Poison, 10^6 MU.
Weight of whole mussels,[a]	kg. 240	5800	13.92
Volume of ground mussel liver,[b]	ml.		
	11,600	MU./ml. 860	9.98

Extraction

Extracts[c]	Volume, ml.	Total solids, g.	Toxicity, MU./mg.	Poison, 10^6 MU.
1	4,010	555	3.37	1.870
2	5,300	411	4.59	1.880
3	6,050	232	4.13	0.960
4	6,600	175	3.12	0.545
5	6,130	113	2.93	0.333
6	3,820	54	2.77	0.150
	31,910	1540	3.73 av.	5.738

[a] The livers were preserved in ethanol containing 4 ml. of concentrated hydrochloric acid per liter. [b] They were ground in a meat grinder and the *p*H of the *brei* was adjusted to approximately 4.5 by the addition of 60 ml. of concentrated acid. [c] Extract no. 1 was made by centrifuging the suspension of ground livers. Subsequent extracts were made in a similar manner after resuspending the residue in ethanol containing 2 ml. of concentrated hydrochloric acid per liter and stirring overnight. The final extracts were made by heating the suspension at 70° during the stirring.

Extracts of comparable toxicity were combined and divided into aliquot portions, each containing about 10^6 MU. of poison. Since the first extract of each day's collection contained large amounts of lipid material, it was advantageous to remove this by extraction with ether before proceeding further. With subsequent extracts it was immaterial whether defatting was done before or after decolorization. The ether extract usually contained less than 1000 MU. of poison.

Decolorization was carried out by adding 10–20 g. of active carbon (Nuchar C or XXX) to the extract and shaking. After standing overnight, the extract was filtered through a layer of the carbon on a Bücher funnel. The first portion of the filtrate was set aside and the remainder passed through a second lot of fresh carbon. This was continued until the desired degree of decolorization (dark red-brown to pale yellow) was attained. As much as 10% of the poison was lost due to irreversible adsorption or actual destruction. For this reason, the minimum weight of carbon (10–60 g.) was used and only partial decolorization carried out.

To remove a large portion of the ethanol, the decolorized extract was concentrated to 400–500 ml. With some extracts it was possible to improve the toxicity greatly by an additional step. The extract was concentrated *in vacuo* at 35–40° to a thin sirup. By the very slow addition of absolute ethanol to the sirup, a white granular material was precipitated. This contained almost none of the poison and was in part taurine. However, the precipitate was often gummy and contained a large proportion of the poison. For this reason the step was omitted in most preparations.

The low concentration of the poison in the crude extract and its chemical and physical properties, insofar as they were known, determined the limited number of methods available for its purification. Evidence pointed to a minimum toxicity of 1 MU./0.001 mg.[8] for the purified poison. In a crude extract with a toxicity of 4–8 MU /mg. the poison would be present at most to the extent of 0.4–0.8%.

Mussel poison has been shown to be soluble in water, methanol, ethanol, glacial acetic acid and aqueous acetone.[4a] The effectiveness of these solvents for extracting the poison appeared to increase if small amounts of hydrochloric acid were added. The poison was insoluble in ether, chloroform, ethyl acetate, butanol and toluene. It could not be extracted from an aqueous alkaline solution by any of these solvents.

Studies were made of the effect of *p*H and temperature on the stability of the poison in aqueous solution as measured by its toxicity (Figs. 1 and 2). The results of these studies set further limits on the methods which might be used in purification. The decrease in toxicity with an increase in *p*H and in temperature showed that the poison must be handled in acid or, under certain conditions, in neutral solution and for all operations the temperature must be kept as low as possible.

Previous attempts to isolate the poison by precipitation with reagents, such as gold chloride, Reinecke salt, and phosphomolybdic, picric and rufianic acids, had been disappointing.[8] In general, either the compound, if it were formed, was too soluble to be obtained as a precipitate, or there was a loss of up to 100% in the toxicity of the poison, or the complex could be decomposed only by the use of alkali which destroyed the poison.

The isolation of betaine from a partially purified extract of mussel poison indicated that the properties of betaine and the poison were enough alike that the substances tended to accompany each other. This fact and the solubility behavior of the poison suggested that it might be a nitrogenous base.[4a] In an attempt to take advantage of the basic properties of the poison, ion exchange was tried as a method of concentration.[8] This proved to be highly successful and experimentally demonstrated the basic nature of the poison.

A commercial zeolite, sodium Permutit, was used by Sommer and Müller, but further studies[9] indicated that the barium compound was more

(8) H. Müller, *J. Pharmacol. Exptl. Therap.*, **53**, 67 (1935).

(9) R. P. Monnier, "Versuche zur Isolierung eines in *Mytilus californianus* enthaltenen Giftes," Thesis, Eidgen. Techn. Hochsch., Zurich, 1938.

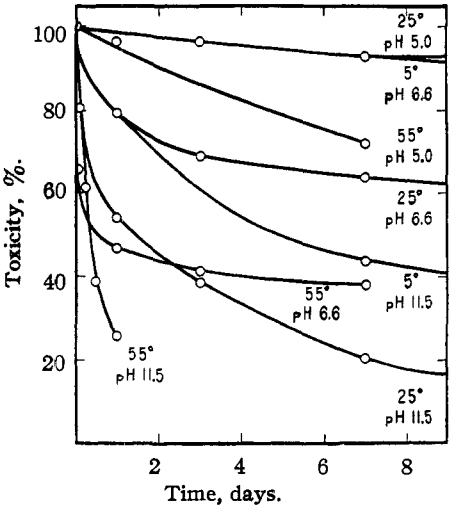

Fig. 1.—The effect of temperature and *p*H on the toxicity of mussel poison in aqueous solutions. No change in the toxicity of mussel poison in 3 *N* hydrochloric acid was observed at 55°; this was true also of an aqueous solution, *p*H 5.0 at 5°.

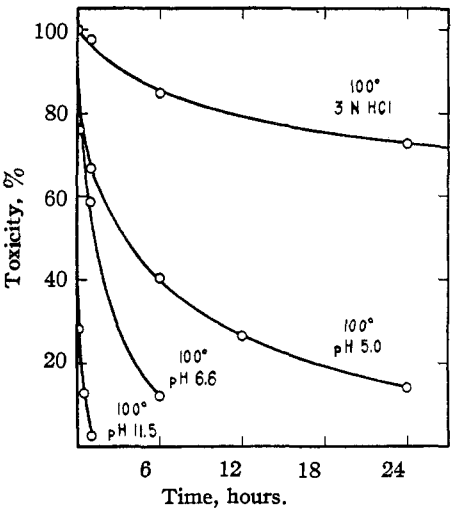

Fig. 2.—The effect of *p*H on the toxicity of mussel poison in aqueous solution at 100°.

suitable. The poison was adsorbed according to the equation

$$BaZ + BCl_2 \rightleftarrows BZ + BaCl_2$$

where BaZ is barium Decalso (Permutit) and BCl_2, mussel poison hydrochloride. After the column had been well washed with water, the poison was eluted with a 20% solution of barium chloride. Concentration of the eluate and treatment with absolute ethanol removed most of the barium chloride.

The conditions for the concentration of mussel poison by ion exchange were determined by the properties of the poison and of the cation exchanger. The *p*H range of the poison extract for

TABLE II

CONCENTRATION OF MUSSEL POISON BY ION EXCHANGE

Preparation		1	2	3	4	5	6[b]
Starting material	Poison, 10^6 MU.	1.000	1.000	1.000	1.070	1.070	1.250
	Toxicity, MU./mg.	8.0	8.0	8.0	6.6	6.6	...
Decolorization	Weight of carbon, g.	60	60	10	20	20	200
	Poison, 10^6 MU.	1.020	0.759	0.895	0.994	1.090	1.250
	Toxicity, MU./mg.	10.1	7.6	7.2	6.3	7.1	...
Extraction with ether	Ether insoluble: Poison, 10^6 MU.	1.060	0.864	0.800	0.940	0.980	1.250
	Ether insoluble: Toxicity, MU./mg.	10.5	8.5	7.6	6.3	6.7	58.8
	Ether soluble poison, MU.	760	200	15,600	500	210	...
Adsorption on Decalso[a]	Decalso cation	barium	barium	barium	barium	sodium	barium
	Extract, *p*H	5.0	2.9	2.9	3.5	3.5	5.0
	Filtrate and washings, poison, 10^6 MU.	0.165	0.140	0.098	0.272	0.075	0.050
	Eluate: Poison, 10^6 MU.	0.557	0.591	0.633	0.547	0.633	1.200
	Eluate: Toxicity, MU./mg.	157	132	151	59.9	59.8	250
Poison recovered, %	Total	72.2	73.1	74.7	76.7	66.2	100.0
	Purified	55.7	59.1	63.3	51.1	59.2	96.0
Toxicity purified poison / Toxicity starting material		19.6	16.5	18.9	9.1	9.1	...

[a] A suspension of 1 kg. of commercial Decalso in water was evacuated to remove the entrapped air. The suspension was poured into a tube provided with a stopcock and a pad of absorbent cotton to form a column of Decalso 7 × 50 cm. It was washed with 3 liters of water, 3 liters of 2% acetic acid, 6 liters of water and 1.5 liters of a 20% aqueous solution of crystalline barium chloride ($BaCl_2 \cdot 2H_2O$). To convert the column from barium to sodium Decalso, it was treated with 2 liters of saturated sodium chloride solution and washed with water. The aqueous poison extract (1.5 liters) was passed through the column followed by 4 liters of wash water. The poison was eluted with 1.5 liters of a 20% solution of barium chloride. [b] The collection of mussels from which this extract was prepared had been made in 1936 at a time when the average mussel liver contained 10,000 mu. of poison. After decolorization, the extract was concentrated to a sirup and treated with 200 ml. of absolute ethanol which precipitated a white insoluble material containing no poison. The filtrate was treated with 1 liter of ether and placed in the cold room overnight. The ether was decanted and the gummy residue was dissolved in 1 liter of water. The *p*H was adjusted to 5.0 with 0.2 *N* barium hydroxide solution before adsorption on Decalso.

adsorption was limited by the fact that the poison is unstable in alkali and the Decalso becomes less stable as the acidity increases. In the early experiments, the *p*H of the extract was adjusted to 5.0 by the addition of 0.2 *N* barium hydroxide solution in the presence of ice (preparation 1, Table II). This introduced barium ions, which tended to drive the above reaction to the left by either preventing the adsorption of the poison or causing its elution. Later experiments showed that, within the limits 2.5–5.0, the *p*H of the extract is not a critical factor. A poison extract, within these limits, may be passed through the column without adjustment of the *p*H (preparation 2).

A comparison of preparations 2 and 3 shows that a decrease from 60 to 10 g. in the amount of carbon used for decolorization of the crude extract may actually increase the poison content and toxicity of the subsequent Decalso eluate. There was no improvement in the toxicity of the poison when the amount of wash water passed through the Decalso column was increased from 4 to 14 liters before its elution.

The amount of poison found in the filtrate and washings depends upon two factors. The first of these is the toxicity, which obviously is inversely proportional to the amount of inert material mixed with the poison. When an extract with a toxicity of 1.93 MU./mg. was passed through barium Decalso, 67.8% of the poison was found in the filtrate and washings; but when one with a toxicity of 27.5 MU./mg. was used, only 9.9% of the poison passed through. The second factor is the cation of the zeolite. After considerable experience had been gained in the use of barium Decalso as the ion exchanger, a second study was made of the properties of sodium Decalso. The change from the divalent barium to the monovalent sodium ion resulted in a decrease from 27.4% to 7.7% of the poison which passed into the filtrate and washings (preparations 4 and 5), and an increase in the recovery of purified poison.

Zeolites using cations other than sodium or barium were also investigated. Copper Permutit adsorbed the poison, which in turn could be eluted with potassium sulfate. It was found unsatisfactory, since the copper ions could be removed only by precipitation with hydrogen sulfide, and this resulted in a loss of about 40% of the poison. Tetramethylammonium Permutit adsorbed the poison quantitatively, but tetramethylammonium bromide was ineffective as an eluant. Concentrated potassium sulfate eluted the poison, but no great improvement in toxicity was obtained.

Lloyd's reagent adsorbs the poison almost quantitatively even in the presence of acid or alcohol. However, until recently it has not been possible to find a satisfactory eluant. This work, which is now in progress, will be reported later.

Synthetic resins prepared by the condensation of polyhydroxy phenols with formaldehyde[10] were investigated as cation exchangers for the concentration of the poison. The condensation product of phloroglucinol and formaldehyde adsorbed the poison, which could be eluted with dilute hydrochloric acid. Since the toxicity of the poison was not improved by this process, it was not further studied.

The following recommended procedure is based on the experience gained in the preparation of more than seventy-five mussel poison concentrates by ion exchange on Decalso. An aliquot portion of the crude extract containing 10^6 MU. of poison with a toxicity of 4–8 MU./mg. is partially decolorized with 10–60 g. of Nuchar. The filtrate is concentrated and extracted with ether to remove the lipids. These steps result in the loss of 10–20% of the poison. The solution of the poison is diluted to 1.5 liters with water and passed through a column containing 1 kg. of sodium Decalso. The Decalso is washed with 4 liters of water and the poison is eluted with 1.5 liters of a 20% barium chloride solution. Concentration of the eluate and extraction with alcohol furnishes a 50–70% yield of the poison with a toxicity of 70–140 MU./mg.

Summary

1. The collection of poison mussel livers and the preparation of the extract are described.

2. Studies show that the toxicity of the poison in aqueous solution decreases with an increase in *p*H and an increase in temperature.

3. The low concentration of the poison and its chemical and physical properties limit the methods available for its purification.

4. A procedure is described for the preparation of mussel poison concentrates by the use of ion exchange on barium or sodium Decalso. It is possible to obtain a 50–70% yield of the poison with a toxicity of 70–140 MU./mg., an enrichment of eighteen-fold.

5. Results obtained by varying the cation of the zeolite or by the use of synthetic resins have proved less satisfactory than those obtained by the method described.

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