(40 ml.), contained in a reaction vessel fitted with a tube containing potassium hydroxide pellets, finely cut metallic sodium (0.1 to 0.2 g.) was added over a period of 15 minutes until a blue color persisted for at least 5 minutes. The excess of sodium was discharged with ammonium chloride, and the ammonia was allowed to evaporate spontaneously. The residue was dissolved in 1 *N* hydrochloric acid (15 ml.). This solution was extracted twice with 20-ml. portions of ether, and the ethereal extracts discarded. The aqueous layer was filtered and neutralized with 1 *N* sodium hydroxide. The precipitate which separated was centrifuged and washed with water until the washings were free of chloride. The poly-L-histidine obtained was dried *in vacuo* over sulfuric acid; yield 0.3 g., $[\alpha]^{22}D$ −20.0° (*c* 4.5, in glacial acetic acid).

The elementary analysis of the polymer, dried to constant weight *in vacuo* over phosphorus pentoxide at 100°, indicated the presence of approximately half a molecule of water per histidine residue.

Anal. Calcd. for $(C_6H_7ON_3)_n \cdot n/2H_2O$: C, 49.3; H, 5.5; N, 28.8. Found: C, 48.7; H, 5.9; N, 28.6.

Poly-L-histidine, $n = 50$, is soluble in dimethylformamide, glacial acetic acid, dichloroacetic acid and trifluoroacetic acid and is insoluble in ether, alcohol and acetone. It is soluble in dilute aqueous acids at *p*H values below 6 and in concentrated aqueous sodium hydroxide (above 3.5 *N*).

Hydrolysis of Poly-L-histidine.—Poly-L-histidine·$n/2H_2O$, $n = 50$, (0.571 g.) was refluxed in 6 *N* hydrochloric acid (6 ml.) for 18 hours and the hydrolysate diluted to 10 ml. with 6 *N* hydrochloric acid. A paper chromatogram of the hydrolysate, using pyridine–water (65:35 by volume) as the mobile phase gave only one spot with ninhydrin with an R_f 0.65, identical with that of an authentic sample of L-histidine. Analysis of the hydrolysate gave an amino nitrogen (Van Slyke) value of 9.1% (calcd. 9.6%), and a specific rotation of $[\alpha]^{22}D$ +14.0° (*c* 6.06, in 6 *N* hydrochloric acid), for the histidine present assuming a quantitative yield.[15]

Poly-L-histidine Hydrochloride.—Poly-L-histidine was dissolved in a slight excess of 2 *N* hydrochloric acid and the hydrochloride precipitated by acetone, washed with acetone and dried for several hours *in vacuo* at room temperature over solid potassium hydroxide and phosphorus pentoxide.

(15) M. S. Dunn, E. H. Frieden, M. P. Stoddard and H. V. Brown, *J. Biol. Chem.*, **144**, 487 (1942), give for L-histidine $[\alpha]^{25}D$ +13.3 (*c* 4.05, in 6.08 *N* hydrochloric acid).

Anal. Calcd. for $(C_6H_8N_3OCl)_n \cdot nH_2O$: C, 37.6; H, 5.3; N, 21.9; Cl, 18.5. Found: C, 37.5; H, 5.6; N, 22.3; Cl, 19.0.

Poly-L-histidine picrate was obtained by the addition of picric acid to an aqueous solution of poly-L-histidine hydrochloride. The picrate was washed with ether and dried *in vacuo* at 100° for four hours before analysis.

Anal. Calcd. for $(C_{12}H_{10}N_6O_8)$: C, 39.3; H, 2.8; N, 22.9; neut. equiv., 366; amino N (after acid hydrolysis), 3.8. Found: C, 39.2; H, 3.0; N, 21.9; neut. equiv., 364, determined by anhydrous titration in dimethylformamide with sodium methoxide using thymol blue as indicator; amino N, 3.9 (Van Slyke), after hydrolysis with 6 *N* hydrochloric acid for 48 hours at 120°.

Complexes of Poly-L-histidine with Metals.—The silver complex was prepared as follows: 5 *N* ammonium hydroxide (2 ml.) was added to a solution containing poly-L-histidine (100 mg.) and silver nitrate (500 mg.) in 0.2 *N* nitric acid (10 ml.). The precipitate formed was centrifuged and washed with dilute ammonium hydroxide until the washings were free of silver. The precipitate was then triturated with acetone and dried *in vacuo* over sulfuric acid. The precipitate contained approximately one atom of silver per histidine residue (found: Ag, 38.4).

The copper complex was prepared in an analogous manner from 100 mg. of poly-L-histidine and 500 mg. of copper sulfate in 10 ml. of 0.2 *N* sulfuric acid. The complex contained approximately one copper atom per histidine residue (found: Cu, 28.6).

Complexes of polyhistidine with zinc, magnesium and cobalt were prepared in the manner described for the silver complex from poly-L-histidine and the sulfates of zinc, magnesium and cobalt. They were found to contain 35.1% Zn, 11.3% Mg and 8.4% Co, respectively.

The mercury complex, containing 34.4% Hg, precipitated from an acid solution of poly-L-histidine in nitric acid upon the addition of mercuric nitrate.

Measurements of *p*H.—Measurements of *p*H were made on a model G Beckman *p*H meter. A standard phosphate buffer (*p*H 7.00) was used for calibration.

Argentometric Titrations.—The argentometric titrations were carried out with a model G Beckman *p*H meter using an uncoated silver wire as the indicator electrode.

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REHOVOTH, ISRAEL

[CONTRIBUTION FROM THE CHEMICAL CORPS, FORT DETRICK, THE CHEMICAL LABORATORY OF NORTHWESTERN UNIVERSITY,[1] AND THE GEORGE WILLIAMS HOOPER FOUNDATION, UNIVERSITY OF CALIFORNIA[1]]

Paralytic Shellfish Poison. VI. A Procedure for the Isolation and Purification of the Poison from Toxic Clam and Mussel Tissues

BY EDWARD J. SCHANTZ, JAMES D. MOLD, D. WARREN STANGER, JOHN SHAVEL, FRANK J. RIEL, JOHN P. BOWDEN, JOSEPH M. LYNCH, RUTH SAVAGE WYLER, BYRON RIEGEL AND HERMANN SOMMER

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A procedure is described for the purification of the poisons from toxic clam and mussel tissues. It involves extraction of the toxic tissues with acidified aqueous ethanol, ion exchange fractionation on carboxylic acid resins (Amberlite IRC-50 and XE-64) and chromatography on acid-washed alumina. The purified poison from either clams or mussels has a toxicity of 5500 ± 500 average lethal mouse doses per mg. and a specific rotation of +130 ± 5°.

The procedures described previously[2] for the purification of the poison from toxic mussels (*Mytilus californianus*) or from the plankton *Gonyaulax catenella* yielded preparations with toxicities up to about 1600 mouse units (MU)[3] per mg. An improved procedure described in this paper resulted in the purification of mussel poison to a toxicity of

(1) The work performed in these laboratories was carried out under contract with the Chemical Corps.

(2) (a) H. Sommer, R. P. Monnier, B. Riegel, D. W. Stanger, J. D. Mold, D. M. Wikholm and E. S. Kiralis, THIS JOURNAL, **70**, 1015 (1948); (b) H. Sommer, B. Riegel, D. W. Stanger, J. D. Mold, D. M. Wikholm and M. B. McCaughey, *ibid.*, **70**, 1019 (1948); (c) B. Riegel, D. W. Stanger, D. M. Wikholm, J. D. Mold and H. Sommer, *J. Biol. Chem.*, **177**, 7 (1949).

(3) The toxicity values given in this paper were determined by the intraperitoneal injection of 1.0 ml. of aqueous solutions of the poison into white mice. The mouse unit is the average lethal dose of poison that will kill a 20 g. mouse in 15 minutes (see Experimental section).

TABLE I

ION EXCHANGE FRACTIONATION OF A TYPICAL CRUDE EXTRACT OF MUSSEL POISON ON THE SODIUM FORM OF AMBERLITE IRC-50[a]

Fraction	Solvent	Vol., l.	Total MU × 10^3	Recovery, %	Solids, g.	Ash, g.	Toxicity, MU/mg. solids
Starting material[b]	Aq. EtOH *p*H 5.6	94.5	5770	100.0	2900	566	2
Filtrate	Aq. EtOH	94.5					
Rinse no. 1	H_2O	4.8	119	2.1	2870	526	0.1
Rinse no. 2	Buffer *p*H 4.0[c]	14.5					
Rinse no. 3	H_2O	3.8	168	2.9	970		
Eluates	0.5 *M* HOAc	1.6	16	0.3	11.7	10.6	1.4
	.5 *M* HOAc	5.3	106	1.8	20.1	18.6	5.3
	.5 *M* HOAc	13.2	5400[d]	93.5	13.9	0.37	388
	.5 *M* HOAc	12.2	51	0.9	4.4	.00	11
	.05 *M* HCl	4.4	5	.1	6.6	.00	0.7
	1.0 *M* HCl	4.5	5	.1	1.2	.18	4
Total				101.7			

[a] A column (9.3 cm. in dia.) was prepared with 6800 ml. of sodium Amberlite IRC-50 and washed with water to remove excess NaOH. The poison solution was filtered through the resin at a rate of about 300 ml./hr./l. of resin. Development and elution of the poison was continued at the same rate. The data presented in Tables I through IV are representative of data obtained on larger or smaller columns. [b] The starting material was a percolate made with 15% ethanol in water at *p*H 3 and adjusted to *p*H 5.6 with 1650 ml. of 1 *M* NaOH prior to absorption on the resin. [c] Buffer, 1.0 *M* HOAc adjusted to *p*H 4.0 with a saturated solution of NaOAc. [d] Fraction transferred to subsequent column.

5500 ± 500 MU per mg. with better yields and greater ease of operation. It also accomplished equally well the purification of the poison from toxic Alaska butter clams (*Saxidomas giganteus*). Although the poison content of clams is usually much lower than that of mussels, toxic clams were important as a source of the poison because the supply was larger and generally more available than from mussels.[4] About two-thirds of the poison found in a clam is contained in the siphon which was used as the starting material in this work. Clams were collected only when the toxicity of the siphons was 5,000 to 10,000 or more MU per 100 g., 0.05 to 0.1 MU per mg. Mussels on the other hand contain 90 to 95% of the poison in the liver or digestive gland, and these were used when the toxicity was 0.5 or more MU per mg. Acidified aqueous extracts from toxic clams usually contained material with a toxicity of about 0.5 to 2 MU per mg. of solids, whereas extracts from mussels usually contained 2 to 8 MU per mg. of solids. The Decalso used in the older procedure[2a] to remove the poison from the crude extracts was very inefficient when the poison was present in concentrations below 3 MU per mg. and therefore ineffective in concentrating the clam poison, whereas the carboxylic acid resins used in the present procedure effected a quantitative recovery of poison from both clam and mussel tissues.

The collection and preparation of the toxic digestive glands of mussels has been described previously.[2] Toxic butter clams were collected in Southeastern Alaska with the coöperation of the Fish and Wildlife Service, U. S. Department of the Interior. The siphons of the clams were removed, canned and frozen until they could be ground with acidified aqueous ethanol. The digestive glands of mussels were removed and ground with acid aqueous ethanol. Under these conditions the poison was stable for long periods.

The aqueous alcoholic suspensions of the ground tissues from toxic mussels or from clams were mixed with Celite 545 to a moist, mealy consistency and the poison extracted by percolation with 15% ethanol acidified to *p*H 2–3 with hydrochloric acid. The recovery of the poison in the extract was 98 to 100%.

Removal of most of the acidic and neutral contaminants was accomplished by adsorption of the poison on columns of the sodium form of the carboxylic acid ion exchange resin, Amberlite IRC-50. A large portion of the sodium ions remaining on the resin were eluted with 1 *M* acetic acid buffered at *p*H 4 with a saturated solution of sodium acetate. Usually less than 5% of the poison was lost by elution in this step. Subsequent fractional elution with 0.5 *M* acetic acid yielded up to 90% of the poison with a toxicity of 100 to 500 MU per mg. Table I gives the results of a typical run on this column. The remaining poison with a lower toxicity was reworked with subsequent batches of poison. The fractions containing material with a toxicity of 100 to 500 MU per mg. were combined and the excess acetic acid removed by vacuum evaporation. Further purification was accomplished by chromatography on the finely divided hydrogen form of the resin, Amberlite XE-64. This chromatogram was developed and the poison eluted with 0.1 *M* acetic acid. Usually two passes through the column brought the toxicity of 70 to 80% of the material to about 2500 or more MU per mg. solids, which appeared to be the practical maximum attainable by the process. Typical runs are presented in Tables II and III. The total recovery of poison from all fractions in any one run was usually 80 to 100%. The less toxic fractions were reworked by the same procedure.

(4) When clams become toxic they usually remain so for at least a year which allows their collection whenever weather and tides permit. In contrast, the source of poison from mussels is limited to a few days when an occasional culture of the toxic dinoflagellate, *Gonyaulax catenella*, grows in the water around the mussel beds and serves as food for the mussels. The primary source of the poison from mussels is in the toxic dinoflagellate *Gonyaulax catenella*, but the primary source of the clam poison is unknown. Many biological properties of clam poison indicated a similarity to mussel poison and the results obtained during the course of purification of both clam and mussel poisons have shown them to be very similar in all biological, physical and chemical properties.

TABLE II

CHROMATOGRAPHY OF MUSSEL POISON FRACTIONS FROM AMBERLITE IRC-50 ON AMBERLITE XE-64[a]

Fraction	Solvent	Vol., l.	Total MU × 10^3	Recovery, %	Solids minus ash,[b] total g.	Toxicity, MU/mg. solids
Starting material[c]	H_2O, *p*H 5.2	6.75	5400	100.0	13.5	400
Filtrate and rinse	H_2O	7.27	136	2.5	2.80	45
Eluates	0.1 *M* HOAc	0.25	36	0.7	1.00	36
	.1 *M* HOAc	.40	334	6.2	0.44	758
	.1 *M* HOAc	.50	720	13.3		
	.1 *M* HOAc	.60	750 [d]	13.9	0.78	962
	.1 *M* HOAc	.50	473	8.7		
	.1 *M* HOAc	.70	1775	32.9	1.19	1500
	.1 *M* HOAc	.85	807	15.6		
	.1 *M* HOAc	1.10	319	5.9	1.54	208
	.1 *M* HOAc	1.30	22	0.4	1.56	14
	.01 *M* HCl	1.00	6	.1	1.50	4
	.1 *M* HCl	1.00	6	.1		
	1.0 *M* HCl	1.00	1	.0		
Total				100.4		

[a] A column (3.3 cm. in dia.) was prepared with 675 ml. of hydrogen Amberlite XE-64 and washed with H_2O. [b] HCl was added to the total solids samples prior to ashing. [c] The starting material consisted of the highest potency eluates from the sodium Amberlite IRC-50 (Table I) which were concentrated somewhat and adjusted to *p*H 5.2 with 10 *M* NaOH prior to absorption. [d] Fractions transferred to subsequent column.

TABLE III

RECHROMATOGRAPHY OF MUSSEL POISON FRACTIONS ON AMBERLITE XE-64[a]

Fraction	Solvent	Vol., ml.	Total MU × 10^3	Recovery, %	Solids minus ash,[b] mg.	Toxicity, MU/mg.
Starting material[c]	H_2O, *p*H 5.0	1000	4800	100.0	5400	890
Filtrate and rinse	H_2O	1300	1	0.3	130	10
Eluates	0.05 *M* HOAc	3500	3	0.8	590	6
	.05 *M* HOAc	250	1	0.1	72	7
	.05 *M* HOAc	550	95	2.2	182	576
	.05 *M* HOAc	220	220	5.0	81	2680
	.05 *M* HOAc	480	625	14.2		
	.05 *M* HOAc	760	1585 [d]	36.0	395	4010
	.05 *M* HOAc	750	1240	28.2		
	.05 *M* HOAc	690	1565	35.6	524	2990
	.05 *M* HOAc	700	4	0.9	175	24
	.05 *M* HOAc	600			144	
	.05 *M* HCl	1000	2	0.6	2100	1
Total				122.8		

[a] A column (3.3 cm. in dia.) was prepared with 376 ml. of hydrogen Amberlite XE-64 and washed with H_2O. [b] HCl was added to the total solids samples prior to ashing. [c] The starting material consisted of the highest potency eluates from the hydrogen Amberlite XE-64 column (Table II) which were concentrated and adjusted to *p*H 5.0 with 10 *M* NaOH. [d] Reassay of this combined fraction indicated a total of 4475 × 10^3 MU of poison which would lower the total recovery to 106%. The specific rotation for this fraction was $[\alpha]^{25}D$ +84°. Part of this combined fraction was transferred to the alumina column (Table IV).

Further purification of the poison to the point where it appeared to be pure was accomplished by chromatography on acid-washed alumina using absolute ethanol as the solvent. The poison was converted to the hydrochloride salt by adding an amount of hydrochloric acid equivalent to the acetate ion present and removing the acetic acid by vacuum distillation. Water was removed from the poison concentrate by co-distillation with absolute ethanol. The ethanolic solution of the poison, after filtration to remove an insoluble precipitate, was applied to the alumina column. Development, of the chromatogram and elution of the poison was accomplished with absolute ethanol and aqueous ethanol (Table IV). Poison with a toxicity of 5500 ± 500 MU per mg. solids, $[\alpha][^{25}D$ +130 ± 5°, was obtained in the first few fractions in 25–45% yields. The remaining fractions of lower toxicity were reworked to increase the total recovery through the alumina to 70–90%.

The recovery of purified poison from the starting material by a single pass through all steps in this procedure ranged from 15 to 30%. The recovery was increased to as high as 50% by reworking the less pure fractions.

Purification of the poison has been achieved in these laboratories by two other procedures which are worthy of note but did not prove practical for several reasons. One of these procedures involved the further purification of the poison in preparations obtained by ion exchange on Decalso and repeated chromatography on acid-washed Norit A[2b] (toxicity about 1000 MU/mg.), by crystallization of the poison several times as the helianthiate followed by further chromatography of the hydrochloride salt of the poison on Norit A. The prod-

TABLE IV

CHROMATOGRAPHIC FRACTIONATION OF MUSSEL POISON ON ACID-WASHED ACTIVATED ALUMINA[a]

Vol., ml.	Solids, mg./ml.	Optical rotation[b] observed (+)	Remarks
Starting material,[c] absolute alcohol			
110	10.0	0.318°	Total MU, 3.4×10^6; $[\alpha]^{25}D$ +79.6°, toxicity 3120 MU/mg.
Eluates, absolute alcohol			
409	0.09		Concn. too low to read
25	0.88	0.228	265 mg. of solids in combined fractions, total MU 1.4×10^6, toxicity 5300 MU/mg.; $[\alpha]^{25}D$ +128°, recovery (MU) 41%
25	1.13	.315	
26		.331	
25	1.32	.353	
50		.333	
25	1.06	.268	
36		.232	
25		.239	
27	0.76	.220	
53		.174	
25	0.68	.152	
Eluates, 95% alcohol			
25		0.095	Recovery (MU) 33%, toxicity 3400 MU/mg., $[\alpha]^{25}D$ +110°
25		.146	
50		.141	
26	1.90	.432	
28	1.47	.359	
50	1.05	.223	
50	0.61	.132	
52	0.30	.078	
Eluates, 50% alcohol			
25	0.16	0.030	Recovery (MU) 6%, toxicity 2200 MU/mg., $[\alpha]^{25}D$ +66°
42		.004	
60	1.64	.208	

[a] A column (1.7 cm. in dia.) was prepared with 100 g. of the acid-washed activated alumina by allowing the powder to settle in absolute ethanol. [b] Optical rotations were determined in a 2-dm. tube. [c] An aqueous solution of the poison was concentrated to about 20 ml. *in vacuo* at 40°, 500 ml. of absolute ethanol added and all but 50 ml. of the solvent distilled off. This operation was repeated again and the volume brought about 100 ml. A flocculent brown precipitate which had separated was centrifuged (48.5 mg., containing 1.1% of the total poison). The clear alcoholic filtrates were applied to the column.

uct obtained had a toxicity of about 5000 MU per mg. and a specific rotation of 122–130°, but the procedure was difficult to carry out and the yields were extremely poor. The other method involved the purification of the poison by countercurrent procedures. These procedures, however, were complicated by the fact that the hydrochloride salt of the poison is soluble only in water, methanol and to some extent in ethanol and quite insoluble in all solvents immiscible in water. A slow decomposition of the poison took place when processing was attempted in alkaline solutions especially at *p*H values greater than 8 or 9. By working at a temperature of 4 to 6° and buffering the solutions at *p*H 8, purification by countercurrent distribution was accomplished with some degree of success when starting with a partially purified preparation (about 3000 MU/mg.) but not with crude preparations. The solvent systems used consisted of a 15% aqueous solution of potassium chloride containing 0.1 *M* potassium carbonate adjusted to *p*H 8 and *n*-propyl alcohol containing 0.5% (v./v.) of α-ethylcaproic acid or one containing *n*-butanol, 95% ethanol, 0.1 *M* aqueous potassium carbonate and α-ethylcaproic acid in a volume ratio of 146:49:200:5 with the aqueous layer adjusted to *p*H 8. Of the two solvent systems the latter proved to be more practical. Although purification from 3000 to 5000 MU per mg. was achieved by countercurrent methods, the preparations were complicated by the presence of large amounts of potassium chloride or the buffer salts in solution with the finally purified poison. Chromatography on acid-washed Norit A[2b] was necessary therefore to remove the salts. The yields of purified poison were quite low. Because of the various difficulties encountered in the latter methods, they were abandoned in favor of chromatography on carboxylic acid resins and alumina.

Some properties of the poison in crude form, such as its strongly basic character, and its solubility and stability have been described previously.[3a,5,6] The purified poisons obtained by the improved procedure were white amorphous solids. Crystallization of the poisons as the chloride, sulfate, acetate or other simple salts was not accomplished, but various physical and chemical tests indicated that they were highly purified when obtained at a toxicity of 5500 ± 500 MU per mg. and a specific rotation of +130 ± 5°. This toxicity places the poisons among the most virulent known to man except for some venoms and certain bacterial toxins. Analysis of the purified clam and mussel poisons, free of ash, and an estimation of their molecular weights indicate that the molecular formula for both is $C_{10}H_{17}O_4N_7 \cdot 2HCl$. Evidence for the purity of the poisons is given in the following paper. Details on the chemical and physical properties of the poisons will be subjects of forthcoming papers.

Experimental

The procedure described below proved to be most satisfactory of those investigated for the purification of the poison found in the siphons of butter clams and in the digestive glands of mussels. The data given in this section are for the largest quantities of material used in the purification procedure. Tables I through IV present data on smaller quantities of material used in developing the purification procedure. These data (Tables I–IV) are presented because a more detailed analysis was made on the various steps than was made when the larger quantities were employed.

Step 1. **Extraction of the Poison.**—The digestive glands (hepatopancreas) of toxic mussels or the siphons of toxic clams were ground in a meat grinder and sufficient 95% ethanol was added to cover the settled mass. This suspension was acidified with concentrated hydrochloric acid to *p*H 2–3 and was well mixed with about one-half the weight of Celite 545 (Johns-Manville) to a moist, mealy consistency. The mixture was packed loosely in a wide diameter filtering device with a pad of glass wool and fresh Celite for support; 15% ethanol in water, acidified to *p*H 2–3, was allowed to filter through the mass under gravity. The purpose of the ethanol in the solvent used for extraction was to reduce the growth of molds and bacteria. Filtrates were collected and assayed at intervals until only negligible amounts of the poison were found. In this way the poison was quantitatively extracted (98 to 100%) from the tissues with a minimum amount of contamination by proteinaceous material. From 600 lb. of poisonous clam siphons of average toxicity (5000 to 10,000 MU per 100 g.), it was possible to extract up to

(5) H. Müller, *J. Pharmacol.*, **53**, 67 (1935).

(6) R. P. Monnier, Thesis, Eidgen. Techn. Hochsch., Zürich, 1938.

27×10^6 MU of poison in 600 liters of solution. This solution contained about 15 kg. of solids regardless of the poison content of the tissues.

Step 2. Ion Exchange on Amberlite IRC-50.—The crude extract obtained in Step 1 was concentrated 15 to 20 times by vacuum evaporation to facilitate handling for fractionation on the sodium form of the carboxylic acid ion exchange resin, Amberlite IRC-50 (Rohm and Haas Co.). The resin, which was obtained in the hydrogen form, was converted to the sodium form by stirring it batchwise with 1 *M* sodium hydroxide[7] until the *p*H remained greater than 10. The sodium resin was then placed in a column and washed with distilled water until the *p*H of the effluent had dropped to about 8. The poison extract was adjusted to *p*H 5.5 with concentrated sodium hydroxide and filtered just prior to adsorption on the ion exchange column. It was passed through a column containing 20 liters of resin in a tube 6 inches in diameter by 6 feet in length at a rate of about 300 ml. per hour per liter of resin. Twenty liters of the settled resin may be expected to absorb the poison from about 750 liters of crude toxic clam extract. The filtrates were assayed periodically to determine when the column had become saturated. When the poison had begun to leak through, the column was rinsed with 5–10 liters of water (rinse 1). Acetate buffer (1.0 *M* acetic acid to which saturated sodium acetate had been added to bring the *p*H to 4.0) was passed through the column (rinse 2) until the *p*H of the effluent had dropped to between 4.0 and 4.5. The column was again rinsed with 5–10 liters of water (rinse 3). Most of the poison was then eluted with 0.5 *M* acetic acid. Up to 90% of the poison was recovered in the acetic acid fractions. The remainder of the poison was found largely in the rinse with the acetate buffer at *p*H 4–4.5 with only 1–2% in the filtrates. The toxicity of the clam and mussel poisons was usually improved about 100 to 200 fold by this treatment. Typical data are presented in Table I.

Step 3. Ion Exchange Chromatography on Amberlite XE-64.—Poison eluates obtained from the ion exchange fractionation in step 2 were evaporated *in vacuo* to a concentration between 500 and 2000 MU per ml. and were absorbed on the hydrogen form of the carboxylic acid resin, Amberlite XE-64 (a finely-divided form of IRC-50). The resin was prepared for use by converting to the sodium form with 1 *M* sodium hydroxide, rinsing with distilled water, reconverting to the hydrogen form with 1 *M* hydrochloric acid and rinsing with distilled water until the *p*H of the rinse was above 4.0. The very fine particles that caused trouble in the flow through the column were removed by decanting the supernatant after the resin had been stirred and allowed to settle for 30 minutes. One liter of settled resin was used for 75 to 150 g. of dissolved solids. The glass column used was 6.5 cm. in diameter and 100 cm. long. Elution was carried out with 0.1 *M* acetic acid chromatographically at a flow rate of about 300 ml. per liter of resin per hr., and the best fractions, as determined by bioassay and solids content, were combined. The combined fractions were rerun in the same way on a fresh column of the resin until the toxicity was 2500 to 3000 MU per mg. of solids and the specific rotation was about +80°. This usually required two passes through the resin with yields of the best fractions totaling about 90% for each pass. The total recovery of poison was nearly quantitative, and most of the side fractions were concentrated and reworked in a similar manner to give increased yields of purified poison. Tables II and III give typical data for this step.

Step 4. Chromatography on Alumina.—The product obtained from step 3 was concentrated *in vacuo* to about 10–15 mg. of solids per ml. after the addition of an amount of hydrochloric acid to replace the acetate ion present. Absolute ethanol was added and the distillation continued until all of the water had been removed and the poison hydrochloride was in an absolute ethanol solution of about 10–15 mg. solids per ml. A small amount of an insoluble precipitate containing some poison was centrifuged from the solution. The clear ethanol solution of the poison was passed into a column of acid-washed activated alumina (40–60 g. of adsorbent per 1 g. of total solids in a column 3.3 cm. in diameter and 90 cm. long) on which all of the solids were adsorbed. The preparation of the acid-washed, activated alumina was carried out as follows: Coarse-powdered, catalyst grade, active alumina (Harshaw Chemical Co., Al-0109P) was stirred with 1 to 2 *M* hydrochloric acid until no change in *p*H was noted when fresh acid was added, and stirring continued for several days (*p*H 0.2 to 0.3). The fines were removed by stirring vigorously then allowing the mixture to settle for 30 minutes and decanting the supernatant. The operation was repeated until the supernatant was clear after settling for 30 minutes. The acid-washed alumina was then rinsed continuously in a column with distilled water until the soluble solids in the effluent were less than 20 mg. per liter of rinse per liter of alumina. The top surface of the column was discarded and the remainder extruded into glass evaporating dishes, dried at 110° for 48 hr. and activated at 260° for 24 hr. The alumina, still hot, was poured into glass-stoppered bottles which were sealed with paraffin until just prior to use. Alumina prepared in this way when suspended in water produced a *p*H of 4.0–4.5. Elution of the poison was carried out with absolute ethanol, 95% ethanol, 50% ethanol and finally water at the maximum flow rate under gravity. The progress of the chromatography was followed by the determination of the optical rotation of the eluates. The use of each solvent was discontinued when the concentration of poison, as estimated from the optical roation, had decreased to about 0.3–0.5 mg. per ml. The poison appeared to be less strongly adsorbed than any of the impurities and purified poison was usually obtained in the early ethanol eluates. In some cases, however, purified poison did not come through until 95% ethanol was used as the eluent. In general, yields of 25–45% of purified poison with a specific rotation of 130 ± 5° and a toxicity of 5500 ± 500 MU per mg. were obtained by this procedure. The total recovery of poison from the alumina column including all side fractions was usually 70–90%. The combined side fractions usually were reprocessed through the column to increase the yield of purified poison.

Step 5. Removal of Alcohol, Acid and Water from Poison.—Evaporation of the alcoholic solutions of the poison to dryness resulted in a loss of toxicity and a decrease in the specific rotation. Mineral acids in strong concentration also reduced the toxicity. The alcohol solution of the poison was diluted with an equal volume of water and the alcohol removed by codistillation under vacuum (about 20 mm. pressure) until the volume was about one-tenth the starting volume. The addition of water was repeated and the volume reduced so that the concentration of poison was 10 to 20 mg. per ml. The solution was then adjusted to *p*H 5 with Amberlite resin IR-45. This solution was allowed to evaporate almost to dryness under vacuum and over P_2O_5 and KOH. It was then diluted 3 to 4 times with water and allowed to evaporate to dryness under the above condition. The dried amorphous material was broken up and powdered and allowed to remain under the drying conditions for at least another week. Material obtained in this manner was a pure white amorphous powder which was quite hygroscopic and had a specific rotation of +130 ± 5° and a toxicity of 5500 ± 500 MU per mg.

Analysis and Molecular Weight.—For elemental analysis and determination of the molecular weight by the cryoscopic method, the purified poison was chromatographed on acid-washed Norit A to remove any traces of salt.[2b] This material had a toxicity of 5500 MU per mg. and $[\alpha]^{20}D$ +133°.

Anal.[8,9] Calcd. for $C_{10}H_{19}N_7O_4Cl_2$: C, 32.26; H, 5.14; N, 26.34; Cl, 19.05; mol. wt., 372. Found for clam poison: C, 32.35; H, 5.08; N (Dumas), 26.42; N (Kjeldahl),[10] 26.83; Cl, 19.33; O (difference), 16.80. Mussel poison: C, 32.39; H, 5.35; N (Dumas), 25.93; Cl, 19.10; O (difference), 17.23; O (direct), 16.79; mol. wt.: (cryoscopic, H_2O), clam poison dihydrochloride, 348; mussel poison dihydrochloride, 342.

The rate of diffusion of the biological activity of both clam and mussel poison was measured in the Northrop dif-

(7) It is desirable at this stage to reverse fresh resin with 1 *M* hydrochloric acid and rinse well with water prior to final conversion to the sodium form for use.

(8) Analysis for C, H, N (Dumas) and Cl were made by J. F. Alicino, Squibb Institute of Medical Research, New Brunswick, N. J.

(9) The direct analysis for oxygen was performed by the Clark Microanalytical Laboratory, Urbana, Ill.

(10) H. A. Lepper, "Methods of Analysis of the AOAC," 6th Ed., Association of Official Agricultural Chemists, Washington, D. C., 1945, pp. 763–765. The ordinary procedure, without addition of reducing agents, was modified by the use of powdered selenium in the digestion mixture.

fusion cell[11] using solutions at concentrations of about 5,000 MU per ml. The diffusion coefficients for both clam and mussel poisons were found to be $4.9 \pm 0.2 \times 10^{-6}$. These diffusion coefficients would indicate a molecular weight of about 300 and in line with that determined by the cryoscopic method.

Bioassay.—Sommer and Meyer[12] published data showing the relation of mouse units to time of death and defined a mouse unit as the amount of poison that would kill a 20 gram mouse in 15 minutes. The most consistent results with the purified poison were obtained when the dilutions were adjusted so that the time of death was in the neighborhood of 5 to 7 min. Previous experiments showed that 0.3 to 0.4 micrograms of poison were required to kill a 20 gram mouse in 5 to 7 minutes. The solutions of shellfish poison for this experiment were therefore diluted to 0.4 microgram of poison per ml. and the *p*H adjusted to 3 to 4. One ml. of each solution was injected intraperitoneally into each of 10 white mice weighing between 19 and 21 grams and the time to death (the last gasping breath) noted. The death times of the ten mice were averaged and this average value used to determine, from the data published by Sommer, the mouse units per ml. of the solution injected into the mice. The dilution factor times this value gave the mouse units per ml. in each prepared solution of poison.

The curve relating time of death to mouse units may be constructed from the following data. Death times of 4, 5, 6, 7 and 8 minutes are equivalent to 2.5, 1.9, 1.6, 1.4 and 1.3 mouse units, respectively, from the Sommer curve.

If the logarithm of the dose is plotted against the reciprocal of the time a straight line is obtained. The dose can be calculated directly from the equation

$$\log \text{dose} = (145/t) - 0.2$$

where t = time of death in seconds and the death occurs between 240 and 480 seconds.

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FREDERICK, MARYLAND
EVANSTON, ILLINOIS
SAN FRANCISCO, CALIFORNIA

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[A CONTRIBUTION FROM THE CHEMICAL CORPS, FORT DETRICK, AND THE CHEMICAL LABORATORY, NORTHWESTERN UNIVERSITY[1]]

Paralytic Shellfish Poison. VII. Evidence for the Purity of the Poison Isolated from Toxic Clams and Mussels

BY JAMES D. MOLD, JOHN P. BOWDEN, D. WARREN STANGER, JOHN E. MAURER, JOSEPH M. LYNCH, RUTH S. WYLER, EDWARD J. SCHANTZ AND BYRON RIEGEL

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The principal evidence for the purity of the paralytic shellfish poisons, with a toxicity of 5500 ± 500 mouse units (MU) per mg. and $[\alpha]^{25}D$ +130 ± 5°, which have been isolated from the siphons of toxic Alaska butter clams and the digestive glands of toxic California mussels is based on the behavior of these compounds and their dihydro derivatives upon countercurrent distribution in the Craig apparatus. Supporting evidence consists of the preparation of biologically identical material by several diverse procedures and evidence that all impurities known to be present in the crude starting extracts have been removed from the purified product. Paper chromatography of the purified clam and mussel poisons and their dihydro derivatives lends strong support to the contention that these two poisons are very similar in structure, if not identical.

Methods for the purification of the paralytic poison found in the siphons of toxic Alaska butter clams, *Saxidomus giganteus*, and in the digestive glands of toxic California mussels, *Mytilus californianus*, have been presented in a previous report.[2] The final product obtained in each case had a toxicity of 5500 ± 500 MU per mg. and $[\alpha]^{25}D$ +130 ± 5°. Crystallization of the poisons as salts of any simple inorganic anion with characteristic melting points was not accomplished, and consequently other methods of establishing their purity were investigated. It is the purpose of this report to discuss the evidence obtained which indicates that the poisons with the above toxicity and specific rotation are in a highly purified state.

Countercurrent distribution of the hydrochloride salts of the poisons in the Craig apparatus is made difficult by their limited solubility in solvents immiscible with water.[3] In strong alkali, which is necessary to liberate the free base, a rapid loss of toxicity was noted. This inactivation was increased when high concentrations of alcohol and some other organic solvents were used in alkaline solutions of the poisons. By working at low temperature (5 to 10°) and by buffering the solutions at *p*H 8, a suitable solvent system was developed for the countercurrent distribution of the shellfish poisons. This system consisted of a mixture of *n*-butyl alcohol, 95% ethanol, 0.1 *M* aqueous potassium bicarbonate and α-ethylcaproic acid in a volume ratio of 146:49:200:5 with the aqueous layer adjusted to *p*H 8. The distribution of some model substances in this system was determined to test the selectivity of the fractionation. The partition coefficient for arginine was 0.54, creatinine 0.37 and betaine 0.45 compared to clam and mussel poisons which had a coefficient of about 3.

The most important evidence for purity was the

(1) The work at this Laboratory was supported under a contract with the Chemical Corps.

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