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II. Some Chemical and Physical Properties of Paralytic Shellfish Poisons Related to Toxicity

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The production of a toxin by a particular species is often explained as a defence mechanism to protect it from other invading or devouring species. Certain species of dinoflagellates of the genera *Gonyaulax*, namely *G. catenella* and *G. tamarensis* produce an extremely potent poison that has no detectable effect on shellfish that consume it as food but has served to a great degree to discourage the human consumption of many species of shellfish because of the accumulation of this poison in various organs of the shellfish. During a series of outbreaks of shellfish poisonings along the California Coast between 1920 and 1936 Sommer, Whedon, Kofoid, and Stohler[1] found that the California sea mussel became toxic only when *G. catenella* was present in the waters bathing the mussels, and that this organism produced a poison which was retained by the dark gland or hepatopancreas of the mussel. The poison causes no observable disturbance of the natural life processes of the mussel but is lethal to persons unfortunate enough to eat the mussels at the time that *G. catenella* is growing in the water bathing the mussels. A historical review of these developments has been presented elsewhere.[2] 'Mussel poisoning' in humans as described by Sommer and Meyer[3] usually develops within $\frac{1}{2}$–3 h after consuming even a few toxic mussels. A numbness in the lips and finger tips is followed by an ascending paralysis and finally death from respiratory paralysis within 3–20 h, depending upon the magnitude of the dose. If a person survives as long as 24 h the prognosis is good, and as far as is known there are no lasting effects of the ordeal. Poisonings have occurred mainly along the Pacific Coast of the United States, Canada and Alaska, the estuaries of the St. Lawrence River, the Bay of Fundy region, areas around the North Sea and the English

Channel, and in South Africa. In these areas mussel poisoning is a local public health problem but has become of greater importance because of the commercial shipment of shellfish to other areas.[4]

The poison has never been isolated in pure form directly from *G. catenella*, but has recently been isolated from California mussels (*Mytilus californianus*) and from Alaska butter clams (*Saxidomas giganteus*) through the combined efforts of workers at the Hooper Foundation of the University of California, Northwestern University, and the Chemical Corps Biological Laboratories.[5] A comparison of the properties of these purified poisons with those of the poison from *G. catenella* indicates that the poison found in this organism is retained by the mussel unchanged. Chromatographic studies carried out by Burke *et al.*[6] have shown that the poison from mussels and that from the organisms move together. Schantz has studied the gross physiological properties, the stability to acid, alkali, and heat, and the diffusion rates of the two poisons; in every respect the poison appears unchanged. Provasoli[7] has achieved the isolation of *G. catenella* free of bacteria and has found that the poison is a metabolic product of the organism and not the result of a symbiotic effect of the bacteria usually associated with it. It appears therefore that the mussel poison, and quite likely other poisons of this type in shellfish, are the products of micro-organisms of the sea.

Many attempts have been made to purify the poison from mussels.[2] In some of the early work, Sommer[8] and Muller[9] pointed out the basic character of the mussel poison and employed Decalso as a cation exchange resin, along with chromatography on carbon for purification. These methods, however, brought about a partial purification only. The Decalso was not efficient in the removal of the poison from crude extracts, and a great portion of it was lost. Studies by Schantz *et al.*[5] have shown that the most practical method for highest yields and purity is by chromatography on carboxylic acid exchange resins, followed by chromatography on acid-washed alumina. Although considerable success was achieved in purifying the poison from California mussels and Alaska butter clams by this procedure, it was not successful for purifying the poison occurring in toxic scallops. The poison found in scallops is believed to have its origin in *G. tamarensis*.[10] This difference is believed to be due to a slightly

different basicity of the scallop poison. The primary source of the poison occurring in butter clams is not known, but some evidence exists that it may be produced by *G. catenella*.[2]

For the purposes of this symposium, only the purified mussel and clam poisons will be discussed. These poisons were not obtained in crystalline form as simple salts from solution, although the dried preparations often appeared crystalline. Evidence for the purity of the poisons was obtained through countercurrent

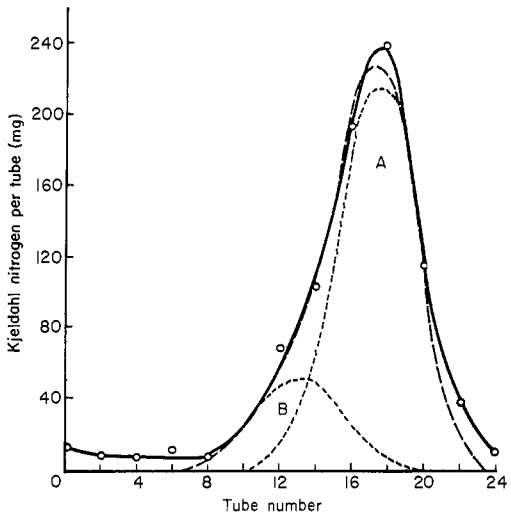

Fig. 1. —— Countercurrent distribution of purified mussel poison: o–o–o, actual distribution of nitrogen; – – – –, calculated distribution of theoretical components; — —, sum of components A and B. The data are representative of the distribution of purified clam poison also

distribution studies. Mold *et al.*[11] found that distribution in a solvent system of *n*-butanol, ethanol, 0·1M aqueous potassium carbonate, and α-ethylcaproic acid in a volume ratio of 146:49:200:5, with the aqueous layer adjusted to pH 8, resulted in the separation of the poisons into two components. The component present in the larger amount, ($K = 2 \cdot 7$), A, Fig. 1, was somewhat more toxic than the smaller component B ($K = 1 \cdot 2$). When either A or B was isolated, allowed to equilibrate in acid solution, and re-run through the distribution, a similar distribution into

two components occurred. It was assumed therefore that the poisons exist in two tautomeric forms. Analyses of the components indicated that the poisons had a purity of at least 95 per cent.

Studies on the two poisons have shown that they must be similar if not identical in chemical structure. Both are basic substances forming salts with mineral acids. They are very stable in acid solution but labile in alkaline solution when exposed to the air. The dihydrochloride salts are white hygroscopic solids, very soluble in water, soluble to some extent in methanol and ethanol, and insoluble in all lipid solvents. They have a specific optical rotation of $+130°$, show no absorption in the ultraviolet, and have the same infrared spectra with strong absorption at 3, 6, and 9 μ (Fig. 2). Two basic functions are present in equivalent amounts, pK_a 8·1 and about 11·5. The molecular formula was found to be $C_{10}H_{17}N_7O_4 \cdot 2HCl$, the molecular weight 372. The toxicity in terms of the average minimum lethal dose is $5 \cdot 5 \times 10^6$ MU* per gram of poison. This amounts to 0·18 μg for a mouse or 9 μg/kg body weight. The intravenous dose for a rabbit is 3–4 μg/kg body weight. The oral dose for a human has been estimated from accidental poisonings by Meyer[12] to be at least 20,000 MU, but Bond and Medcof[13] and Tennant *et al.*[14] believe that the dose may be as low as one-third of this value. In terms of the purified poison, the minimum lethal oral dose for man would be between one and four mg.

Because of the strongly basic group one might expect the poison to be an amine or a quaternary ammonium compound. Methylation studies on the poison indicate that such structures are not present. Certain colour tests have been helpful in yielding information about the molecule. The poisons react with certain aromatic nitro compounds to form coloured complexes in much the same way as creatinine reacts with dinitrophenol in the Jaffe test and with dinitrobenzoic acid in the Benedict–Behre test. A comparison of the absorption spectrum of the colour complex formed when the poisons and creatinine are reacted with the Jaffe or Benedict–Behre reagents suggests that at least some part of the

* The mouse unit (MU) is defined as the minimum quantity of poison to kill a 20-g white mouse (Webster Strain) in 15 min when one ml of poison solution is injected intraperitoneally.

structure of the poison must be similar to that of creatinine Other compounds such as hydantoin and certain barbituric acid derivatives that give the test showed marked differences from the

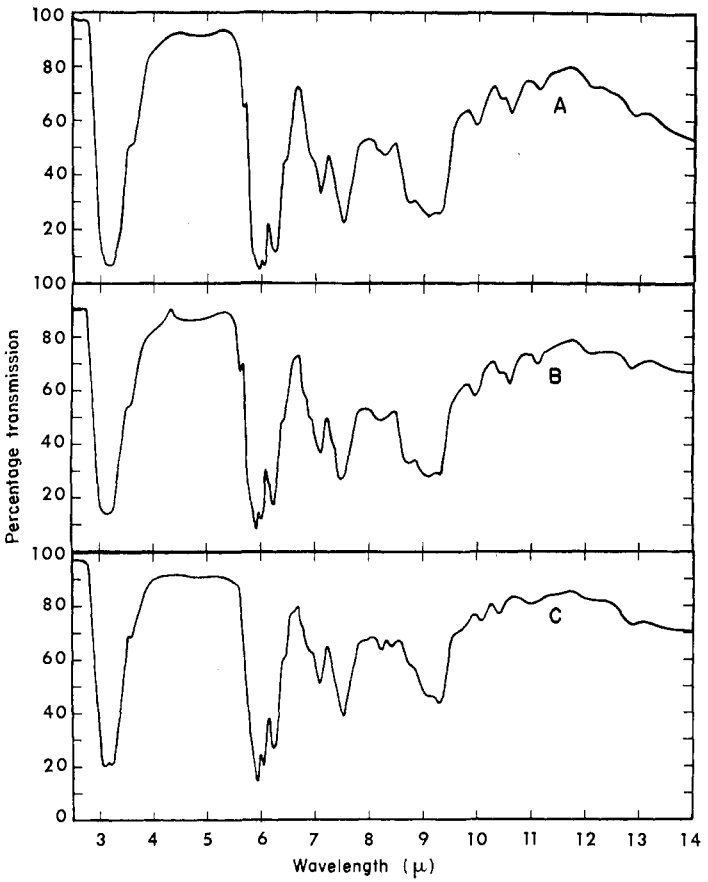

Fig. 2. Infrared spectra of clam and mussel poisons. A. Clam poison. B. Mussel poison. C. Dihydro clam poison dihydrochloride. Comparable spectra were obtained for films of the poisons on KRS–5 (potassium thallium bromide) and sodium chloride and in pellets of potassium bromide

poisons and creatinine in absorption characteristics of the colour complexes. The Irrevere–Sullivan test for creatinine-like compounds was found to be negative. The Weber nitroprusside test

commonly used for substituted guanidines was positive for the poisons but the Sakaguchi test was negative, indicating that a free guanidinium group such as exists in arginine is not present in the poison. Negative tests were also obtained for the Weygand and Csendes test for enols of 1,3-diketones, the Fearon and Mitchell test for primary and secondary alcohols, and the Benedict test for reducing sugars.

Howard *et al.*[15] found that the poisons were hydrogenated at 1 atm pressure in the presence of a platinum catalyst to produce a completely nontoxic dihydro derivative, $C_{10}H_{19}N_7O_4 \cdot 2HCl$. The loss in toxicity was directly proportional to the uptake of hydrogen. This derivative no longer reacted with the dinitro compounds to yield a colour complex. The presence of the unsaturated bond therefore appears to be a vital part of the toxic structure. The same bond is involved also in the reactions with the Jaffe and Benedict–Behre tests. The Weber nitroprusside test, however, remained positive for the nontoxic reduced derivative.

Distribution studies on the dihydro derivative showed that the tautomeric forms were no longer present and that the substance moved as a single component as illustrated in Fig. 3. These data are additional evidence for the purity of the poisons.

Upon oxidation of the poison in mild alkaline solution [0·25M Ba $(OH)_2$] exposed to the oxygen of the air, as carried out by Wintersteiner *et al.*,[15] the toxicity decreased in direct proportion to the oxygen uptake, and at the consumption of one mole of oxygen all toxicity was gone. In this case, too, the Jaffe and Benedict–Behre tests were negative and the Weber test positive. In addition, other breakdown products were formed that gave a positive Sakaguchi test. The mixture possessed a molecular extinction of 6,000 to 7,000 in the ultraviolet at 235 and 333 mμ (Fig. 4). None of these products was isolated in pure form, but the absorption and fluorescent properties were suggestive of pteridine-like compounds. The reduced derivative was not oxidized in alkaline solution under the above conditions. This fact again indicates that the unsaturated bond is at least one of the vital links in the toxic structure of the poison and appears to be the point at which oxidation of the poison starts when exposed to air at pH values higher than seven. The good correlation of the

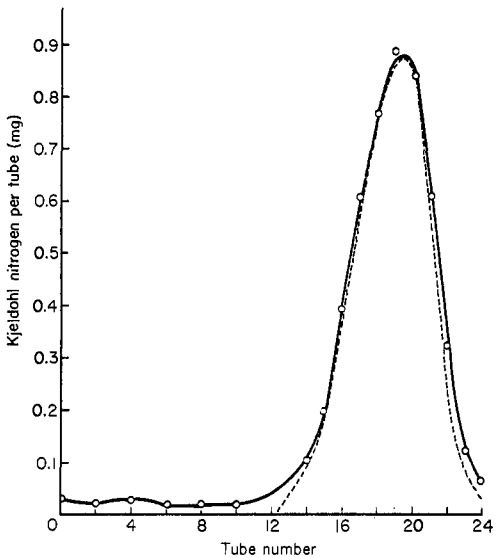

Fig. 3. —— Countercurrent distribution of dihydro clam poison: o–o–o, actual distribution of nitrogen; – – – –, theoretical distribution calculated for a single component. The data are representative of the distribution of dihydro mussel poison also

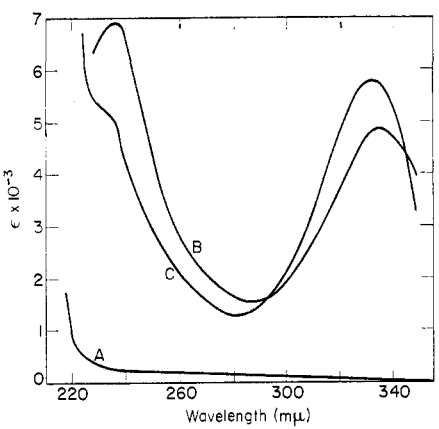

Fig. 4. Ultraviolet spectra of clam poison and its oxidation products. A. Clam poison. B. Clam poison after mild oxidation in alkaline solution exposed to air. C. Products of B in acid solution at pH 1

Jaffe test with toxicity suggested its use as a chemical test for the poison, and methods have been worked out for its use in this respect.[11,16]

Stronger oxidation, such as with periodate and permanganate or strong acid hydrolysis, yielded Sakaguchi-positive compounds. Guanidopropionic acid, urea, ammonia, carbon dioxide, and guanidine have been isolated and identified among the resulting oxidation products.

The relation of optical rotation to the toxicity of the molecule is not exactly clear, but all indications are that the structure responsible for toxicity is not involved in the optical centre. Reduction of the poison to the nontoxic dihydro derivative did not change the optical rotation. When the poison was placed in alkaline solution the optical rotation dropped from $+130°$ to $+50°$. Under anaerobic conditions the toxicity did not change and the optical rotation stayed in this region. However, under aerobic conditions at room temperature the toxicity dropped to zero in about 24 h, and the optical rotation dropped to $-40°$ followed by a rise in rotation to $0°$ as the ultraviolet absorption reached a maximum. These points appear important from the standpoint of probable synthesis of the poison. If the optical centre is not involved in the toxic structure, the synthesis of the structure responsible for biological activity should be a greater possibility.

One of the difficulties encountered in work on the chemical structure of the poison was the isolation of a degradative product that could be easily identified and would still represent a fair portion of the intact molecule. Past attempts met with only limited success. All products were either too large and too close to the parent structure or too small to be of great value in postulating a structure. Rapoport[17] has recently reported the isolation of an eight-carbon fragment, probably a pyrrolo-[1,2-*c*]-pyrimidine, from a reduction with phosphorus and hydriodic acid in glacial acetic acid. A structure of this size would be a great step toward elucidating the structure of the poison.

Certain properties of the poisons allow additional conclusions on the structure. The ultraviolet and infrared spectra indicate no aromatic structures, conjugate unsaturation, or isolated carbonyl groups. The elemental ratios, of course, require that

ring structures be present. The high nitrogen content and the absence of nitro and nitrogen-to-nitrogen bonds as indicated by Kjeldahl analyses strongly suggest that several of the nitrogen atoms must be involved in a heterocyclic structure. Titration suggests that one basic function (pK_a 11·5) may be a guanidinum group and the other (pK_a 8·1) an amine.[18, 19]

Although the physiological action of the poisons has sometimes been described as curare-like, those substances that inhibit the action of curare have no inhibitory action on these poisons.[20] The action, however, appears to be mainly at the myoneural junction. No antidote for the poisons is known. The administration of atropine to mice or rabbits challenged with shellfish poison did not change the symptoms or death time and this fact would indicate that the poison is not a cholinesterase inhibitor. Bolton *et al.*[21] have published similar conclusions from studies on end-plate potentials. No effect of the poisons has been found on the biochemical mechanisms of muscular contraction. Isolated muscle fibres (psoas from rabbit) in the presence of ATP and magnesium ions, as described by Szent-Györgi,[22] contract in the normal manner even when suspended in a solution of the poison at a concentration of 1 mg/ml.[23] When the poison was introduced into a Warburg flask containing respiring diaphragms, freshly removed from mice, no change in the rate of oxygen consumption was noted. O'Neill *et al.*[24] have found that the mechanism converting ADP to ATP from creatinine phosphate is not inhibited by the poison.

The value of studies on toxins and poisons lies in the use of these substances for the elucidation of biochemical and physiological mechanisms. To date no other known chemical structure has been shown to produce the same physiological action as that of the shellfish poisons. This would suggest that the unique structure of these poisons offers a novel tool for the elucidation of some biochemical and physiological mechanisms at the myoneural junctions.

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