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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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.

(2) E. J. Schantz, J. D. Mold, D. W. Stanger, J. Shavel, F. J. Riel, J. P. Bowden, J. M. Lynch, R. S. Wyler, B. Riegel and H. Sommer, THIS JOURNAL, **79**, 5230 (1957).

(3) H. Sommer, R. F. Monnier, B. Riegel, D. W. Stanger, J. D. Mold, D. M. Wikholm and E. S. Kiralis, *ibid.*, **70**, 1015 (1948).

data obtained from countercurrent distribution of dihydro clam poison. The dihydro poison was prepared by reduction of the poison with hydrogen at one atmosphere of pressure over Adams platinum catalyst. The distribution of the dihydro clam poison with the solvent system described above accurately fit the theoretical distribution curve calculated for a single component (Fig. 1).[4]

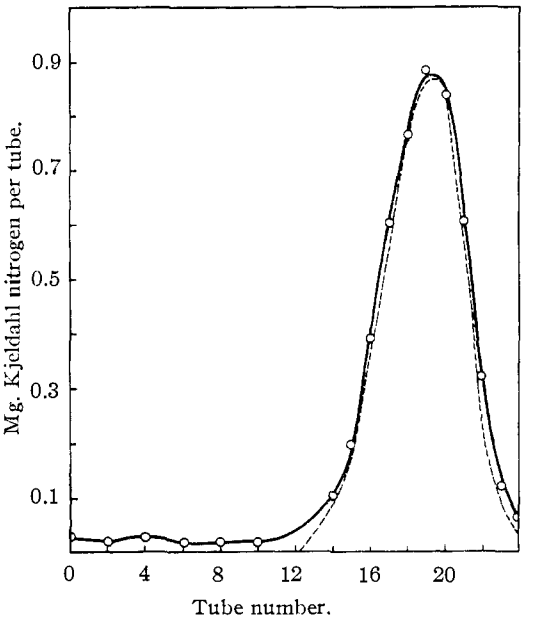

Fig. 1.—Countercurrent distribution of dihydro clam poison: O—O, actual distribution of nitrogen; ---------, theoretical distribution calculated for a single component. The data are representative of the distribution of dihydro mussel poison also.

The data used in plotting the curve were determined by micro-Kjeldahl analysis for nitrogen. The distribution of non-reduced purified clam poison by the Craig technique also provided evidence for the purity of the compounds, but the data indicated that the poison consisted of a mixture of at least two components. These two components, designated A and B, are believed to be tautomeric forms of the poison, because after reduction the molecule apparently was stabilized and exhibited only one component on countercurrent distribution. The possibility that the distribution obtained for the poison was a result of chemical change produced by the solvent system during the extraction was ruled out since this should have produced a skewed curve rather than one which could be represented by the sum of two theoretical curves. Mussel poison and dihydro mussel poison exhibited behavior similar to that of clam poison and dihydro clam poison. Only one component was observed on countercurrent distribution of the dihydro mussel poison and two components, A (K = 2.7) and B (K = 1.2) Fig. 2, on distribution of the non-reduced poison. When the contents of tubes containing the component A present in largest amount were combined, concentrated and redistributed with this solvent system, a distribution pattern was obtained which was comparable to that obtained originally. Similarly, when the contents of tubes containing the component B present in lesser amount were combined, concentrated and redistributed, with this solvent system the reappearance of the original pattern made it apparent that an equilibrium of the components A and B had been partially re-established. A third component with a maximum at tube 4 sometimes appeared after prolonged handling of the poison. This substance was non-toxic and did not occur in any fixed ratio with respect to either A or B. It was assumed to be an irreversible decomposition product of the poison resulting from the long exposure to the conditions under which the distributions were carried out.

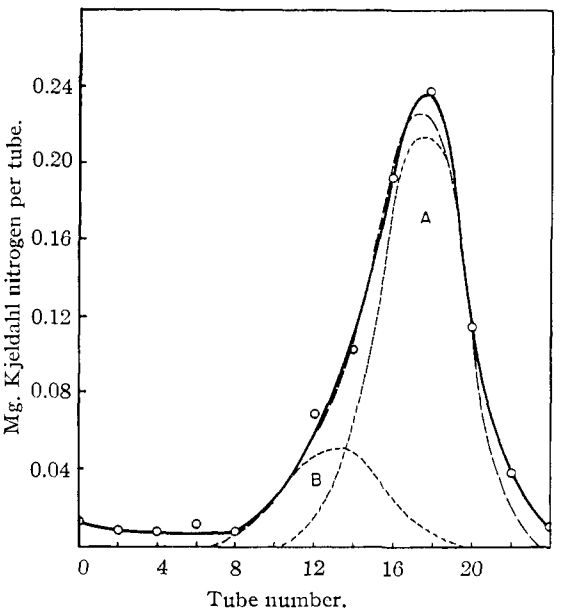

Fig. 2.—Countercurrent distribution of purified mussel poison: O—O, actual distribution of nitrogen; -----, calculated distribution of theoretical components; — —, sum of components A and B. The data are representative of the distribution of purified clam poison also.

The distribution of the poison on the basis of MU followed the distribution of nitrogen for combined components A and B, when a period of equilibration was allowed. The reduced poison was not toxic and of course could not be followed by bioassay. The bioassay when carried out immediately after the distribution was complicated to some extent by the fact that the assays in tubes containing mainly component A were considerably higher in the number of MU than the nitrogen content would indicate and tubes containing mainly component B were somewhat lower. The conversion from the nitrogen analysis to MU was based on 26.3% nitrogen in the poison and a toxicity of 5500 MU per mg. of poison. However, when the solutions of each tube were acidified and allowed to stand 16 to 24 hr., both components showed the number of MU to be in agreement with the nitrogen content.

Since the average error of the nitrogen content, as determined by micro-Kjeldahl analysis, was about 5%, the Craig countercurrent distribution procedure indicates that the purity of clam and mussel

(4) B. Williamson and L. C. Craig, *J. Biol. Chem.*, **168**, 687 (1947).

TABLE I

PAPER CHROMATOGRAPHY OF CLAM POISON, MUSSEL POISON AND THEIR CORRESPONDING DIHYDRO DERIVATIVES

	R_f values for:							
	Clam poison as indicated by			Mussel poison as indicated by			Dihydro clam poison as indicated by Weber[11]	Dihydro mussel poison as indicated by Weber[11]
Solvent system	Weber[11]	Jaffé[9]	Assay	Weber[11]	Jaffé[9]	Assay		
Phenol, water (4:1)	0.29	0.26	0.29	0.29	0.28[a]	0.30	0.38	0.36
t-Butyl alcohol, acetic acid, water (2:1:1)	.30	.30	.28	.29	.30[a]	.30	.28	.31
t-Amyl alcohol, pyridine, water (7:7:6)	.44	.43	.49	.47	.45	.50	.43	.41
n-Butyl alcohol satd. with water ctg. 2% *p*-toluenesulfonic acid	.40	.42	.41	.38	.34	.38	.35	.32
Tetrahydrofurfuryl alcohol, water (17:3)	.57	.59	.60	.57[a]	.57[a]	.58	.57[a,b]	.58[a,b]
Chloroform, *i*-butyl alcohol, water (1:3:1)	.00	.00	.00	.00	.00	.00	.00	.00

[a] A weak spot was observed with R_f 0.10–0.15. [b] A weak spot was observed with R_f 0.29–0.35.

poisons, with a toxicity of 5500 ± 500 MU per mg., should be within 95–100%.

Both clam and mussel poison hydrochlorides, purified by (a) ion exchange chromatography on Amberlites IRC-50 and XE-64 followed by chromatography on alumina, (b) chromatography on Norit A and crystallization as the helianthate and (c) by countercurrent distribution procedures, resulted in a product with a toxicity of approximately 5000 MU/mg.[2] The fact that a product of the same toxicity was obtained by each of a variety of procedures and that no significant enhancement of the purity could be achieved by a repetition of any of these procedures is additional evidence for the purity of the poison.

A number of substances which have been identified as constituents of crude extracts of mussel poison are: taurine, tyrosine, betaine, choline and homarine.[5] An unknown substance with a characteristic ultraviolet absorption maximum at 273 mμ was present in partially purified poison concentrates.[5] Glutamic acid, alanine and tryptophan, as well as some unidentified ninhydrin and Sakaguchi-positive substances, were detected in the impure poison extracts by chromatography on paper and on carbon columns. Arginine was found in the product prior to the final step in the purification of mussel poison by the routine procedure mentioned above. It was identified by the microbiological assay procedure of Henderson and Snell,[6] paper chromatographic studies with several solvents and by comparison of the crystalline flavianate and benzylidine derivatives prepared from arginine. Several substances of unknown structure were present in various fractions from chromatography of the poison. These were detected by the Sakaguchi test, ninhydrin test or by their characteristic ultraviolet absorption maxima.

All of these impurities, known to be present in crude mussel poison extracts, have been removed from the purified product. This was demonstrated by: (a) the absence of sulfur in the purified product, (b) negative ninhydrin test,[7] (c) negative Sakaguchi test[8] and (d) the absence of any maximum beyond 230 mμ in the ultraviolet absorption spectrum of the purified poison. Betaine and choline, which would not be detected by any of the above tests, were not adsorbed by Norit A under the conditions used for chromatography of the poison and were undoubtedly removed by this treatment.[2] These substances also showed rather widely different values for partition coefficients from those of the poison in the solvent system used for Craig countercurrent distribution and should have been detected by this method.

Both mussel and clam poison dihydrochlorides behaved similarly upon paper chromatography with several solvent systems. This similarity was also observed for the dihydro derivatives of both poisons (Table I).

A positive reaction is given by the purified poison with the Jaffé,[9] Benedict–Behre[10] and Weber oxidized nitroprusside[11] reagents. It has not been found possible to separate from the purified poisons any non-toxic impurities which give positive reactions with any of these reagents.

Experimental

Countercurrent Distribution of Dihydro Clam Poison.—A portion of dihydro clam poison dihydrochloride weighing 22.1 mg., prepared by hydrogenation of purified clam poison dihydrochloride in aqueous solution with hydrogen gas at atmospheric pressure over Adams platinum catalyst,[12] was dissolved in 8 ml. of the aqueous or stationary phase of the solvent system and placed in the first tube of a conventional Craig apparatus (stainless steel, 24 tubes, 8 ml. per phase per tube). The alcoholic phase was added, and the remaining tubes were filled with 8 ml. of each phase. The solvent system was prepared by equilibrating *n*-butyl alcohol, 95% ethanol, 0.1 *M* aqueous potassium bicarbonate and α-ethylcaproic acid mixed in a ratio of 146:49:200:5 (v./v.) and adjusted to *p*H 8. Twenty-four transfers were applied at +6°. At the conclusion of the extraction each tube was acidified immediately and the contents analyzed by a micro-Kjeldahl procedure for nitrogen. The distribution of nitrogen is illustrated in Fig. 1. In this and subsequent countercurrent distribution experiments, the nitrogen values were based on an average of 2 to 4 determinations per tube. Dihydro mussel poison was similarly treated.

(5) B. Riegel, D. W. Stanger, D. M. Wikholm, J. D. Mold and H. Sommer, *J. Biol. Chem.*, **177**, 1 (1949).

(6) This assay was performed by Miss Ann Gerhard according to the procedure outlined by L. M. Henderson and E. E. Snell, *J. Biol. Chem.*, **172**, 15 (1948).

(7) A negative test was obtained with 50 μg. of purified mussel poison dihydrochloride under the same conditions that positive tests were obtained for 0.5 μg. of alanine, tyrosine, tryptophan and glutamic acid.

(8) The method used was essentially that of A. A. Albanese and J. E. Frankston, *J. Biol. Chem.*, **159**, 185 (1945). A negative test was obtained with 1.62 mg. of mussel poison dihydrochloride under conditions which gave a positive reaction with 5 μg. of arginine hydrochloride.

(9) R. W. Bonsnes and H. H. Taussky, *J. Biol. Chem.*, **158**, 581 (1945).

(10) S. R. Benedict and J. A. Behre, *ibid.*, **114**, 515 (1936); W. D. Langley and M. Evans, *ibid.*, **115**, 333 (1936); J. J. Carr, *Anal. Chem.*, **25**, 1859 (1953).

(11) C. J. Weber, *J. Biol. Chem.*, **78**, 465 (1928).

(12) The hydrogenation was performed by Dr. William L. Howard.

Countercurrent Distribution of Mussel and Clam Poison.—A 6.5-mg. sample of purified mussel poison dihydrochloride (5500 MU per mg.) was dissolved in 8 ml. of the lower phase of the solvent system and placed in the first tube of the Craig apparatus with an equal volume of the upper phase. Twenty-four transfers were applied as described above. The distribution of poison was determined by bioassay[13] and the nitrogen distribution determined as described above. Results on the distribution are presented in Fig. 2. A portion of clam poison weighing 31 mg. also was distributed in the Craig apparatus and similar results were obtained.

The distribution coefficients of the two components of clam and mussel poisons sometimes varied as much as 25% in different experiments. This variability may be due in part to the difficulty in fitting the best theoretical curve to the observed data. An error of one tube in the placement of the maximum of curve A would produce a variation of about 25% in the calculated distribution coefficient. Some variation was also believed to be due to difficulties in controlling the temperature of the room in which the countercurrent distributions were performed.

Redistribution of Fractions A and B.—A sample of purified mussel poison dihydrochloride weighing 25 mg. was distributed through 24 transfers in the Craig apparatus at +6° with the solvent system described previously. At the conclusion of the experiment, the content of each tube was acidified prior to bioassay and analysis for nitrogen by the micro-Kjeldahl procedure. The poison appeared to be partially separated into a major component with a partition coefficient of 3.2 and a minor component with a partition coefficient of 1.2. The poison contained in tubes 18 through 24 had a toxicity of 23,200 MU per mg. of nitrogen or 5500 MU per mg. of poison. The bioassay on all tubes showed a quantitative recovery of the poison.

The contents of tubes 16 through 21 from the extraction described above, containing only the major component A, were concentrated to 5 ml. The *p*H of the poison solution was adjusted to 8 with concentrated potassium hydroxide, and the appropriate amounts of ethyl alcohol, *n*-butyl alcohol, α-ethylcaproic acid and aqueous potassium bicarbonate were added to give 32 ml. of the two-phase solvent system. This was divided equally between the first two tubes of the Craig apparatus and equal amounts of fresh solvent and aqueous layers added to the other tubes. Potassium chloride was added to each of the tubes which did not contain the poison in order to adjust the salt content to 1.4%, the amount present in the tubes containing the poison. Twenty-four transfers were applied.

The contents of tubes 9 through 15 from this extraction containing predominantly the minor component B were treated in a similar manner as described above for component A.

Paper Chromatography of Clam and Mussel Poison Dihydrochlorides and of Their Dihydro Analogs.—Four microliters of a solution of the test compound containing 50 mg./ml. was applied near the bottom of strips of Whatman No. 1 filter paper (12.5 × 38 cm.). After drying in air, the strips were dipped into the appropriate solvent contained in a cylindrical glass battery jar (13 × 45 cm.) to a depth of about 0.25 inch. The upper edge of the paper was attached by means of spring clips to a glass rod fastened to a ground glass plate which served as the cover. Yellow petrolatum was used to seal the cover. The solvent was allowed to ascend the paper for 16 hr. The strips were air-dried and sprayed with the Weber reagent[11] and Jaffé reagent[9] or cut into 2-cm. sections and eluted with water for bioassay (Table I).

Determination of Microgram Quantities of Nitrogen by a Kjeldahl Procedure.—The following digestion procedure was used for micro-Kjeldahl nitrogen determinations on samples containing up to 300 mg. of salt. Five-tenths ml. of 9 *N* sulfuric acid, containing 0.1% red mercuric oxide, was added to 2 ml. or less of a sample containing 0.5 to 10 μg. of nitrogen. The mixture was heated in an 18 × 150 mm. Pyrex test-tube at 150° on a sand-bath covered with an asbestos plate through which holes were bored to admit the tubes. Heating was continued until all of the water was driven off, then the tubes were covered with small beakers and digested at 270–300° for 18 hr. The entire operation was carried out in an ammonia-free atmosphere. After cooling to room temperature the colorimetric determination of the ammonia nitrogen produced was carried out directly on the digestive mixture according to the Borsook method,[14] or the solutions were made strongly alkaline (*ca.* 2 *M* sodium hydroxide), the ammonia diffused into one drop of 1 *N* sulfuric acid[15] and then determined either by the Borsook procedure or by Nesslerization.[16]

(13) Bioassay was carried out as described previously.[2] It was necessary to correct for the effect of the inorganic salts, alcohol and *p*H upon the bioassay in order to obtain reliable values. This was accomplished by the use of standard curves determined for the purified poison containing known amounts of these contaminants. After acidification of the contents of the tubes from the Craig extraction, it required several hours before the bioassay increased to its equilibrium value.

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(15) R. C. Hawes and E. R. Skavinski, *Ind. Eng. Chem., Anal. Ed.*, **14**, 917 (1942).

(16) M. J. Johnson, *J. Biol. Chem.*, **137**, 575 (1941).

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[CONTRIBUTION FROM THE KETTERING-MEYER LABORATORY,[1] SOUTHERN RESEARCH INSTITUTE]

Synthesis of Potential Anticancer Agents. IX. 9-Ethyl-6-substituted-purines[2]

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Several 9-ethyl-6-substituted-purines have been prepared from 6-chloro-9-ethylpurine. This compound was prepared by reaction of 5-amino-4,6-dichloropyrimidine with ethylamine and cyclization of the resulting 5-amino-6-chloro-4-ethylaminopyrimidine to the purine by the use of diethoxymethyl acetate.

Recently Robins and Lin reported[3] the synthesis of several 9-methyl-6-substituted purines. One of them, 6-chloro-9-methylpurine, has shown the same order of activity against Adenocarcinoma 755 in C57 black mice as 6-chloropurine,[4] and two other 9-methylpurines have shown lesser activity[4] against this tumor. These results make the investigation of other 6,9-disubstituted purines of great interest. This paper, the first of a series dealing with the synthesis of 6,9-disubstituted purines, is concerned with 9-ethyl-6-substituted purines.

Few 9-ethylpurines are reported in the literature,[5] and these are not prepared by methods which are generally applicable. For this series of 9-ethyl-6-substituted purines a procedure similar to that of

(1) Affiliated with the Sloan-Kettering Institute. This work was supported by funds from the C. F. Kettering Foundation.

(2) Part VIII, B. R. Baker and Kathleen Hewson, *J. Org. Chem.*, in press.

(3) R. K. Robins and H. H. Lin, THIS JOURNAL, **79**, 490 (1957).

(4) H. E. Skipper, J. R. Thomson and R. K. Robins, unpublished data.

(5) (a) E. F. Armstrong, *Ber.*, **33**, 2314 (1900); (b) H. Biltz and K. Strufe, *Ann.*, **423**, 1215 (1921); (c) B. R. Baker, R. E. Schaub and J. P. Joseph, *J. Org. Chem.*, **19**, 639 (1954).