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Chemical Studies of Paolin II, an Antiviral Substance from Oysters. (31514)

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The antimicrobial activity of substances named "paolins" isolated from marine animals has been described(1-7). The "paolins," active *in vitro* as well as *in vivo* are perhaps a new class of substances concerned with defensive mechanisms of animals against invading microorganisms including viruses(6, 8). These substances seem to be normal constituents of water or acetic extracts of all mollusc species thus far studied. This report concerns further chemical purification and characterization of an antiviral substance (paolin II) isolated from oysters.

Material and methods. Fresh frozen oysters bought at a local market were weighed and then homogenized with 1 volume of 50% acetic acid in a Waring blender for 5 minutes. The homogenate was allowed to extract overnight at room temperature, after which it was adjusted to pH 5.0 with NaOH. The extract was recovered by centrifuging the homogenate for 30 minutes at 2000 $\times$ *g*. The clear supernate was dialyzed against distilled water for 4 days in the cold room with 2 changes of water daily. The extract was then filtered through a K-5 clarifying pad (Hercules Filter Corp., New York) and lyophilized. The methods for assay of antiviral activity *in vitro* and *in vivo* have been described(5).

Ethanol separation: The procedure for the ethanol precipitation is shown in Fig. 1. Briefly, the lyophilized acetic extract from oysters was dissolved in distilled water in a 10% solution, precipitated with one-half volume of cold 95% ethanol and the mixture held at 4°C overnight. The precipitate which formed was collected in a refrigerated centrifuge, dissolved in 4 times the volume of cold distilled water and then lyophilized. This material constituted the one-half volume ethanol precipitate designated (A). Three subsequent precipitates formed with increasing amounts of ethanol to the supernatant (1, 2 and 4 vol), treated similarly as A. The precipitates which resulted from this treatment were designated B, C and D, respectively, while the evaporated supernatant of D represented fraction E. The most active antiviral agent was concentrated into a single fraction (D). The yield of active material was small—usually 0.5 to 1% of the acetic acid product.

Paper chromatography: The active material was analyzed for amino acid content by means of descending paper chromatography using the procedure of Wolfe(9) substituting the ninhydrin reagent of Barrollier(10) for that of Wolfe. Carbohydrates were identified according to the method of Colombo *et al*(11) using a 2.5% aniline hydrogen phthalate as a spray.

Purine and pyrimidine analysis: Purines and pyrimidines were determined by heating a sample of material in N-HCl in a boiling water bath for 1 hour. The hydrolysate was applied to Whatman No. 1 filter paper for descending paper chromatography(12).

Ultracentrifugal analysis: Sedimentation studies were performed at 20°C in a Spinco Model E ultracentrifuge at 60,000 r.p.m. for 65 minutes.

Results. Chemical properties of fraction D: The antiviral product is a white powder, water soluble, non-dialyzable, thermostable at a

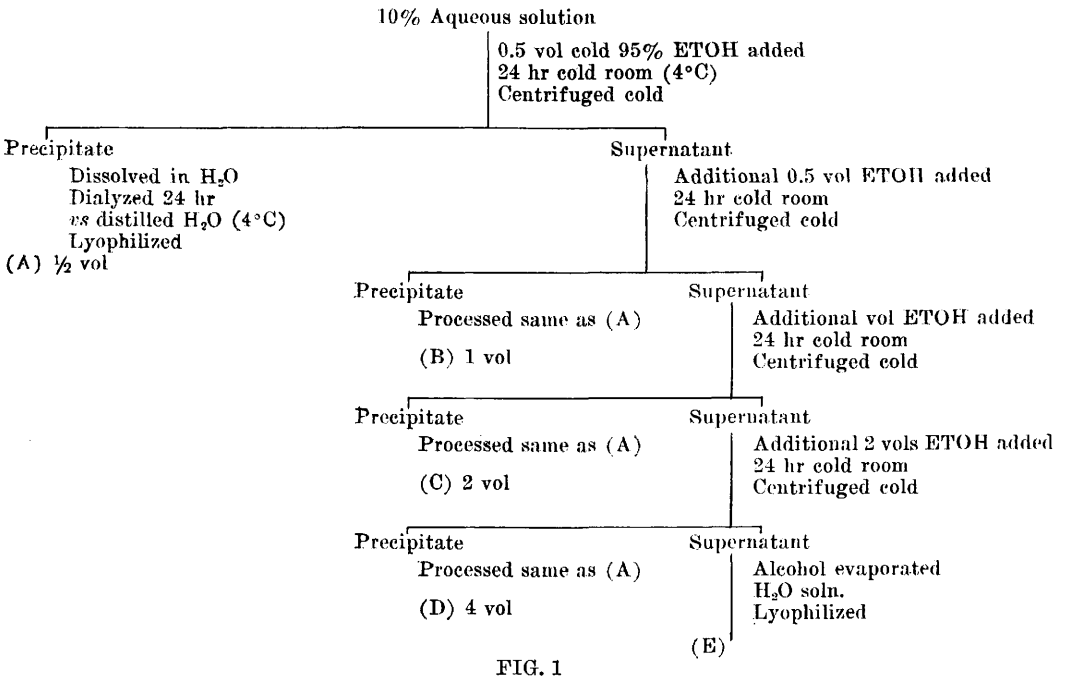


FIG. 1

temperature of 70°C for 30 minutes at pH 7.0 and gave positive reactions with all the well known protein tests. Analytical studies were performed on fraction D to determine the chemical composition and its relationship to antiviral activity. The antiviral agent was found to consist of carbohydrate, polypeptide and an unidentified material. The composition and some chemical properties of the material are shown in Table I. Chromatography of an acid hydrolysate for quantitative analysis of amino acids showed a full spectrum of amino acids (Table II). Since histones similar to calf thymus histone were isolated in earlier studies from abalone and oysters, studies were performed to determine if the paolin II and calf thymus histone were structurally related. In addition, lysozyme was also compared with paolin II, since this antimicrobial substance has been found present in a number of animal tissues. Fraction D was found to be different from lysozyme or calf thymus histone reported by previous workers to possess microbial activity(6,13,14). Certain chemical differences are shown in Table I. It is evident from the data that the nitrogen and sulfur contents of the 3 preparations are different. When samples of fraction D were hydrolyzed and paper chromatographed the carbohydrate component was found to consist of a high concentration of glucose (23.4%). There was a marked difference when compared with lysozyme and calf thymus histone which contain only trace

TABLE I. Comparison of Antiviral Paolin II with Lysozyme and Histone.

Constituent	Paolin	Lysozyme	Histone*
N — %	8.6	18.6	17.05
P — %	.3	.0	Trace
S — %	1.6	2.5	.38
Carbohydrates — %	23.4	Not detectable	Not detectable
Purines	Not detectable	Not detectable	Not detectable
Pyrimidines	Not detectable	Not detectable	Not detectable
Molecular wt	10,000	17,000-30,000	1. 10,000- 16,000 2. 100,000-140,000

* From calf thymus.

TABLE II. Amino Acid Composition and Some Chemical Constants of Antiviral Paolin II from Oyster.

Amino acid	Spot on Fig. 2	% Amino acid*	% Nitrogen†
Alanine	9	2.60	.41
Arginine	5	2.81	.91
Aspartic acid	2	4.18	.44
Cysteine and cystine	1	1.62	.49
Glutamic acid	3	7.33	.70
Glycine	7	3.10	.58
Histidine	6	.91	.25
Isoleucine	15	2.24	.24
Leucine	16	2.96	.32
Lysine	4	5.12	.98
Methionine	not det.‡		—
Phenylalanine	14	.60	.05
Proline	10	4.01	.49
Serine	8	2.43	.32
Threonine	13	3.81	.42
Tyrosine	11	2.32	.18
Valine	12	3.10	.37
Total		49.14	7.13
NH_3N			.82
Humin N			.05
Total nitrogen			8.00

* On the basis of dry wt.
† Amino acid nitrogen is expressed as a percentage of total nitrogen present in the sample after hydrolysis.
‡ Not detectable.

amounts of the carbohydrate. The high content of carbohydrate in the paolin II molecule indicates clearly the mucoprotein character of the preparation.

Fraction D was hydrolyzed with 6N-HCl for 18 hours at 120-130°C, and the hydrolysate was chromatographed on paper. A trace of 2-dimensional chromatogram of the paolin is shown in Fig. 2. The figure reveals at least 16 ninhydrin stained spots. A comparison of the amino acid composition of the antiviral paolin with that found in lysozyme and calf thymus histone showed differences in these materials. The data presented in Table III indicate that there were several differences in the amino acid composition of paolin with only 25% the amount of arginine and 50% the amount of alanine found in the latter two products. Other differences in the molecules were found in the amounts of lysine, aspartic acid, cystine and serine. The amino acids in the paolin account for almost all the protein nitrogen (90%) (Table II), but only 50% of the weight of the product. This suggests that the non-protein moiety actually represents about 50% of the molecule. With approximately 75% of the paolin molecule accounted for, the structure of the prosthetic group is being elucidated. The fraction D was hydrolyzed with N-HCl for 1 hour, then chromatographed on Whatman No. 1 paper and eluted descendingly in an all glass chromatography jar with isopropanol-concentrated hydrochloric acid and water (130:33.4:33.6 ml) for 30-48 hours. Standard solutions of purines and pyrimidines were applied to the same paper and were run parallel with the unknowns. The papers were dried and observed under ultraviolet light. No purines or pyrimidines were detectable in the active antiviral fraction.

Sedimentation studies: Ultracentrifugal analysis of the paolin (fraction D) revealed only one peak (symmetrical) at a concentration of 2 mg/ml after 65-minute centrifugation at approximately 60,000 r.p.m. in a synthetic boundary cell (Fig. 3). The material has a sedimentation constant of 1.6 to 1.8 and a diffusion coefficient of 17×10^{-7}, leading to a calculated molecular weight of about 10,000.

Antiviral activity of the alcohol fraction oyster extract "D" was demonstrated both *in vitro* and *in vivo*. In primary rabbit kidney cell monolayer cultures, the extract at a concentration of 10 μg/ml inhibited 10-100 $TCID_{50}$ of herpes simplex virus, HF strain, during the early phase of multiplication, reducing the virus yield by 4 logarithmic units. The *in vivo* antiviral activity is shown in 2 typical mouse experiments as illustrated in Fig. 4 and 5. In the former experiment a single intraperitoneal dose of 5 mg/kg of the extract given 5 hours after intranasal inoculation of influenza B virus reduced the death rate of 30 mice to 47% as compared to

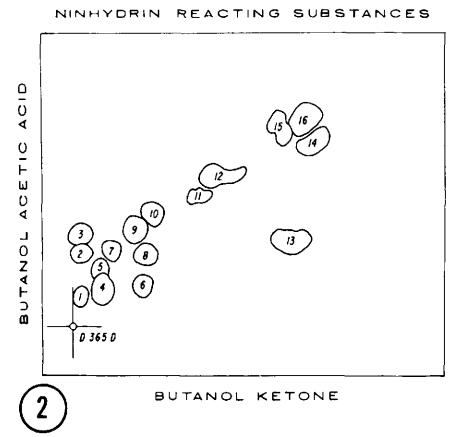
TABLE III. Differences in Content of Amino Acids Between Antiviral Paolin II, Lysozyme and Histone. Results in g per 100 g protein.

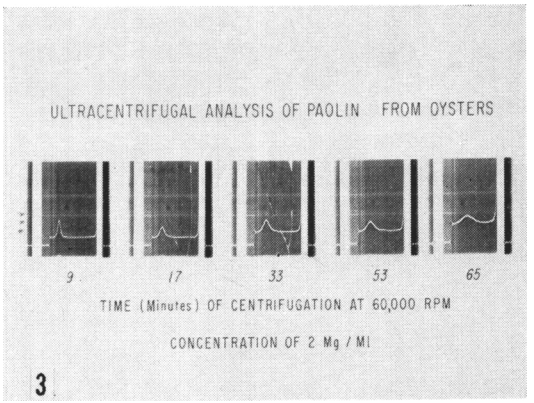
Amino acid	Paolin	Lysozyme	Histone*
Alanine	2.6	5.8	6.5
Arginine	2.8	12.7	12.6
Aspartic acid	4.2	18.2	4.7
Cysteine + cystine	1.6	6.8	.3
Lysine	5.1	5.7	13.5
Valine	3.1	4.8	8.6

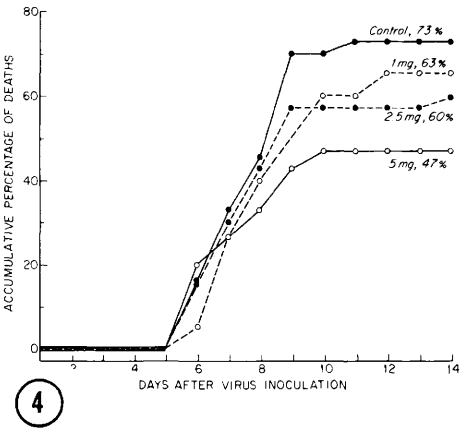
* From calf thymus.

73% in the same number of untreated control animals. Smaller doses were less effective, indicating a dose response. In the latter experiment (Fig. 5) carried out under similar conditions the death rate of poliovirus-infected mice was reduced to less than half of that of the untreated control animals.

Discussion. An antiviral paolin was separated from crude material of an acetic acid extract of oysters and found to be predominantly protein in nature. On the basis of analytical results of amino acids, carbohydrate components and molecular weight, it is apparent that there is little positive evidence that shows any definitive correlation existing between this antiviral agent and lysozyme or histone. In addition, the preparation of paolin studied contained only trace amounts of phosphorus and appeared to be free from nucleic acid as tested by UV absorption of a chromatogram of an acid hydrolysate, indicating that the antiviral activity was not due to any adhering nucleic acid. Furthermore, from studies performed, it would also







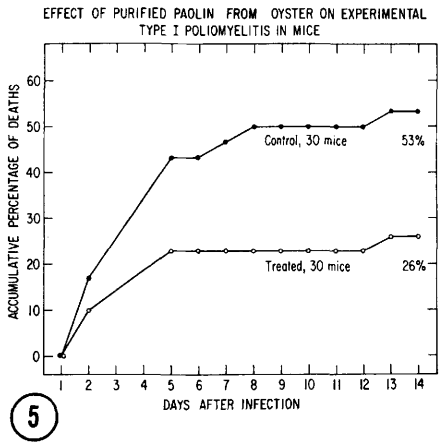


FIG. 2. Two dimensional chromatography of an acid hydrolysate of antiviral paolin from oysters.

FIG. 3. Ultracentrifugal analysis of antiviral paolin from oysters at 60,000 r.p.m. and 20° C. Sedimentation boundary moves from left to right.

FIG. 4. Effect of alcohol fractionated oyster extract "D" on influenza in mice. The extract was given to Swiss white mice in a single intraperitoneal injection of the indicated dose (mg/kg) 5 hr after intranasal infection of influenza B virus. Mice which received saline injections served as controls. Thirty mice were used for each group.

FIG. 5. Effect of alcohol fractionated oyster extract "D" on experimental poliomyelitis in mice. The extract was given to Swiss white mice in a single intraperitoneal dose of 2.5 mg/kg hr prior to intracerebral infection with type I poliovirus, mouse-adapted LSa strain. Mice which received saline injections served as controls.

appear that the size of the molecule has no bearing upon its antiviral activity. The variation in amino acids upon hydrolysis of samples of material from some without demonstrable cysteine to those having as high as 2% and with molecular weights from 700-30,000(7) indicates that the activity may be due to an active component so far unidentified. Analytical studies of the carbohydrate content of the active fraction indicated in most instances yields which ranged from 20-25%. In earlier experiments in which different methods of isolation were used, active antiviral fractions were obtained with one-fourth the carbohydrate content. These findings suggest that the carbohydrate is not involved in the antiviral activity of the material. Differences in carbohydrate content may reflect differences in quantitative yields of the active component in the antiviral material. The main function of the mucoprotein appears to be that of serving as a carrier for the active component. Studies are currently in progress to determine the molecular configuration of the active group.

Summary. An antiviral thermostable substance (paolin II) is isolated from oysters by extraction with acetic acid followed by fractional precipitation with ethanol. The substance shows one peak on ultracentrifugation, has a sedimentation constant of 1.6-1.8 and a molecular weight of approximately 10,000. The material yields 16 amino acids on hydrolysis with a composition of 8.6% nitrogen and 23.4% carbohydrate, indicating the mucoprotein nature of the substance.

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Identification of Isomers Differing from 9,*a*, in the Early Labelled Bilirubin of the Bile.* (31515)

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The "early labelling" of the fecal stercobilin was first demonstrated by London and associates(1). This subject has recently been reviewed with particular reference to the basic

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