Reports

Isolation from Human and Pork Lung of an Inhibitor of Virus Hemagglutination

Aqueous extracts of egg white (*1*), blood-cell stromata (*2*), various animal tissues (*3*), and excretory fluids (*4*) are known to inhibit the hemagglutination of chicken red blood cells by influenza, mumps, and Newcastle disease viruses. Certain plant products such as apple pectin (*5*) will also inhibit viral hemagglutination. It is generally considered that the material responsible for inhibitor activity is a mucoprotein (*3, 4, 6*). However, to the best of our knowledge, no information designed to elucidate the detailed chemistry of these inhibitors has been published.

In an attempt to obtain sufficient material for studies that might help to clarify the chemistry of the virus-inhibitor substances, considerable material of a mucopolysaccharide nature and with high inhibitor activity was isolated from both human and pork lung (*7*). Attempts to increase the purity of our materials, however, led to the isolation of a substance that, although it was not mucopolysaccharide in nature, was apparently responsible for the inhibitory activity of our crude material. The substance was found to contain phosphate, but not sulfate. Incubation of this substance with allantoic fluid containing PR8 virus resulted in a loss of activity, together with a liberation of free phosphate. The Salk method (*8*) was used to measure inhibitor activity.

Three pairs of fresh, normal human lungs were cut into small pieces, dipped in Celite, passed through a mechanical grinder, covered with acetone, and stored at −25°C overnight. The acetone was removed by filtration on a Buchner funnel, and the solid residue was extracted with 3-percent ammonia water for ½ hour at 60°C. The inhibitor material was separated by filtration through Celite on a Buchner funnel, and the *p*H of the filtrate was adjusted with acetic acid to approximately 7. The precipitate that formed was removed by centrifugation, redissolved in 200 ml of 3-percent ammonia water, filtered, adjusted to *p*H 7 with acetic acid, and centrifuged. The supernatant fluids containing material soluble at *p*H 7 were combined, and six volumes of absolute ethanol were added. The precipitate was collected by centrifugation. Weight, 52 g; activity, 4 μg inhibited two units of PR8 virus.

The biologically active precipitate was dissolved in distilled water containing a few drops of 10-percent ammonia water (*p*H 7 to 8), and the protein was removed by shaking the solution a number of times with a 1/4 mixture of amyl alcohol and chloroform after the manner of Sevag (*9*). The mixture was centrifuged to break the emulsion, the clear aqueous layer was acidified (*p*H 6 to 7) with acetic acid, and the active material was precipitated by the addition of six volumes of absolute ethanol. The precipitate was washed several times with absolute ethanol and with ether, then dried at room temperature under a high vacuum. Weight, 20 g; activity, 1 μg inhibited two units of PR8 virus.

The dried powder was extracted with 25 ml of formamide and filtered, and the active material was precipitated from the formamide solution by the addition of 200 ml of absolute ethanol and 25 ml of ether. The precipitate was washed with absolute ethanol and with ether. Weight, 2 g; activity, 0.1 μg inhibited two units of virus.

The inhibitor substance was further purified by dissolving it in 50 ml of distilled water acidified with acetic acid (*p*H 6 to 7) and adding a saturated solution of lead acetate drop by drop until precipitation was complete. The precipitate was collected by centrifugation and washed twice with distilled water. Weight, 200 mg.

One gram of this lead salt was stirred with 10 ml of a freshly prepared 3-percent solution of ammonium sulfide, and the mixture was centrifuged and filtered through a fritted glass filter. The excess ammonium sulfide was removed by warming the solution under reduced pressure at 30 to 35°C. Ten milliliters of absolute ethanol were added to the solution, which was then filtered. After the addition of 10 ml of ether, the solution was allowed to stand at 0°C overnight. Additional material could be obtained by recycling the insoluble fractions.

The precipitate was collected by centrifugation, washed with ethanol and ether, dissolved in 10 ml of distilled water, and treated with 10 ml of a saturated solution of calcium hydroxide. The ammonia was removed by cautiously warming at 40°C under reduced pressure. The excess calcium hydroxide was removed by treating the solution with carbon dioxide gas and then filtering. The calcium salt precipitated as an amorphous powder on the addition of five volumes of ethanol. Weight, 600 mg; activity, 0.01 μg inhibited two units of virus. A crystalline product could be obtained by adding absolute ethanol to turbidity and allowing the solution to stand for several days at 4°C. Elemental analyses (*10*) showed: C, 43.34; H, 5.37; N, 12.01; P, 2.37; and Ash (as sulfate) 10.11 percent. Optical rotation; $[\alpha]^{28}_{D}$, −33° (H_2O, *C*, 0.1).

An ammonium salt could be prepared by treating the calcium salt with ammonium carbonate, adding an equal volume of ethanol, filtering, and then adding ether until precipitation occurred.

Both salts gave a positive test with the Molish and anthrone (*11*) reagents, reduced Fehlings solution after but not before hydrolysis, and gave negative xanthoproteic, Millon and ninhydrin tests for amino acids. The biuret test was positive. The test for hexosamine (*12*) was positive after hydrolysis. The substance was stable at *p*H 8 to 9 but readily lost biological activity at *p*H 4 to 6. The ultraviolet absorption spectrum showed a maximum at 260 mμ. The infrared spectrum showed absorption at 3.0, 6.05, and 6.45 μ, which may be considered to indicate the *R*—CO—NH*R'* grouping (*13*). Electrophoretic analysis showed one component at *p*H 8. At *p*H 4, however, after 15 minutes, four components were observed. We consider this to be due to degradation at low *p*H. No inhibition of the infectivity of PR8 virus in the chick embryo could be found.

When pork lung (*14*) was subjected to the afore-mentioned isolation procedure, the material, so far as could be ascertained by a comparison of biological activity, chemical, electrophoretic, and infrared analysis, was the same as that obtained from human lung. The approximate yield, in both cases, was 100 mg from 10 kg of fresh lung.

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References and Notes

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Concentration of Visual Purple in a Retinal Rod of Rana pipiens

The density of the photosensitive pigment in the distal segments of the retinal rods and cones determines the effectiveness of radiation incident upon the retina in evoking a visual response. Only the light that is absorbed by the pigment in the visual cells can be effective in initiating a visual stimulus. Since there are significant losses of light in the transparent media of the refractive apparatus and in the neural layers of the retina, it is of importance to know what portion of the light that finally reaches the distal segments will be absorbed. This can be evaluated by a knowledge of the density of the pigment within the distal segments of the retinal rod.

Several methods have been applied to the determination of the density of visual purple in the distal segment of the retinal rod. One procedure has been to determine the maximum amount of pigment that is extractable from a known number of retinas and to estimate how much light this quantity of pigment would absorb if it were dispersed in an area corresponding to the retinal distribution of rods (*1*). A second method is to estimate the total visual pigment in the outer limb by measuring the minimum molecular weight per chromophore and computing the total content by assay of the carotenoid component (*2, 3*). An additional and completely unrelated method is to compare the scotopic luminosity curve and the spectral absorption curve of visual purple as used by Hecht *et al.* (*4*) in order to estimate the absorption by rod segments in the retina.

A more direct method is to assay the extracted visual purple and to reduce the data to the dimensions of a single distal rod segment, as was attempted by Broda *et al.* (*5*) and Denton (*6*). The problem of light absorption in the distal segments has become increasingly important in the consideration of the mechanism of the initiation of visual response.

In the series of estimations reported here (*7*), *Rana pipiens* were used. The animals were adapated to the dark for 24 hours, the eyes were removed and sectioned, retina and pigment layer were removed, and the pigment layer was separated from the retina. The retinas were washed in Ringer's solution and then very gently shaken in it. This served to detach the distal segments of the rods. The rod segments were separated from the remainder of the retinal debris by straining through fine wire mesh and recovered from the Ringer's solution by low-speed centrifugation. The accumulated rods were resuspended in a known volume of Ringer's solution, and the total number of rod segments in the solution was estimated by means of a hemocytometer (Table 1, column 3). The visual purple was extracted in 5 ml of digitonin solution after the suspended rod segments had been collected by centrifugation at high speed. The absorption spectrum of the resulting solution was measured with a Beckman DU spectrophotometer. The density at 500 mμ is given in Table 1, column 4. The residue remaining after the centrifugation of the first digitonin extract was extracted a second time with fresh digitonin, but it did not contain a measurable quantity of visual purple.

The dimensions of the distal segments of the rods were measured by means of an ocular micrometer. Fifty separate measurements gave an average length of 52.1 μ and a diameter of 7.6 μ. From these figures, the cross-sectioned area of a single rod was computed to be 45.36 μ^2.

Since the number of rod segments in each preparation is known, the contribution of a single rod segment to the density of the total visual purple solution can be computed. This is shown in Table 1, column 5.

Assuming that Beer's law is valid over the ranges of concentration encountered, we can estimate the density of the visual purple in a single rod segment by reducing the cross-sectional area of the solution to that of a single rod. Since the density of the solution for a depth of 1 cm is known from direct measurement, this manipulation is in effect equivalent to computing the density of the solution when its cross-sectional area is equal to that of a single rod and when its depth is permitted to increase until the volume is identical with that of the original 5-ml extraction. This procedure yields a concentration factor by which the contribution of a single rod segment to the density is multiplied to yield the density of pigment in a distal segment of the rod. The concentration factor and the computed density of the visual purple in the rod segment are shown in Table 1. The density of pigment in the rod segment is 0.642 at a depth equivalent to the axial length of the rod, 52 μ, and the density per micron along the axis of the rod is 0.012.

This value is higher than that reported by Broda *et al.* (*5*) or that computed by Hubbard (*3*), and it is of the same order of magnitude as that reported by Denton for another species of frog (*6*). Since this method of assay requires the solubilization of the pigment, the unavoidable losses during processing mean that these are minimal values. In addition, if the distal segments of the rod are not removed intact, there must be a portion of the visual pigment which remains in the retinal layers. The density per micron depth of a distal segment of a rod is a more general and useful value and is obtained by using the value of 52.1 μ for the length of the segments analyzed. This value of 0.01233 per micron is also shown in Table 1.

An additional consideration in estimating the maximal absorption by visual purple in a single rod is the degreee of orientation of the anisotropic molecules (*8*). The absorption of unpolarized light by a solution of randomly oriented anisotropic molecules would be lower than that of a rod that consisted of a series of parallel planes of optically oriented

Table 1. Density of visual purple in retinal rods. The concentration factor is 0.1102×10^8; the computed density of the pigment in the rod segment along the axis (density per rod) (factor) is 0.642; and the density per micron along rod axis is 0.012.

Preparation No.	Number of retinas	Total rods	Optical density at 500 mμ for 1-cm depth of solution	Density per single rod in 5-ml solution at 1-cm depth
1	25	4.06×10^6	0.256	0.063×10^{-6}
2	100	18.175×10^6	1.049	0.058×10^{-6}
3	100	26.18×10^6	1.410	0.054×10^{-6}
			Av.	0.0583×10^{-6}