8. Results of detailed NMR studies of other methylated and N^{15} cytosine derivatives will be published at a later date.
9. Made by Space Avionics, Inc., Alexandria, Va.
10. G. E. Hilbert, *J. Am. Chem. Soc.* **56**, 192 (1934).
11. More than 95 percent N^{15}; Merck of Canada, Ltd.
12. C. A. Dekker, *Ann. Rev. Biochem.* **29**, 453 (1960).
13. See, for example, L. M. Jackman, *Application of Nuclear Magnetic Resonance Spectroscopy in Organic Chemistry* (Pergamon Press, London, 1959), p. 85.

30 August 1963

Preparation of Retine from Human Urine

Abstract. *Human urine contains "retine." Its partial isolation is described.*

We have reported (*1*) that certain fractions of the urine of children have an inhibitory action on the growth of transplanted malignant tumors in mice. This action was similar to the inhibitory action of analogous extracts of different tissues. The substance responsible for this action was called "retine". We have since found a similar activity in the urine of adults of the age group of about 20 to 25 years. The availability of this material opens the way to large-scale preparation. Since there is less extractable material in urine than in tissues, urine offers the most propitious material for attempts at isolating retine.

Figure 1 shows the effect of retine, prepared from urine, on the transplanted Krebs-2 ascites tumor of Swiss albino mice. The top row, left, shows the size of the tumors on the 4th day after inoculation with 30 million cancer cells subcutaneously into the shoulder region, without treatment. The bottom row shows the tumors of untreated animals a week later. The third row shows the size of the tumors after the animals had been treated from the 4th to the 11th day with one unit of retine daily (a unit was called the quantity which inhibits growth by 50 percent). The second row shows the tumors after a similar treatment with three units of retine. The top row, right, shows the tumors under similar condition after treatment with six units daily. These tumors contained very little live cancer tissue and consisted chiefly of necrotic cancer cells. Injected daily in this quantity, retine produced no observable toxic effect.

As Fig. 1 shows, while smaller doses inhibit growth, bigger ones make the tumors regress. However, our present studies are not aimed at the detailed study of the action of retine but at its final isolation.

The methods used in our test have been described earlier (*1*). The method of purification used in preparing the substance, the action of which is shown in Fig. 1, is as follows.

One thousand liters of urine were concentrated to 80 liters in a Turba Film Evaporator at moderate temperature; the concentrate was chilled to 2° to 4°C, the *p*H was adjusted to 1, and the fluid was extracted four times with 10 liters of chloroform and filtered through Whatman No. 3 paper. The filtrate was cooled to −20°C and filtered again. At this stage the total dry weight was 61.2 g. The chloroform extract was concentrated in a Turba Film Evaporator to 3.5 liters, cooled overnight at −20°C and filtered in the cold to eliminate the hydrochloric acid. Then the chloroform solution was concentrated at reduced pressure to 100 ml and extracted five times with an equal volume of 0.1*N* NaOH. The final *p*H was 11 to 12. The watery extracts were collected, cooled in a bath of 0°C and centrifuged after the *p*H was adjusted to 7.5 to 7.6. The watery extract was filtered through 200 g of Sephadex G-100 gel at 2° to 3°C. The column was buffered with 0.1*M* phosphate buffer of *p*H 7.38.

The void volume (V_0) of the column was 930 ml. The first five volumes contained 30.5 g of material (dry wt.) with about 10 percent of the total retine activity. The effluent was collected from the sixth V_0 up to the tenth (Fig. 2), cooled to 2°C, acidified to *p*H 1, and extracted three times with 1 liter of chloroform. The chloroform extract contained 0.52 g of material (dry wt.) It was cooled to −20°C and filtered. After the filtration it was concentrated to 50 ml and extracted three times with 50 ml of 0.01*N* NaOH. The watery extract was chilled to 2°C and extracted three times with 30 ml of benzene after the *p*H was adjusted to 1. The benzene extract was filtered through Whatman No. 3 paper. This "benzene filter" was washed with benzene and saved. The benzene extract contained 152 mg of inactive material and it was discarded. The cloudy, watery extract was shaken three times with 100 ml of chloroform. Then the chloroform extract was filtered through the "benzene filter," shaken once again with one-third its volume of 1*N* HCl, cooled and filtered at −20°C. It contained 34.2 mg of material with the total activity of 8000 retine units. This corresponds to about 4 μg per

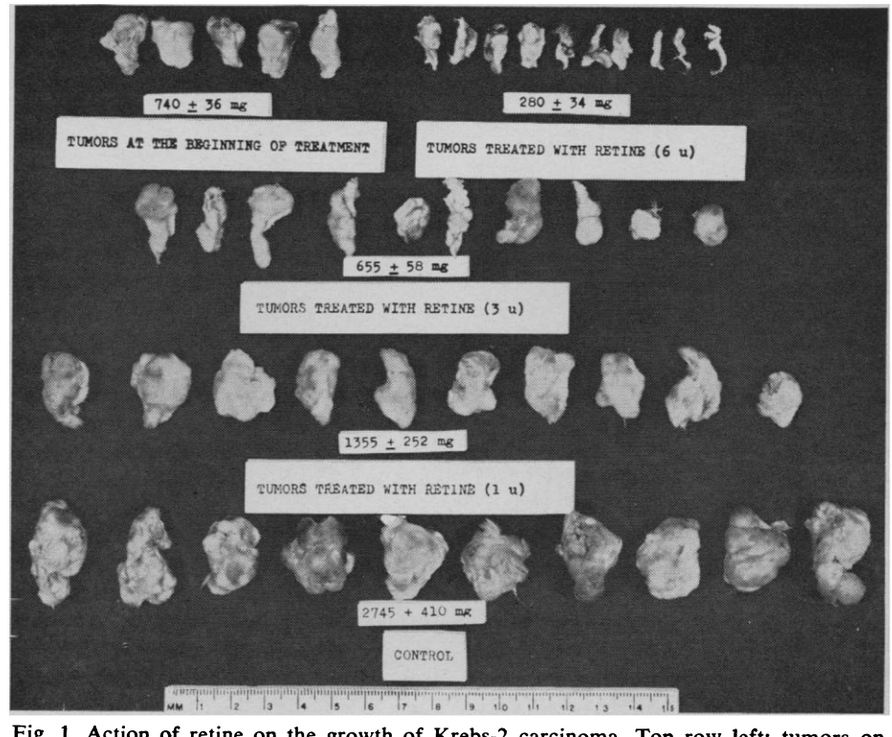

Fig. 1. Action of retine on the growth of Krebs-2 carcinoma. Top row left: tumors on the 4th day after inoculation, untreated. Top row right: treated daily from the 4th to 11th day with six units of retine. Second row: similar treatment with three units. Third row: treatment with one unit. Bottom row: untreated.

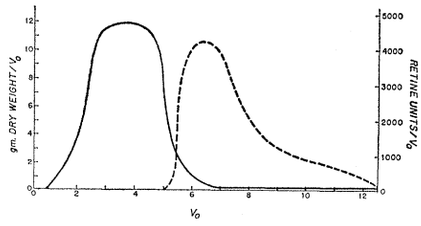

Fig. 2. Filtration of an extract of 1000 liters of urine through Sephadex G–100 gel column. Solid line, distribution of the total dry weight in the eluate. Dotted line, the distribution of the biological activity in the void volumes, expressed in retine units.

unit. The purified preparation of retine dialyzes through cellophane, indicating a molecular weight of less than 1000. The behavior on various Sephadex columns suggests a molecular weight of about 400 (*2*).

ANDREW HEGYELI
JANE A. MCLAUGHLIN
ALBERT SZENT-GYÖRGYI
Institute for Muscle Research,
Marine Biological Laboratory,
Woods Hole, Massachusetts

References and Notes

1. A. Szent-Györgyi, A. Hegyeli, J. A. McLaughlin, *Science* **140**, 1391 (1963).
2. Supported by grants from the National Institutes of Health (GM–10383) and the National Science Foundation (G–5835).

7 October 1963

Clearance of Bacteria by the Lower Respiratory Tract

Abstract. *The disappearance of inhaled bacteria from the lungs of mice is rapid and predictable over an 8-hour period following pulmonary implantation by an aerosol. This indicates that important clearance mechanisms are active in the bronchopulmonary tree. The constant pattern of clearance offers an experimental system in which the effects of various conditions relevant to respiratory infection can be estimated quantitatively. Herein, the effects of hypoxia, alcohol, cigarette smoke, and cortisone are described.*

The fact that the normal lower respiratory tract is free of viable bacteria (*1*) is indeed remarkable, since bacterial contamination by inspired air and the aspiration of upper respiratory tract secretions is a common and almost inevitable occurrence. In contrast, bacterial pathogens can be isolated from the bronchial secretions of patients with chronic bronchitis during periods of relative well-being (*1*). These findings indicate that antibacterial mechanisms which are effective in the normal bronchial tree are markedly impaired in subjects with chronic bronchitis. This report describes a quantitative method for studying the capacity of the lower respiratory tract to dispose of inhaled bacteria.

Aerosols were generated from a buffered suspension (*p*H 7.3) of bacteria contained in eight glass nebulizers. The spray from the nebulizers was directed into mixing cylinders through which a secondary airflow of 2830 lit./minute was drawn. The larger air volume mixed the aerosol and carried it into a plexiglass chamber holding 200 white mice. Swiss Webster male mice were exposed for a period of 30 minutes to aerosols of a coagulase-positive *Staphylococcus aureus* (FDA 209P) which has relatively low virulence for the mouse. It was ascertained that the lungs of this strain of mice were normally sterile. After exposure, one group of mice was killed immediately, and the others were grouped and killed at hourly intervals thereafter; the lungs from the mice in each group were removed aseptically, homogenized, and diluted. Nutrient agar pour plates were made of the lung homogenates. After incubating the plates for 48 hours at 37°C, the numbers of viable bacteria in the lungs of each mouse was calculated from the product of the number of plate colonies and the dilution factor. Deposition refers to the numbers of viable organisms retained in the lungs at the end of the exposure time; and mean deposition values for groups of mice correlated directly with nebulizer and air concentrations of bacteria, and lung and body weights.

As illustrated in Fig. 1, the lungs of mice killed during the postdeposition period showed a rapid decrease in the numbers of viable staphylococci. The mean numbers of viable bacteria retained was determined at the designated time intervals; and by subtracting these values from the mean numbers originally deposited, the mean numbers of staphylococci cleared were derived. In addition, the numbers retained were expressed as a percentage of the deposition, and the percentage cleared was obtained by subtracting the percentage retained from 100 percent. At the end of 1 hour, 45 percent of the bacteria deposited had cleared; after 2 hours, 70 percent; 3 hours, 80 percent; 4 hours, 88 percent, and after 6 hours, 95 percent of the staphylococci had cleared. The clearance curve was remarkably constant for bacterial depositions (at 0 hours postdeposition) from 1.0 to 2.5 $\times$ 10^5, and it is identical with the pulmonary clearance pattern for smaller numbers of bacteria (*2*). It is emphasized that the term "clearance" refers to that number of deposited bacteria whose presence in a viable state can no longer be demonstrated. During the experimental period they might have been exhaled, killed, or actually removed from the lungs. Lungs which were removed after deposition and incubated at 37°C showed bacterial colonization. These results indicate that clearance is an active process and does not represent the natural death rate of the organism in pulmonary tissue.

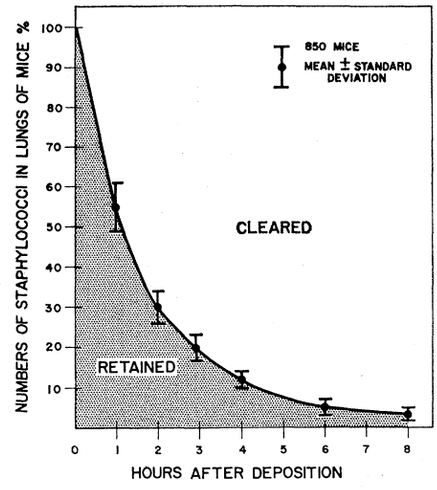

Fig. 1. The clearance of staphylococci from the lungs of mice. Each point on the curve represents the mean percentage of three or more studies with an average of 50 mice in each study. Example: deposition 100,000 ± 13,000; retained at end of 1 hour, 55,000 ± 7000; 2 hours, 30,000 ± 5000; 3 hours, 20,000 ± 3500; 4 hours, 12,000 ± 2500; 6 hours, 5000 ± 1000; and 8 hours, 4000 ± 1000.

The pulmonary clearance of staphylococci was then determined by this method in mice subjected to hypoxia, cigarette smoke, alcohol, and cortisone. The percentage of deposited organisms which were cleared in 4 hours by the normal (control) Swiss Webster male mice was compared with corresponding results obtained with mice subjected to each experimental condition. However, the individual and comparative effects of the different variables are made more apparent, quantitatively, by relating the experimental and control results in each circumstance in terms