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Inactivation by Bromine of Single Poliovirus Particles in Water

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Quantitative electron microscopy shows that Freon-extracted poliovirus, velocity banded in a sucrose gradient, contains over 95% single particles. This well-dispersed virus reacts quite rapidly with bromine in turbulent flowing water, losing plaque titer at the rate of one $\log_{10}$ unit in 10 s at pH 7, 2 C, and at a bromine concentration of 2.2 μM. At 10 and 20 C the rate of disinfection ($\log_{10}$ plaque-forming units per second) is faster, and at both temperatures it increases in approximately linear fashion with increasing bromine concentration. At 2 C such a linear relationship is not observed.

The kinetic features of the reaction of disinfecting agents on viruses in water have usually revealed a significant departure from the simple straight line expected from a reaction of the first order. Several authors have suspected (1, 2, 4, 6) that aggregation among the virions is responsible for some if not all of this irregularity. This seems very likely because several enteric viruses, including polio, are generated in large, tightly packed, cytoplasmic crystals (3) whose release into water suspension is not likely to be accompanied by complete dispersion. Then, too, laboratory preparation of virus suspensions for halogen-inactivation experiments have often involved concentration by pelleting in the ultracentrifuge, a process that can induce aggregation even among previously well-dispersed particles.

Now that means are available for selecting a fraction of single polio particles by velocity banding in a density gradient and for verifying by appropriate quantitative procedures for electron microscopy that they are indeed monodisperse (8), it seems that the kinetics of single-particle reactions can be examined, perhaps for the first time. This is essential if the inactivation kinetics of virus in the complex environment of polluted water are to be analyzed and understood.

The reaction rates encountered here are very fast. A special apparatus has been used for accurate determination of survival titers and reaction times as short as 2 s, and all virus suspensions have been monitored by electron microscopy to insure a high degree of dispersion at the time of bromine contact.

MATERIALS AND METHODS

Preparation, purification and plaquing of poliovirus. Poliovirus type I, Mohoney strain, was obtained from Gerald Berg, Environmental Protection Agency, Cincinnati, Ohio, and was serially passaged in human epidermoid carcinoma cells, HEp-2, supplied by Dr. Hoff. The cells were grown in medium 199 containing 0.105% $NaHCO_3$ and 5% fetal calf serum. After the cells reached confluency, they were maintained on the same medium with 2% fetal calf serum.

Stocks of virus were produced in HEp-2 cells at 37 C by infection at a multiplicity of 10 to 20 plaque-forming units/cell under a maintenance medium of 199 + 0.105% $NaHCO_3$ and 2% fetal calf serum. After 18 to 24 h, when the cytopathic effect was 100%, the cells were frozen and thawed three times, and the cell debris was removed by low-speed centrifugation (~800 × *g*) for 10 min. The supernatant fluids had titers of 4×10^{10} plaque-forming units/ml and were kept frozen at −70 C.

Purified virus was produced in the same cells grown for 3 days in 32-ounce (ca. 0.95-liter) prescription bottles inoculated with the above stock virus in Dulbecco phosphate-buffered saline (PBS) containing 12.5 mM $MgCl_2$, at a multiplicity of ~100 plaque-forming units/cell. The virus was allowed to adsorb for 1 h at 37 C, and then 40 ml of maintenance medium containing 12.5 mM $MgCl_2$ was added to each bottle and the cells were further incubated at 37 C for 11 h. The cells were chilled to 4 C, and the supernatant fluid was decanted. The monolayers were washed two times with PBS, and the cells of each bottle were scraped into 10 ml of PBS and pelleted at 250 × *g* for 10 min. Cells remaining in the supernatant fluid were pelleted similarly and pooled with the scraped cells and pelleted again. The pellet of cells was resuspended in 6 ml of PBS not containing Mg^{2+} or Ca^{2+}. Four milliliters of Freon 113 was

added, and the cells were homogenized at one-half speed in a Sorvall Omni-Mixer for 2 min. The phases were separated at 800 × *g* for 10 min, and the upper aqueous phase was removed and held in an ice bath.

The Freon phase was re-extracted with another 6 ml of PBS and the phases were again separated. This aqueous phase was pooled with the first, and the Freon phase was re-extracted again with 6 ml of PBS. All three aqueous phases were combined and made to a volume of 20 ml. A 10-ml amount of this extract was placed on each of two 10 to 30% (wt/wt) sucrose gradients made in 0.05 M phosphate buffer, pH 7.2. The gradients were centrifuged at 25,000 rpm in a Beckman SW27 rotor at 4 C for 2 h and 15 min. Fractions of 2 ml each were collected from the tubes and examined by the kinetic attachment method (7) for the presence of the virus. Twenty fractions were obtained from each tube, and the highest count of virus was most frequently found in fraction 14. All relevant fractions were pooled, and the virus was stored at refrigerator temperature without any attempt to remove the sucrose.

Plaque assays of poliovirus were performed on 4-day-old monolayers of HEp-2 cells in 1-ounce prescription bottles under an overlay of 1% agar (Difco) containing medium 199 plus 5% fetal calf serum, 0.210% $NaHCO_3$, 0.003% neutral red, and 5 mM $MgCl_2$. The plaques were read after 3 days of incubation at 37 C.

Physical assay of the virus. Purified virus preparations, recovered from the sucrose gradient usually at 22% concentration, can be assayed directly by the kinetic attachment method previously described (7). A minimum dilution of 1/5 is required to reduce the sucrose concentration to a level at which it does not interfere with the process. If further dilution is required to reduce virion concentration for optimum numbers per electron microscope picture, it is helpful to include 5% sucrose in the diluent. We have obtained the most uniform results in this way.

Water. Preparation of demand-free water, phosphate buffered to pH 7, and adjustment to proper bromine concentration have been described in a previous paper (8).

Apparatus. The apparatus for exposure of virus to bromine action and removal of samples for titration after 1 to 20 s of exposure is described in a previous paper on reovirus (9).

RESULTS

When the virus used in these experiments is extracted from the infected HEp-2 cells with Freon and velocity banded in a sucrose gradient, the particle content of several successive fractions from such a gradient is as shown in Fig. 1. The concentration of particles calculated from counts from such pictures is plotted against radial position in Fig. 2. Many particles smaller than virus, probably ribosomes, can be seen in fractions 11 and 12 just above the virus peak. These were not counted. At no point below the main virus peak was there any secondary peak in the region where pairs, triplets, etc. would have been expected had they been present in substantial numbers. It appears from this that the Freon extraction process has yielded a virus preparation with a very high proportion of single particles.

The strip pictures of Fig. 1 were taken at high particle concentration, presenting enough particles so the reader may judge their relative number and degree of purity. Other pictures made with more dilute preparations were used to determine the true degree of aggregation, free of the effect of one particle falling upon another by accident. Calculation of the number of such accidental pairs will be made later (see Discussion). They indicate that the starting virus for these experiments contains at least 95% single particles.

Poliovirus inactivation by bromine at 2 C and pH 7. Six experiments were made at 2 C, three of which are shown graphically in Fig. 3. All were characterized by an initial linear phase and constant decline in log plaque-forming unit survivor ratio per unit time of exposure. None of these reactions showed any tendency toward delay during the first time interval, which was 4 s. The slopes of all the reactions, ranging from 0.6 to 22 μM bromine concentration, have been plotted in Fig. 4, where it can be seen that increasing bromine concentration does not produce a proportionate increase in reaction rate. The increase becomes progressively less with increasing bromine concentration, indicating a progressive decrease in efficiency.

In several inactivation experiments at bromine concentrations of 3.5 μM or greater, there has been a slight increase in the reaction rate after a substantial period of linearity (see the 22 μM line in Fig. 3). We have not been able to produce this effect regularly. Slopes for Fig. 4 were taken from the linear part of each survival graph.

Poliovirus inactivation by bromine at 10 C and pH 7. At 10 C the inactivation rate was constant at 1.9 μM bromine concentration (Fig. 5). There is no indication, in this experiment, that there is any time delay before inactivation begins. However, at higher bromine concentrations the linear part of the graph begins only after the first 4-s time interval (Fig. 5). It seems unlikely that the initial rate at the higher concentrations could be less than that of the straight line for 1.9 μM bromine, so the lower curves have been drawn tangent to it at zero time. The reaction rates, slopes of the linear parts of the three kinetic experiments, are shown (Fig. 4) to be a linear function of bromine

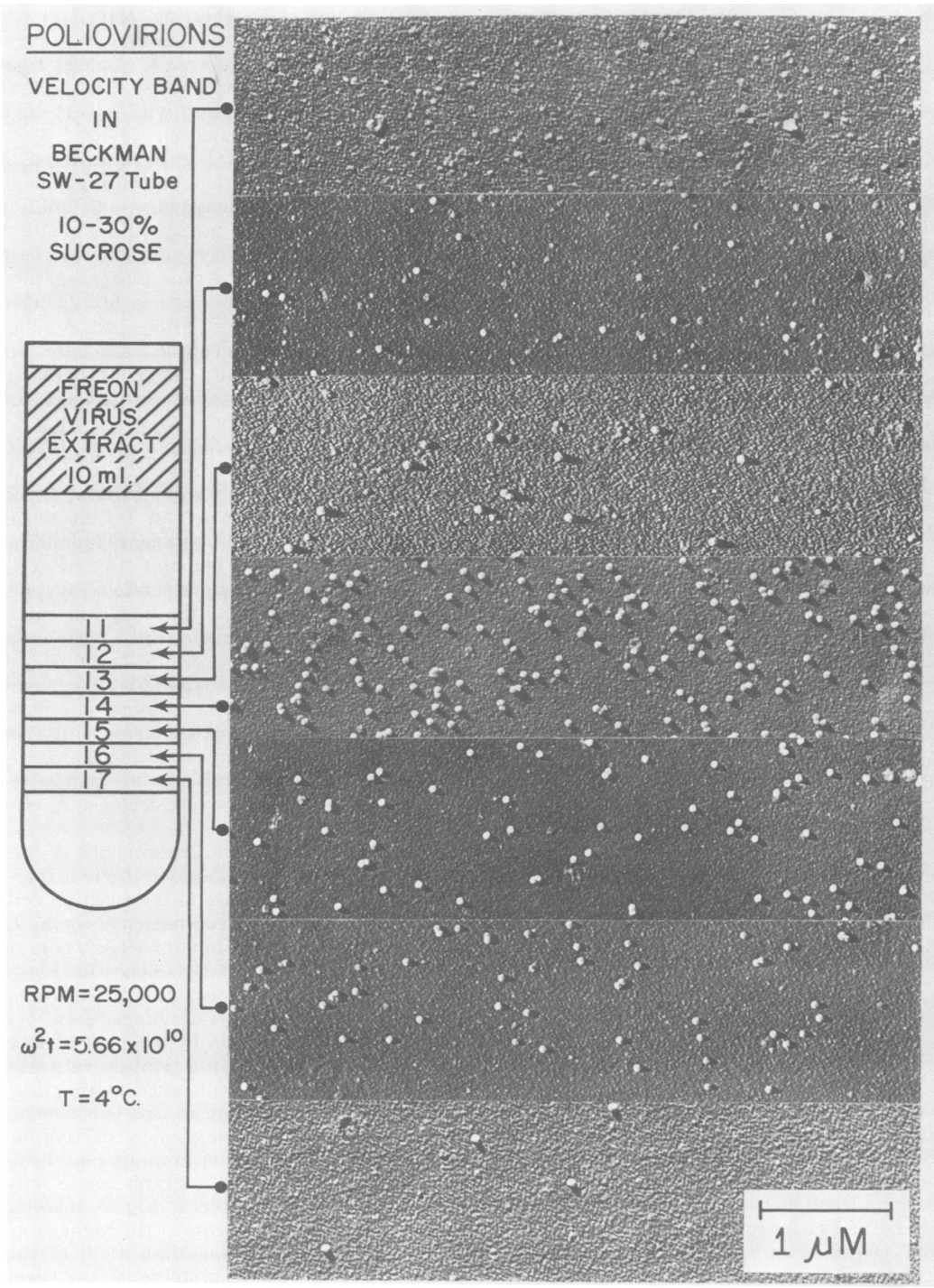

FIG. 1. *The banding of Freon-extracted poliovirus in a 10 to 30% (wt/wt) sucrose density gradient is shown here in seven strip pictures of fractions taken from the position indicated in the centrifuge tube. Prepared for quantitative electron microscopy by the kinetic attachment method, fraction 11 has many of what are probably ribosomes. Peak virion count occurs in fraction 14, as shown in Fig. 2.*

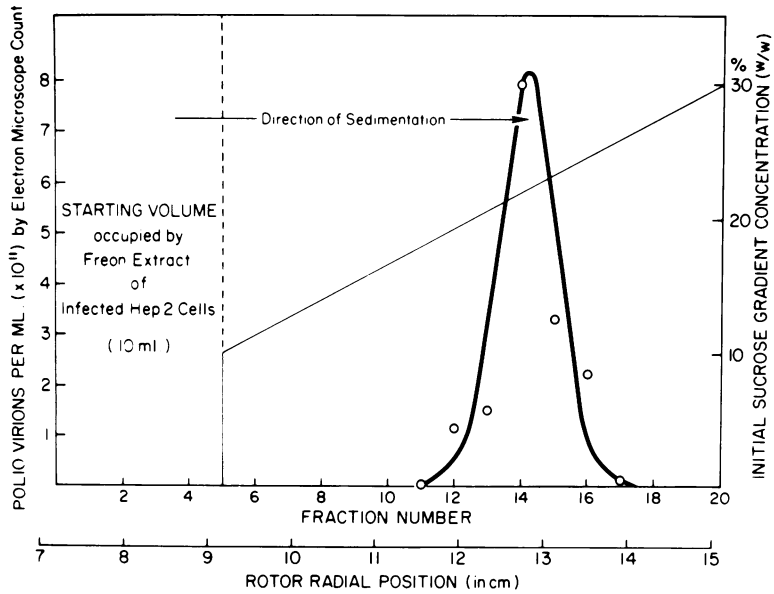

FIG. 2. *Graphical display of poliovirus particle count by electron microscopy, taken from the fractions and pictures shown in Fig. 1.*

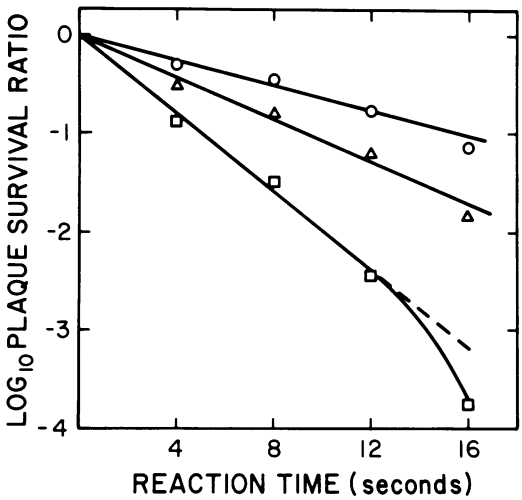

FIG. 3. *Inactivation of poliovirus at three different concentrations of bromine at pH 7 and 2 C: (○) 0.6 μM; (△) 2.2 μM; (□) 22 μM.*

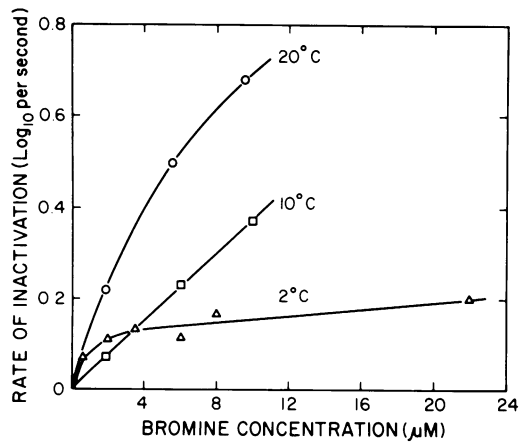

FIG. 4. *Inactivation rates (slopes in logs per second taken from Fig. 3, 5, and 6) for poliovirus at pH 7 as function of temperature and bromine concentration.*

concentration, with an intercept indicating zero reaction for zero bromine.

Poliovirus inactivation by bromine at 20 C and pH 7. At 20 C the inactivation of the virus proceeds faster at all bromine concentrations than it did at 10 C (Fig. 6). There is some indication in the graph for the 9.5 μM experiment that aggregation is showing its effect at several levels below 10^{-3}. Reaction rates were determined from the straight part of each line.

DISCUSSION

At 2 C the inactivation of poliovirus by bromine shows no evidence of delay even during the first 4 s under conditions leading to 99.9% plaque reduction in 16 s. The logarithm of plaque survival ratio decreases linearly with exposure time, as might be expected from the electron microscope data that indicated over 95% of single particles in the preparation. At 10 C only the slowest reaction, the one at 1.9 μM bromine concentration, was strictly linear.

The faster reactions at higher bromine concentrations showed a slight delay before linear decline rates were established (Fig. 5). Speculation in the past about such delay in the inactivation curve has often invoked aggregation as one of the possible explanations (1). Now it is clear that the small amount of aggregation in this preparation can have no perceptible effect on the course of the reaction above the 10% survival level. Such a delay may be just the time required for penetration of the bromine to the critical point within the virus capsid.

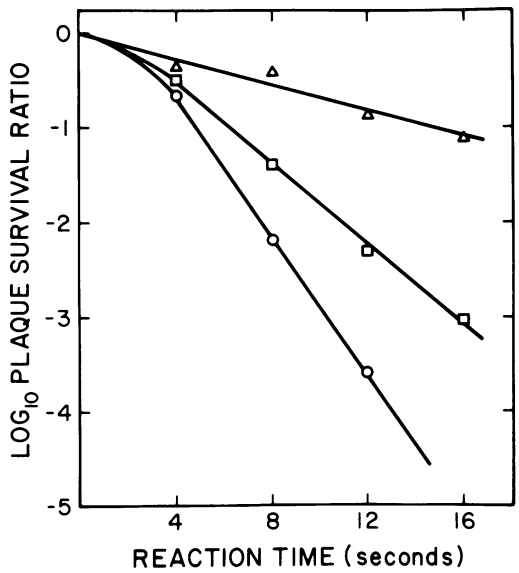

FIG. 5. *Inactivation of poliovirus at three concentrations of bromine at pH 7 and 10 C: (△) 1.9 μM; (□) 5.9 μM; (○) 10 μM.*

Inactivation at 20 C was essentially first order over a fivefold range of bromine concentration, and it was substantially faster than that at 10 C, but the summary (Fig. 4) showing the dependence of reaction rates on both bromine concentrations and temperature reveals an unexpected feature. The approach to zero rate at 2 C is quite different from that seen at the two higher temperatures. From bromine concentrations of 22 down to 3.5 μM the rate falls slowly by only 36%. Beyond that point it must plunge steeply to zero or intersect the vertical axis, but not before crossing the 10 C line at a 3.5-μM bromine level. This seems to indicate that below 3.5 μM bromine concentration the rate of poliovirus inactivation is greater at 2 C than at 10 C. At this time we have no mechanism to propose that would explain these facts. We are not aware of any significant error in measurement of bromine concentration even at the 1-μM level. However, there is evidence in the work of Weidenkopf (10) with chlorine on poliovirus that at 0 C the inactivation rate depends on chlorine concentration in much the same way as we have seen at 2 C with bromine. With

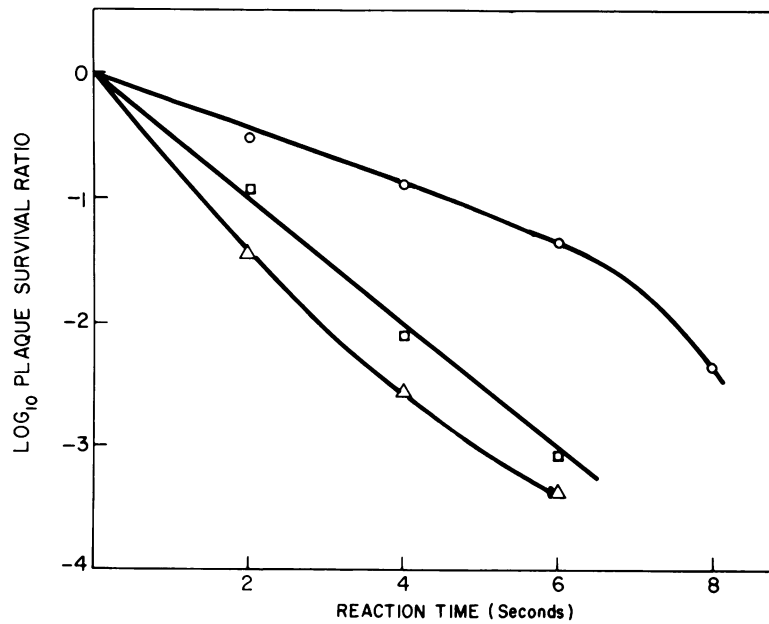

FIG. 6. *Inactivation of poliovirus at three concentrations of bromine at pH 7 and 20 C: (○) 1.9 μM; (□) 5.5 μM; (△) 9.5 μM.*

Dr. Weidenkopf's kind permission we have plotted the data from Table 1 of his publication. Figure 7 shows a linear decrease in rate from 24 μM chlorine down to somewhat below 4 μM, beyond which it must intersect the vertical axis or fall rapidly to zero as drawn. Careful calculations made from the published graphs of Scarpino et al. (5) with chlorine at 5 C definitely do not show this effect. It would appear to occur only at low temperatures and it may be related to changes in virion hydration near the transition point of water, which is at 4 C.

A comparison of rates of bromine and chlorine inactivation of poliovirus seems appropriate at this point, but it can be misleading. The data have been obtained here with essentially single virus particles, and they indicate reaction rates much faster than those reported for chlorine. The data of Weidenkopf (Fig. 7) show a decline in survival titer at 0 C of 1.44 $\log_{10}$/min at 10 μM chlorine (pH 6). In Fig. 4 the rate for bromine at 2 C was six times faster. From Scarpino et al. (5) we have estimated that 10 μM chlorine (pH 6 and 5 C) produces a 0.8-$\log_{10}$/min drop in plaque survival titer. Neither paper (5, 10) provides any data on the temperature coefficient of the reaction, so it is not possible to compare them directly, but both indicate a reaction rate for chlorine at pH 6 that is 1/5 to 1/10 of what we find for bromine. A more accurate comparison cannot be made until the effects of chlorine have been observed on essentially monodisperse virus preparations. We expect to report this in a subsequent paper.

It is obvious that any method used to collect the particles suspended in a volume of fluid on a plane surface must reveal some few aggregates by coincidence, even though there may have been none in the suspension. Thus, when none or a very few pairs exist in the suspension we have assumed that the chance of one particle falling upon another and producing an accidental pair in the electron micrograph is just equal to the quotient of total area occupied by the particles divided by the total area of the surface photographed. The area excluded by a round particle of radius (r) already on the surface would be just $4\pi r^2$. The difference between this, the number calculated, and the number of pairs observed has been taken to be the true number of pairs present in the suspension, and it has been so reported.

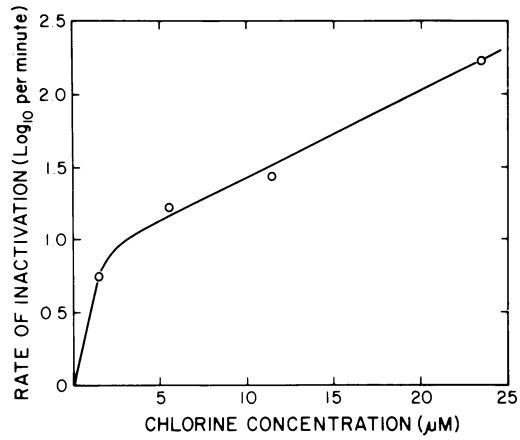

FIG. 7. *Concentration dependence of the reaction rate of HOCl at 0 C on poliovirus. These data have been taken with the permission of Dr. Weidenkopf from Table 1 of his publication (10).*

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