material could be extracted by the process used. When the isolation procedure was carried out rapidly at −2° C, no active hemolysin was found. These ethanol extracts were evaporated under reduced pressure, taken up in ether and precipitated in acetone. This inactive acetone soluble fraction was suspended in phosphate buffer at pH 7.0 and incubated for 2 hr at 37° C with an inactive saline extract of acetone-dried tissue. The incubation mixture of the two inactive fractions yielded an active hemolysin that could be extracted in the manner described above.

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Inactivation of Vaccine Virus by Preparations of Hyaluronic Acid with or without Hyaluronidase: Experiments on Cell Cultures[1]

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The purpose of this preliminary note is to report on the effects of preparations rich in hyaluronic acid, with or without the addition of hyaluronidase, on vaccine virus grown in media of the Maitland type. The materials were added to the cell-containing media, together with the virus, in an attempt to imitate the ground substance of the mesenchyme and thus duplicate, more or less remotely, the conditions that one can imagine prevail during infection (*1*).

We worked with 14 different preparations of either hyaluronic acid or potassium hyaluronate, often highly purified, made from either human umbilical cord or cattle vitreous humor.[3] Saline solutions of these materials were employed (*a*) fresh, after filtration through Berkefeld N candles, (*b*) steamed for 15 min, and (*c*) autoclaved at 120° for 15 min; and at each passage they were added to the cultures, containing 10 ml of medium, generally at the amount of 1 ml of a 1% solution, so that a final concentration of 1:1100 resulted. All the preparations, whether or not they had an inactivating effect on the virus, had a pH ranging from 4.0 to 5.0. Upon addition to the medium the pH of the latter dropped to around 6.8, with some preparations, to return to its normal of 7.2–7.5 shortly after, and remaining so until the end of the culture growth 5 days later. The hyaluronidase preparation[4] was used at a 1% dilution in saline after filtration through Berkefeld N candles. About 200 different lines of cultures, mostly with the Levaditi and dermal strains,[5] were obtained, each line consisting of 1–30 passages. The cultures were titrated on rabbits by intradermal inoculation of progressive tenfold dilutions of the supernatant fluid.

The significant results obtained ranged from a slight decrease in the virus titer to its complete inactivation. This variation on the effects of the polysaccharide preparation depended on the following factors:

a) Source of the preparation: preparations from cattle vitreous humor had less inactivating power than those from human umbilical cord.

b) Concentration of the solution: the inactivating effect was in direct relation to the strength of the solution.

c) Simultaneous addition of hyaluronidase: all preparations that failed to inactivate the virus did so upon addition of hyaluronidase.

d) The culture material employed, whether supernatant fluid or cells, in carrying the passage: the cells afforded extreme protection to the virus.

e) Strain of virus used: dermo vaccine was far more resistant than the Levaditi neuro vaccine.

The order of the neutralizing effect was as follows: Vitreous humor preparations at a final concentration of 1:1100, whether fresh, steamed, or autoclaved, had no effect on the dermo virus other than to reduce the average titer by approximately a one-tenth dilution below the control, whereas the 3 preparations inactivated the neuro vaccine in 3–6 passages. All preparations of umbilical cord at a final concentration of 1:1100 inactivated both strains of virus in 1–5 passages.

Steamed hyaluronic preparations from vitreous humor used at a final concentration of 1:600 completely inactivated the neuro virus, usually in one passage and caused a reduction of two-tenths dilution in the titer of the dermo virus. On the other hand, preparations from both vitreous humor and umbilical cord at final concentrations of 1:10,000 and 1:100,000 did not affect virus growth, or affected it very little.

When the hyaluronic acid preparations inactive at a concentration of 1:1200 were added to the cultures together with the hyaluronidase preparation, also at the final concentration of 1:1200, the virus was totally inactivated after 1–3 passages. The phenomenon is all the more remarkable if one considers that free hyalu-

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[2] With the collaboration of Phyllis Johnson.

[3] Seven of the preparations, supplied by the Wyeth Institute of Applied Biochemistry of Philadelphia, were prepared by H. E. Alburn and E. C. Williams (*2*); another five were prepared and supplied by Z. Hadidian (*3*) from the Tufts Medical School of Boston, and the other two were obtained in our laboratory by R. H. Pearce.

[4] Supplied by J. Seifter, of the Wyeth Institute of Applied Biochemistry. The enzyme contained 1,000 TRU/mg.

[5] The dermo virus, secured through F. L. Horsfall, was the strain cultured by Rivers and Ward (*4*) in 1933 and kept lyophilized since.

ronidase is still present in the cultures when tested on rabbit skin, and the resulting spreading of any virus left in the inoculum should lead to enhanced lesions.

In all the cases where total inactivation of the virus was obtained the effect was noticeable from the first or second passage by progressive reduction of titer and of size of the lesions till complete suppression. It is important to point out that we have occasionally obtained unpredictable results with some of the steamed preparations of hyaluronic acid. These preparations inactivated the virus in some experiments but failed to do so in others, the same happening when hyaluronidase was added. An effort is being made to elucidate the cause of the phenomenon, keeping in mind the possibility that steaming might affect the hyaluronic acid in such a way as to permit the development of resistant variants; in one instance, at least, we suspect this to be the case.

When passing the cultures by cells, we obtained only complete inactivation of the neuro vaccine with autoclaved preparations from umbilical cord in 2 out of 3 cases. All the other preparations, with or without hyaluronidase, were ineffective, in the course of perhaps 20 passages, except for a decrease in titer of 1–2 tenfold dilutions below the control.[6]

The protection thus afforded the virus by the cell seems to indicate that the effect of the polysaccharide preparation is directly on the virus and not on the cells, resulting in interference with the process of virus multiplication. To investigate this point further the hyaluronic acid preparation was added to the media from 30 min to 24 hr before or after being seeded with the virus, with no differences in the results. Still another test was to inject intradermally in the rabbit the washed cells of cultures the supernatant fluid of which, undiluted, had become inactive through the effect of the hyaluronic acid preparation: vaccinial lesions were obtained of about the same size and severity as those induced by the cells of control cultures.

Several of the conditions in our experiments and phenomena observed therein duplicate what takes place during infection of the animal. First of all, the concentration of hyaluronic acid, at about 1:1000, was not above what can be roughly assumed is present in the ground substance of several tissues, even disregarding a probable increase of the polysaccharide during inflammation. Further, it is well known that the hyaluronic acid of this ground substance is depolymerized and hydrolyzed by hyaluronidase as well as by other means, and it is this specific enzyme that, attacking its substrate in our cultures, brought to a maximum the inactivating effect of hyaluronic acid on vaccinia. Finally, the generally recognized fact in animal infection of the protection against injurious agents afforded to viruses by cells was also clearly observed in our tests regarding the effects of the hyaluronic acid preparations.

This investigation should obviously be expanded before what is above suggested becomes a conclusion, but if the results obtained so far do in any way duplicate what happens in the organism during infection it could be assumed that hyaluronic acid, and conceivably other polysaccharides, of the ground substance, especially after its degradation during infection, act to inactivate vaccinia and other infectious agents.[7] If this were so the ground substance besides being a *mechanical* barrier (*1*) would also be a *sterilizing* barrier to the progress of infection.

[6] The cell passages were carried out by transfer of most of the sediment obtained by light centrifugation of the cultures, so that in the successive passages cells progressively accumulated in amounts high above those generally employed in virus cultures. Incidentally, this method of cell passage has resulted in the practically indefinite maintenance of ordinary cultures, which, if passed by transfer of the supernatant fluid, would soon have been lost.

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[7] Alice E. Moore, of the Sloan-Kettering Institute, kindly agreed to test some of our preparations of hyaluronic acid on the virus of Russian encephalitis. Her experiments, although preliminary, confirmed with that virus the results we obtained on vaccinia.

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Reversal of the Diabetogenic Action of Alloxan by Sulfhydryl Compounds

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Large doses of sulfhydryl compounds, given prior to intravenous diabetogenic doses of alloxan, prevented diabetes in rats (*1, 2*). Prevention failed to occur, however, when the sulfhydryl compounds were given 5 min after the alloxan. Thus the action of alloxan could not be reversed.

The fundamental action of alloxan is completed within a few minutes after it reaches the blood, the necrosis of the β-cells of the islets of Langerhans being the ultimate result of this initial action (*3*). Attempts to reverse the action of alloxan should therefore be made after the completion of the action but before the onset of β-cell degeneration. The action of alloxan carried to its final stage of cell necrosis is not likely to be reversed. In rats changes in the β-cells only become evident microscopically 24 hr after intraperitoneal injection of alloxan as contrasted to their early appearance (5 min to 1 hr after injection) when alloxan is given intravenously (*4*). This suggested reinvestigation of the problem after intraperitoneal or subcutaneous administration of alloxan. The subcutaneous route was preferred, as it produced diabetes more consistently. A 175 mg/kg dose of alloxan produced diabetes in 100% of young rats

[1] Lady Tata Memorial Research Scholar.