of mucopolysaccharides from epiphyseal cartilage into the metaphysis.

Summary. Measurements of the uptake of $^{35}SO_4$ in epiphyseal cartilage and metaphyseal spongiosa of young rabbits given a short-term treatment with aminoacetonitrile (AAN) are reported. When $^{35}SO_4$ was administered previous to AAN, the results suggested an inhibited "transfer" of chondroitin sulfate from cartilage to bone. Injection of $^{35}SO_4$ during or after AAN-treatment resulted in a depressed uptake in the growth zones. The results are correlated to previously observed alterations in mucopolysaccharide concentrations. The arrested weight increase of lathyritic animals may affect the results.

1. Karnovsky, M. J., Karnovsky, M. L., J. Exp. Med., 1961, v113, 381.

2. Engfeldt, B., Tegner, B., Bergquist, E., Acta Path. Microbiol. Scand., 1960, v49, 39.

3. Shintani, Y. K., Taylor, H. E., Canad. J. Biochem., 1962, v40, 565.

4. Berntsen, E., Proc. Soc. Exp. Biol. and Med., 1966, v123, 588.

5. Conklin, J. L., Am. J. Anat., 1963, v112, 259.

6. Moltke, E., Acta Endocrinol. (Kbh.), 1957, v25, 179.

7. Marckmann, A., Proc. Soc. Exp. Biol. and Med., 1965, v119, 557.

8. Dziewiatkowski, D. D., diFerrante, N., Bronner, F., Okinaka, G., J. Exp. Med., 1957, v106, 509.

Received September 2, 1966. P.S.E.B.M., 1967, v124.

Specificity of a Neuraminidase Activity of Sendai Virus. (31839)

HIDEKI TOZAWA, MORIO HOMMA, AND NAKAO ISHIDA
(Introduced by S. Baron)
Department of Bacteriology, Tohoku University School of Medicine, Sendai, Japan

Myxoviruses are known to liberate sialic acid from various substrates, such as glycoproteins, glycolipids and oligosaccharides. A constituent or a fraction bearing the enzymatic activity was first isolated from the Asian and PR8 strains of influenza A virus after disrupting the virus by trypsin(1). Trypsin was also used for disrupting Newcastle disease virus (NDV)(2).

When Sendai virus (Parainfluenza type 1) was disrupted with ether in the presence of emasol 1130 (polyoxyethylene sorbitan monolaurate). The resulting aqueous fraction had neuraminidase activity, hemagglutinability and S-antigenicity. The fraction which had neuraminidase activity was recovered from the supernatant of ultracentrifugation at 100,000 *g* for 120 minutes, though the fraction still contained the small hemagglutinins.

The present communication describes the comparison made between the neuraminidase activity of the intact Sendai virus grown in eggs or L cells and that of the neuraminidase fraction obtained by the disruption of the virus in relation to the pH optimum and the inhibition of antiserum. In addition to that, the comparison is also made among the influenza viruses, Sendai virus and NDV, and the specificity of the neuraminidase activity is discussed.

Materials and methods. Viruses and L cell culture: The Fushimi strain of Sendai virus was grown at 36°C in the allantoic sac of 10-day-old chick embryos and harvested 72 hours after inoculation of the stock virus. The same strain of virus was also grown in stationary cultures of L cells(3) in a medium containing 90 parts of YLE consisting of 0.5% lactalbumin hydrolysate (Difco) and 0.1% yeast extract (Difco) in Earle's solution with 0.45% glucose and 10 parts of bovine serum. Upon infection, the cultures were washed twice with Hanks' balanced salt solution (BSS), and inoculated with egg-grown virus at a multiplicity input of 100 in a maintenance solution which consisted of 98 parts of YLE and 2 parts of inactivated horse serum (MS). After 1 hour adsorption at 36°C, the cells were washed twice with BSS, refed with MS, and the incubation was continued up to 72 hours. The 1,500 ml

total amount of culture fluid was harvested and the virus was collected by the aid of centrifugation as described below. The pellet was resuspended with 10 ml of 0.01 M phosphate buffered saline at pH 7.2 (PBS) and the hemagglutinin (HA) titer of the suspension was 5×10^4/ml.

In addition to Sendai virus, the PR8, FM1, NWS and Japan/305 strains of influenza A virus, the Lee strain of influenza B virus, and the Miyadera strain of NDV were also examined for their neuraminidase activity. They were all grown in the allantoic cavity of 11-day-old chick embryos at 36°C and harvested after 48 hours of incubation.

The viruses were purified and concentrated by means of two cycles of alternating centrifugations at 5,000 *g* for 15 minutes and 53,000 *g* for 30 minutes except for Sendai virus and NDV for which 43,000 *g* instead of 53,000 *g* was used. The pellets were resuspended in an appropriate amount of PBS. These suspensions containing around 5×10^4 HA units/ml were preserved at −30°C until use.

HA titration: For HA titration, the usual Salk pattern method was employed by using 0.5% chicken red blood cell suspensions.

Disruption of Sendai virus: Ether-emasol was used to disrupt Sendai virus according to the technique described by Hosaka *et al*(4). Emasol was a commercial sample of Kao Chemicals, Tokyo, Japan. To 30 ml of Sendai virus suspension in PBS containing approximately 5×10^4 HA units/ml, was added 2 ml of emasol solution (50 mg/ml in dist. water) and 15 ml of Squibb's anesthetic ether. The mixtures were agitated at 4°C for 20 minutes by a magnetic stirrer. The aqueous and ether phases were separated by low speed centrifugation at 1,000 *g* for 5 minutes. The aqueous phase was taken and excess ether was removed by gentle bubbling of nitrogen through the suspension. The preparation thus obtained increased in HA titer consistently to around 10 times the original.

Preparation of Sendai virus neuraminidase fraction: The aqueous phase containing ether-emasol disrupted virus (5×10^5 HA units/ml) was centrifuged at 10,000 *g* for 15 minutes and the pellet which did not contain any biological activities was discarded. The supernatant which contained S-antigen, small HA and neuraminidase was centrifuged at 100,000 *g* for 2 hours to recover the neuraminidase fraction from the rapidly sedimenting HA as well as the S-antigen. The resulting supernatant which still contained 10^3-10^4 HA units per ml was employed as a Sendai virus neuraminidase fraction in the following experiments. Since the intact Sendai virus should not be contained in this fraction, it may represent the neuraminidase either freed of or associated with small HA. The preparation could be stored at −30°C without deterioration of the neuraminidase activity.

Antibody against egg-grown Sendai virus neuraminidase fraction (anti-Sendai-GL): The neuraminidase fraction described above was emulsified with an equal volume of incomplete Freund adjuvant and this was used for immunization. Three rabbits were inoculated with this preparation according to the following schedule: 0.25 ml was injected into each of the 4 foot pads of each rabbit and this was followed by 2 additional 1 ml boosters injected into the back on 14th and 18th day after the first injection. One week after the last inoculation, the rabbits were bled and the serum was separated. The antiserum was precipitated by one-third saturation with ammonium sulfate and the precipitates were centrifuged at 5,000 *g* for 10 minutes and dissolved with PBS in one-third of the original volume. This solution was subjected to another cycle of precipitation. The precipitates were finally dissolved in physiological saline in one-tenth of the original volume and dialyzed against the same solution for 72 hours at 4°C. This solution contained globulin at a concentration of 55 mg per ml and was stored frozen at −30°C. The solution at a concentration of 1 mg per ml had a hemagglutination inhibition titer of 128. In parallel with this, the globulin of normal rabbit serum (N-GL) was also prepared by the same method as described above.

Assay of neuraminidase activity: As a substrate, orosomucoid prepared from human plasma(5) was used. One-tenth ml of the substrate in an appropriate buffer was mixed with 0.1 ml of the specimen to be tested for neuraminidase activity, and incubated at 37°C

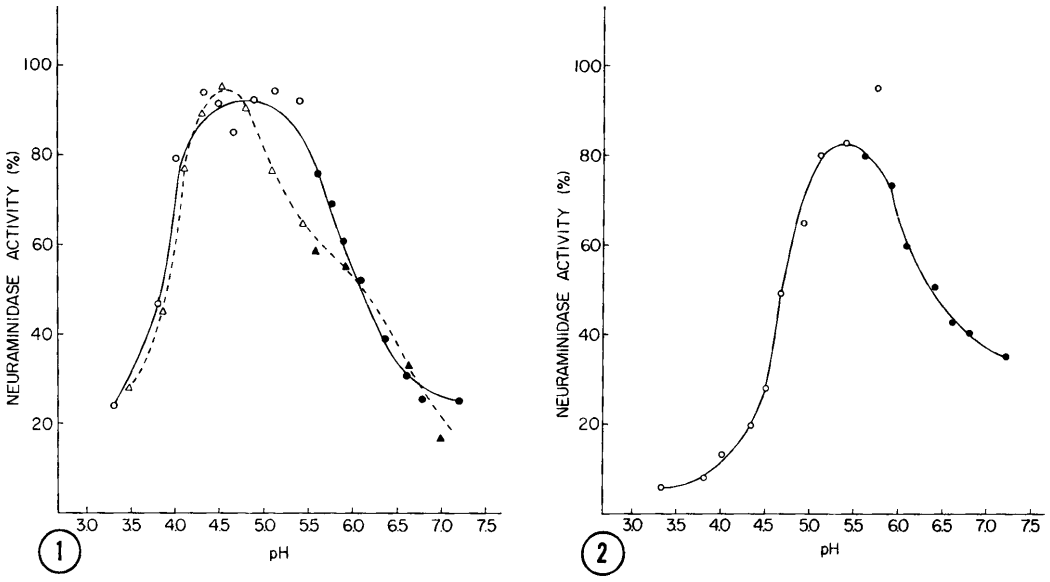

FIG. 1. A comparison of the neuraminidase activity of the neuraminidase fraction prepared from the egg-grown Sendai virus with that of the L cell-grown virus at various pHs. Neuraminidase fraction was incubated with orosomucoid in acetate or phosphate buffer. After 30 min incubation at 37°C, the amount of sialic acid liberated was measured by thiobarbituric acid method(6), and the maximum amount was taken as 100%. ○, egg-grown Sendai and acetate buffer; ●, egg-grown Sendai and phosphate buffer; △, L cell-grown Sendai and acetate buffer; ▲, L cell-grown Sendai and phosphate buffer.

FIG. 2. Effect of pH on neuraminidase activity of intact PR8 virus. Experimental conditions and expression of activity were the same as FIG. 1. ○, acetate buffer; ●, phosphate buffer.

for 30 minutes. The liberated sialic acid was assayed by the thiobarbituric acid method(6).

Results. pH optimum for neuraminidase action of Sendai virus: L cell-grown Sendai virus, possessing adsorbing and eluting activity for chicken red blood cells, is known to be infectious for eggs but not for L cells(7). As it was also shown to have neuraminidase activity (7), it was of interest to know if there was a difference between the L cell-grown and the egg-grown Sendai viruses in that activity. A comparison of pH optima for the activity was made with either intact viruses or the neuraminidase fractions extracted from both of them by the method described above.

For determination of the pH optimum for the neuraminidase activity, two buffer systems were employed. One was 1/5 M acetate buffer (Walpole) which covers pH between 3.5 and 5.5 and the other was 1/5 M phosphate buffer (Sörensen) which covers pH between 5.5 and 7.5. When the neuraminidase activity of either the intact viruses or the neuraminidase fractions was assayed at pH 5.5 in the two different buffer systems, almost concordant values were consistently obtained.

For the test, 0.2 ml of each buffer was mixed with 0.2 ml of the test material, either virus or neuraminidase fraction, which had been dialyzed against physiological saline just prior to use, and then 0.1 ml of the mixture was reacted with an equal volume of the substrate.

Fig. 1 shows the pH optimum curve for the neuraminidase fraction of the egg-grown Sendai virus in comparison with that for the same fraction of the L cell-grown Sendai virus. One can not find significant differences between them, particularly at pH 4.0-5.0 region. The higher activity was found, however, with the preparation from the egg-grown virus at pH 5.0-5.5 region, the reason for which has to be studied in the future.

With the neuraminidase activity of both the intact egg-grown and the L cell-grown Sendai viruses, exactly the same results were obtained.

Comparative study of pH optima for neur-

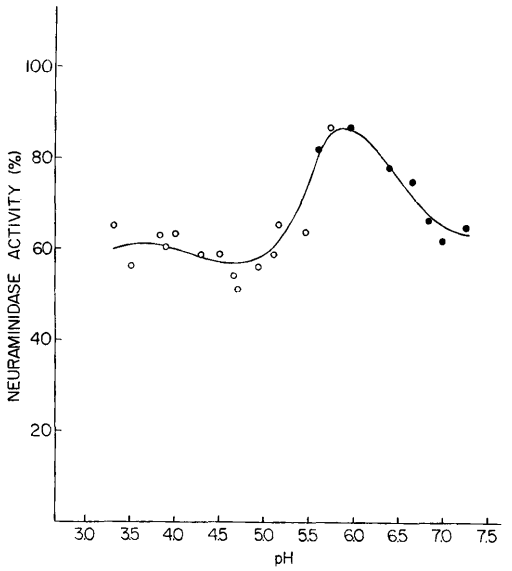

FIG. 3. Effect of pH on neuraminadise activity of intact Japan/305 virus. Experimental conditions and expression of activity were the same as FIG. 1. ○, acetate buffer; ●, phosphate buffer.

aminidase activity among myxoviruses: Although the experiment described above revealed the similarity of pH optima for the neuraminidase activities of both the egg-grown and the L cell-grown Sendai virus, a further study was made to determine the pH optima for the neuraminidase activity of other myxoviruses. Since in the preceding section, no significant differences were found between pH optima for the intact Sendai virus and neuraminidase fraction, only the intact viruses were used with the other viruses.

Result of one such experiment with the PR8 strain of influenza A virus is illustrated in Fig. 2, which shows, in contrast to Sendai virus, the pH optimum at around 5.5.

Another example was obtained with the Japan/305 strain of influenza A2 virus and the results are shown in Fig. 3. The pH optimum, which was close to, but a little higher than that of PR8, was between 5.5 and 6.0. In this case, however, a definite activity was detected even at lower pH of 3.5, giving the plateau between 3.5 and 5.0.

Among the viruses tested in this study, the Lee strain of influenza B virus and the NWS strain of influenza A virus did not give a definite neuraminidase activity on the substrate used here.

Fig. 4 is a composite of Fig. 1 (omitted from which is the result with L cell-grown Sendai virus), Fig. 2 and Fig. 3 and the results with neuraminidase activity of the NDV and FM1. Two strains of influenza A virus, PR8 and FM1, gave similar curves and pH optima. The peak obtained with the Japan/305 strain of influenza A2 virus, however, was shifted to a higher pH. Another example of similarity was found between Sendai virus and NDV.

Immunological specificity of the Sendai virus neuraminidase activity: The studies on pH optima for viral neuraminidase have clearly shown that the neuraminidase activity of Sendai virus can be differentiated from that of influenza A, but not from that of NDV. Thus the immunological specificity of neuraminidase activity of Sendai virus was examined. For this, serial 2-fold dilutions of anti-Sendai-GL and N-GL were made on saline and 0.2 ml of the solution was mixed with an equal amount of neuraminidase fractions of both the egg- and L cell-grown Sendai viruses as well as with the intact viruses including PR8 and NDV. After incubation at 37°C for 30 minutes, to 0.1 ml of the mixture was added an equal volume of substrate in a buffer at appropriate pH for each test system and the neuraminidase activity was measured by the method described in *Materials and methods*. The globulin concentrations in the test systems required for the 50% reduction in the amount of sialic acid released from the substrate were determined as compared with

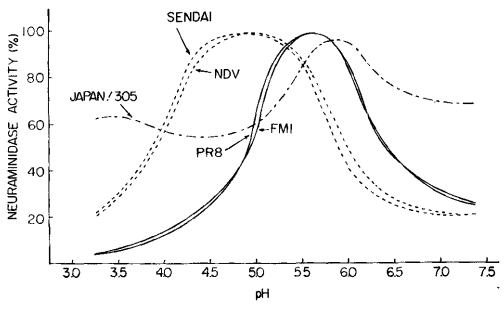

FIG. 4. A comparison of pH curves of neuraminidase activity among myxoviruses. Composite of FIG. 1-3 (with omission of results obtained with L cell-grown Sendai virus) and results with neuraminidase activity of intact NDV and FM1 virus obtained from the experiment conducted along the same line as FIG. 1-3 are summarized.

TABLE I. Inhibition of Neuraminidase Activities by Specific Antiserum.

Neuraminidase preparation	Globulin concentration producing 50% inhibition* (mg/ml)	
	Anti-Sendai-GL	N-GL
Neuraminidase fraction		
Egg-grown Sendai	.12	>5.0
L cell-grown Sendai	.1	>5.0
Intact virus		
Egg-grown Sendai	.12	>5.0
L cell-grown Sendai	.1	>5.0
NDV	>5.0	>5.0
PR8	>5.0	>5.0

* See text.

the control systems where the physiological saline was substituted for the globulins.

The results are illustrated in Table I. One can notice the specific inhibitory activity of the anti-Sendai-GL on the enzyme action of both the intact egg- and L cell-grown Sendai viruses, as well as on that of their neuraminidase fractions. Whereas, even the highest concentration, such as 5 mg per ml, did not inhibit the heterologous neuraminidase activity of PR8 and NDV. With N-GL at 5 mg per ml, no inhibitory effect was found on these test systems.

Discussion. Although orosomucoid acts as a potent substrate for the neuraminidase of Sendai virus, NDV, PR8, FM1 and Japan/305, it does not act so for the Lee strain of influenza B virus and NWS strain of influenza A virus as far as tested in the pH range between 3.0 and 8.0. While, using submaxillary gland mucin as a substrate, Jameson and Levine(8) have shown a difference between the neuraminidase activity of the WS strain and those of the NWS and FNWS strains of influenza A virus.

Rafelson *et al*(9) have shown the different pH optima for the neuraminidase activity of PR8 and that of Japan/305 using orosomucoid as a substrate, with fairly good agreement with our results obtained in this study though the difference is not so remarkable in our case. They also found that the neuraminidases isolated from the PR8 and Japan/305 viruses have the same pH optima as the respective intact viruses. In the present study, the neuramidase fractions, which were freed of intact virus but still contained small HA, derived from egg- and L cell-grown Sendai viruses had the same pH optima for the neuraminidase action as did the intact viruses.

Using collocalia mucoid as a substrate, Darrell and Howe(10) have shown that the pH optimum for the neuraminidase of parainfluenza type 2 is 4.5-5.5. This was close to our results obtained with Sendai virus and NDV which are closely related to the parainfluenza viruses.

Although the neuraminidase activity of the normal chorioallantoic membrane (CAM) (Ada(11) and our unpublished data) and Sendai virus grown in either egg or L cell have the same pH optima, it does not necessarily mean that the neuraminidase activity of Sendai virus is derived from the CAM as a contaminant. In fact, no neuraminidase activity was found in L cells.

From the immunological point of view, Rafelson *et al*(9) and Ada *et al*(12) have shown a specific inhibitory effect of antibody on the neuraminidase action. We also confirmed their results in the present study where anti-Sendai-GL had an inhibitory effect on the neuraminidase action of the homologous viruses regardless of their host origin but not on the neuraminidase action of the heterologous viruses such as PR8 and NDV grown in the CAM.

These results suggest that the neuraminidase of Sendai virus is one of the products which were formed in the host cells by the programming of the virus genetic code.

Summary. The pH optima for the neuraminidase activity of several myxoviruses were determined. The same pH optimum was found for Sendai virus grown in either egg or L cell and for NDV which is closely related to the former virus. However, the antibody prepared against the neuraminidase fraction of egg-grown Sendai virus showed an inhibitory effect only on the neuraminidase activity of egg-grown and L cell-grown Sendai virus but not on that of NDV.

The authors wish to thank Dr. Y. Watanabe for helpful advice in this work, and Mr. K. Tada for able technical assistance.

1. Mayron, L. W., Robert, B., Winzler, R. J., Rafelson, M. E., Arch. Biochem. Biophys., 1961, v92, 475.

2. Drzeniek, V. R., Rott, R., Z. Naturforsch., 1963, v18b, 1127.
3. Ishida, N., Homma, M., Tohoku J. Exp. Med., 1960, v73, 56; Virology, 1961, v14, 486.
4. Hosaka, Y., Hosokawa, Y., Fukai, K., Biken's J., 1960, v3, 27.
5. Schmid, K., J. Am. Chem. Soc., 1953, v75, 60.
6. Warren, L., J. Biol. Chem., 1959, v234, 1971.
7. Homma, M., Tohoku J. Exp. Med., 1961, v73, 215.
8. Jameson, P., Levine, A. S., J. Bact., 1965, v90, 563.
9. Rafelson, M. E., Schneir, M., Wilson, N. W., Arch. Biochem. Biophys., 1963, v103, 424.
10. Darrell, R. W., Howe, C., Proc. Soc. Exp. Biol. and Med., 1964, v116, 1091.
11. Ada, G. L., Biochim. Biophys. Acta, 1963, v73, 276.
12. Ada, G. L., Lind, P. E., Laver, W. G., J. Gen. Microbiol., 1963, v32, 225.

Received September 6, 1966. P.S.E.B.M., 1967, v124.

Labeling of Serum, Liver, and Testicular Lipids Following the Injection Of Arachidonic-1-C^{14} Acid.* (31840)

LEON SWELL AND M. D. LAW
Veterans Administration Hospital and Department Biochemistry, Medical College of Virginia, Richmond, Virginia

It has been demonstrated that arachidonic acid is synthesized from linoleic acid and that arachidonic acid is the precursor of prostaglandin E_2, highly polar metabolites, and certain long chain polyunsaturated fatty acids (1-4). Recent studies(5,6) have shown that arachidonic acid is well absorbed in the rat and appears in the lymph principally as triglyceride. Examination of the liver lipids from several animals fed arachidonic-1-C^{14} acid showed that 65-95% of the C^{14}-arachidonic acid was present in the phospholipid fraction. Also, conversion of arachidonic acid to CO_2 was shown to be slower than that of linoleic acid, suggesting a slower rate of metabolism of arachidonic acid. In view of the limited information available on the metabolism of arachidonic acid, it was of interest to study the handling of injected arachidonic-1-C^{14} acid. Data are presented on the turnover of arachidonic acid and the appearance of arachidonic-1-C^{14} acid in the different lipid fractions of serum, liver, and testes.

Methods and materials. Methyl arachidonate-l-C^{14} (4 μc/mg) was the generous gift of Hoffmann-LaRoche, Inc., Nutley, N.J. It was checked for purity by gas-liquid chromatography and gas-liquid radiochromatography (7). Purity by mass was 97% and radioactive purity was 96%. Fasting, male rats, (Wistar strain) maintained on Purina pellet chow, and weighing 150-185 g were injected into the saphenous vein with 1 μc of methyl arachidonate-1-C^{14} in 0.5 ml rat serum. The solution for injection was prepared by dispersing (by ultrasonic disintegration for 2 minutes at 5°C) 0.25 mg (1 μc) methyl arachidonate-1-C^{14} in 0.5 ml rat serum. The arachidonate was found to be uniformly dispersed in the serum and no chemical changes in the arachidonate-1-C^{14} was noted when the serum was subsequently extracted and analyzed by thin-layer and gas-liquid radiochromatography. The animals remained fasting following the injection until time of sacrifice. Groups of 5 animals were sacrificed at 1, 4, and 24 hours after the injection. At time of sacrifice, the liver, testes, and blood were obtained. The liver and testes were quick-frozen with dry ice, pulverized, and extracted with 2:1 chloroform-methanol. The serum was separated and extracted with 20 volumes 2:1 chloroform-methanol. The tissue lipids were separated by silicic acid column chromatography(8). Cholesterol esters were eluted with 1% diethyl ether-petroleum ether. Glycerides and fatty acids with 25% diethyl ether-petroleum ether, and phospholipids with methanol. The C^{14}-activity of the lipid fractions was determined in a liquid scintillation

* Supported in part by grants from USPHS HE-09039 and HE-09040.