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A Method for the Determination of Neuraminidase Activity in the Presence of Added Neuraminic Acids or Potential Inhibitors

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A new method for the determination of neuraminidase activity has been developed. The method is based on determination of lactose instead of *N*-acetylneuraminic acid, the compounds released by the enzymatic hydrolysis of *N*-acetylneuraminyllactose. All charged substances present in the incubation mixture are adsorbed on a mixture of DEAE- and SE-Sephadex. Lactose is eluted with deionized water. The method makes it possible to determine neuraminidase activity in the presence of added *N*-acetylneuraminic acid and other charged substances which interfere with the reagents available for the determination of neuraminic acids. The method for carbohydrate determination described by Park and Johnson gave good results with amounts of 1 to 15 μg lactose in the sample. Sodium diallyloxyacetate and sodium bis(2,3-dihydroxy)propoxyacetate have been synthesized. These substances, glyoxylic acid and sodium glyoxalate polyvinyl acetal had no inhibitory effect on neuraminidase of *Vibrio cholerae*.

It is a well established fact that neuraminidase, an enzyme found in myxovirus and in bacteria such as *Vibrio cholerae*, splits off *N*-acetylneuraminic acid (and *N*-glycolylneuraminic acid) from glycolipids and mucoproteins.[1] In order to determine the activity of different types of neuraminidase, several substances containing neuraminic acid have been used as substrates, for example mucoproteins from different sources [2,3] and erythrocyte stromata from animals [5–7] and man.[4] Several reagents are available for the determination of neuraminic acids.[8] Ehrlich's *p*-dimethylaminobenzaldehyd reagent,[13,14] Bial's orcinol reagent,[11,12] and other phenols,[10] Dische's diphenylamine reagent,[17] thiobarbituric acid and sodium periodate [9] and tryptophan in perchloric acid.[15] The thiobarbituric acid method as described by Aminoff [18] and Warren [16] discriminates free and bound *N*-acetylneuraminic acid in contrast to the other methods where it is necessary to separate free and bound neuraminic acid, for example by precipitation of the substrate.[6,7] All previously used

reagents are sensitive to the presence of carbohydrates, commonly found in the substrates, and dichromatic reading is recommended.[8]

Svennerholm has described a method for the determination of split off *N*-acetylneuraminic acid from tissues and proteins after separation of the acid with the aid of an anion exchange resin.[10] The discovery of *N*-acetylneuraminyllactose,[19] a well defined substrate for neuraminidase, has broadened the usefulness of Aminoff and Warren's thiobarbituric acid method for the determination of the activity of neuraminidase. However, these methods are not applicable when large amounts of *N*-acetylneuraminic acid,[20] derivates of neuraminic acid, or other substances which interfere [16,18] with the reagent, are present.

In order to meet the need of determining the effect of substances of the above described type on the activity of neuraminidase and at the same time to use a well defined substrate,[21] a new method for the determination of neuraminidase activity has been developed. An aliquot from an incubation mixture containing *N*-acetylneuraminyllactose is applied to a semi-micro ion exchange column filled with a mixture of a cation and an anion exchange resin. Split off lactose is then eluted with deionized water. All charged compounds will be retained by the ion exchange mixture. The lactose in the eluate can be determined by extremely sensitive methods, such as that described by Rogers *et al.*[22] which operates in the nanogram range. This would make it possible to use very small amounts of substrate and enzyme. In this work the method for the determination of carbohydrate described by Park and Johnson [23] has been chosen on account of its simplicity, accuracy, and extreme sensitivity.

RESULTS AND DISCUSSION

Recovery of lactose after passing ion exchange mixture. Sugars are strongly adsorbed on strongly basic polystyrene ion exchange resins.[24] A somewhat weaker adsorption occurs with acidic polystyrene ion exchange resins. To overcome this problem, mixtures of different types of Sephadex ion exchangers were tested. The best results were obtained with the weakly basic ion exchanger

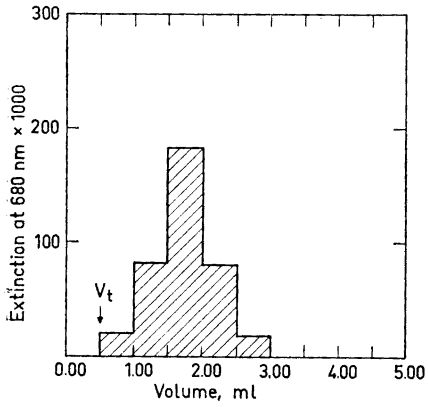

Fig. 1. Extinction at 680 nm of eluate, treated with Park and Johnson's reagents, from an ion exchange column charged with lactose. Deionized water was used as eluent.

in OH^--form, DEAE-Sephadex A-25, mixed with the strongly acidic ion exchanger SE-Sephadex C-25 in H^+-form. 3.0 ml of deionized water was necessary to elute lactose completely from a column with a total volume of 0.50 ml. (Fig. 1.) This retardation of lactose is probably due to the acidic function of the hydroxyl groups.

In order to test the recovery of lactose after elution with distilled water, 1 μg, 5 μg, and 10 μg of lactose in 20 μl of deionized water were applied to the ion exchange columns filled with 0.25 ml of ion exchange mixture. The columns were then eluted with 3×0.50 ml of deionized water as described above. The same amounts of lactose pipetted directly into 1.50 ml of water are assumed to represent the true values. As can be seen from Table 1 the recovery

Table 1. Analysis of lactose according to Park and Johnson, directly, and after passing an ion exchange column. $V_t = 0.25$ ml. Deionized water was used as eluent. $\bar{x}$ is based on 3 measurements. NAN = *N*-acetylneuraminic acid, NAN-lactose = *N*-acetylneuraminyl-lactose.

Sample	μg	$\bar{x}_{E(680\ nm)}$	$s \times 10^3$	$V_x = \frac{s}{\bar{x}_{E(680\ nm)}} \times 100$ (%)
μg lactose in 1.50 ml H_2O	1	0.056	6.56	11.70
	5	0.239	8.02	3.36
	10	0.478	5.86	1.23
μg lactose in 20 μl H_2O applied to 0.25 ml ion exchange mixture and eluted with 3×0.50 ml water	1	0.056	4.55	8.15
	5	0.241	8.49	3.51
	10	0.470	7.08	1.50
μg lactose in 20 μl Tris-HCl, 40 mM, pH 7.4, 10 mM in resp. to $CaCl_2$, applied to 0.25 ml ion exchange mixture and eluted with 3×0.50 ml water	5	0.245	13.43	5.50

Sample	μg	$E_{680\ nm}$
μg NAN-lactose + μg NAN in 20 μl Tris-HCl, 40 mM pH, 7.4, 10 mM in resp. to $CaCl_2$, applied to 0.25 ml ion exchange mixture and eluted with 3×0.50 ml water	10	0.051
	10[a]	0.045
μg NAN-lactose + μg NAN in 20 μl Tris-HCl, 40 mM, pH 7.4, 10 mM in resp. to $CaCl_2$, in 1.50 ml water	10	0.330

[a] The column had been charged once with a sample of the same composition which had been eluted with 3×0.50 ml water.

of lactose is almost quantitative, and the standard deviation is about the same for eluted and directly pipetted lactose. The coefficient of variation V_x lies between 1.2 and 11.7 % which also reflects the distortion of the spectrophotometer in the lower range. This result indicates that it would be possible to estimate inhibition of neuraminidase with at least ± 5 % accuracy. Tests were also performed with incubation buffer and 5 μg of lactose in order to investigate whether the components of the buffer would disturb the elution of lactose; no such disturbance could be detected. The columns contained 0.25 ml of ion exchange mixture and were eluted with 3×0.50 ml of deionized water as above. Table 1 also shows that when a mixture of incubation buffer containing 10 μg of *N*-acetylneuraminic acid and 10 μg of *N*-acetylneuraminyllactose in an volume of 20 μl was applied to the ion exchange column nothing could be detected in the eluate except a small amount of carbohydrate. *N*-Acetylneuraminyllactose from Sigma was reported to contain approximately 5 % of free lactose. The same ion exchange column could be charged with a second sample of the same composition. Tests with NaCl in deionized water showed that the 0.25 ml ion exchange columns had a capacity of at least 4 μequiv.

Lactose determination. The method of total carbohydrate determination modified by Park and Johnson [23] is rapid, accurate, and extremely sensitive, and has been chosen in this investigation for the determination of lactose. The method in based on the reduction of ferric to ferrous iron. The lack of specificity of the method is of no disadvantage in this case. No pH adjustment of the eluate is necessary since the eluent is pure water. The method shows linearity between 1 and 15 μg of lactose. The molar extinction coefficient has been estimated to 118 000 for lactose. The molar extinction coefficients of the thiobarbiturate method, the direct Ehrlich method and the resorcinol method are reported to be 57 000, 2030, and 4700 respectively for *N*-acetylneuraminic acid.[16]

Application of the method. Different amounts of neuraminidase have been incubated with the described incubation medium at 37°C. Aliqots were removed after different periods of time, applied to the ion exchange columns and

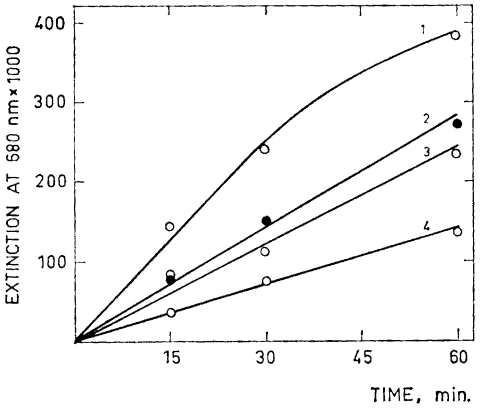

Fig. 2. Split off lactose, expressed as extinction at 680 nm from *N*-acetylneuraminyllactose by different amounts of neuraminidase and after different lengths of time.

1. 40 μl of neuraminidase solution, standard incubation mixture.
2. 40 μl of neuraminidase solution, incubation mixture 10 mM with resp. to *N*-acetylneuraminic acid.
3. 20 μl of neuraminidase solution, standard incubation mixture.
4. 10 μl of neuraminidase solution, standard incubation mixture.

eluted with 3×0.50 ml of deionized water. The extinction at 680 nm after treatment of the eluate with Park and Johnson's reagents was plotted against the different times. (Fig. 2.) All values were corrected for the extinction obtained from a sample at $t=0$, since the *N*-acetylneuraminyllactose contained a small amount of free lactose. Mohr has shown that *N*-acetylneuraminic acid inhibits neuraminidase from *Vibrio cholerae*.[7] In that case, erythrocyte stromata were used as substrate, which was precipitated after the incubation. The remaining amount of bound neuraminic acid was then determined. An inhibition of 50 % was achieved in these experiments, which were performed at pH 6.4 when the relation between bound and added *N*-acetylneuraminic acid was 1:9. Neuraminidase from influenza virus is also reported to be inhibited by *N*-acetylneuraminic acid using *N*-acetylneuraminyllactose as a substrate.[20] It was possible to demonstrate this by the fact that free neuraminic acid could be reduced by $NaBH_4$ to a compound which does not interfere with Ehrlich's *p*-dimethylaminobenzaldehyde reagent. These authors claimed that the affinity of viral neuraminidase at pH 6.8 was about eight times greater for *N*-acetylneuraminic acid when bound to lactose in *N*-acetylneuraminyllactose than when not bound. Using the method described in this paper, *N*-acetylneuraminic acid was found to be inhibitory at pH 7.4. (Fig. 2.) Thus with 20 μl of enzyme solution and an incubation time of 60 min, where the amount of enzyme is proportional to the released amount of lactose, 40 % inhibition was obtained at a concentration of 10 mM *N*-acetylneuraminic acid. In the present case, this is equal to a relation of 1:3 between bound and added *N*-acetylneuraminic acid. Sodium glyoxalate polyvinyl acetal, glyoxylic acid, and sodium bis(2,3-dihydroxypropoxy)acetate, which have structural similarities with *N*-acetylneuraminic acid, were found to have no effect on neuraminidase with a concentration of 10 mM.

EXPERIMENTAL

Materials. *N*-Acetylneuraminic acid, synthetic, free acid, anhydrous (Sigma). *N*-Acetylneuraminyllactose from bovine colostrum, free acid, anhydrous (Sigma). Neuraminidase from *Vibrio cholerae* was purchased from Behringwerke, Marburg, Germany. The activity was reported to be 500 units per ml. (500 μg of *N*-acetylneuraminic acid released in 15 min at pH 5.5 from acid α-glycoprotein). The solution of enzyme was concentrated in the following way: To 1 ml solution was added 200 mg of dry Sephadex G-25 coarse. After 10 min at 4°C the mixture was centrifuged and the supernatant liquid was collected. This supernatant liquid was stored at 4°C and used throughout the investigation.

Uvicord (LKB products, Stockholm, Sweden). Ultra Turrax (Janke & Kunkel KG, Staufen i. Br., Germany).

NMR analyses were carried out in D_2O with TMS as external reference, using a Varian A-60A analytical NMR spectrometer. The substances were, immediately before analysis, twice dissolved in D_2O and evaporated at 40°C (10–15 mm Hg). Extinction measurements were made with a Zeiss PMQ II spectrophotometer.

Synthesis. *Sodium glyoxalate polyvinyl acetal* was prepared by a modification of the method described by Nakajima *et al.*[25] 1.6 g polyvinylalcohol (Mowiol N 30–98) was dissolved in 20 ml of water and 0.4 ml of conc. HCl was added. 3.7 g of glyoxylic acid monohydrate was dissolved in this mixture, which was allowed to react at 60°C for various lengths of time. The gelled reaction mixture was then neutralized to pH 7.5 with 2 M NaOH with magnetic stirring which caused the gel to dissolve. The solution was then evaporated at 50°C (10–15 mm Hg) to about 20 ml and dialyzed against

distilled water until negative reaction for Cl^- (HNO_3+AgNO_3) and reducing substances. (Neutral solution+$AgNO_3$ gives a black colour with glyoxylic acid). The contents of the dialysis tube were then evaporated to dryness as described above. The white solid was washed with hot methanol. 24 h reaction gave 55 % acetalization and 48 h reaction gave 100 % acetalization. The charged polymers were found to move as uniform spots when analysed with thin layer electrophoresis. With 0.10 M ammonium carbonate pH 8.9 and 100 V/cm the polymers acetalized to 55 % moved half the distance of those acetalized to 100 %. (Spray reagent: Iodine in chloroform).

Sodium diallyloxyacetate. 4.6 g of metallic sodium was dissolved in 60 g of dry allyl alcohol by adding the metal in small pieces. Then 7.0 g of dichloroacetic acid was added and the mixture was heated in an oil bath at 130°C for 6 h with stirring. The mixture was then evaporated to dryness at 50°C (10—15 mm Hg) and 50 ml of water was added. After filtration, the solution was evaporated again as above to a thick syrup. To 1.0 g crude product was added 15 ml of methylethylketone containing 0.4 % of water and the syrup dissolved. After drying with Na_2SO_4, 15 ml of ethyl ether was added. The mixture was filtered and the filtrate was evaporated. The remaining hygroscopic white solid was stored over P_2O_5. Yield 8.8 g (83.9%) d: 179—181°C (uncorr.). (Found: C 49.5; H 5.75. Calc. C 49.5; H 5.71). MNR: $\tau=3.70-4.90$ ppm. Group of signals. ($CH_2=CH$) 6H. $\tau=5.20$ ppm. Singlet. (CH) 1H. $\tau=5.78$ ppm. Doublet. (CH_2) 4H. Hydrolysis with warm diluted HCl released allyl alcohol.

Sodium bis(2,3-dihydroxypropoxy)acetate. 500 mg of sodium diallyloxyacetate was dissolved in 15 ml of water and the mixture was cooled to exactly 5°C. 700 mg of $NaMnO_4 3H_2O$ dissolved in 15 ml of water was then added dropwise with vigorous stirring. The temperature was kept as close to 5°C as possible. The reaction mixture turned brown. When all permanganate solution had been added the stirring was stopped, and the temperature of the mixture was allowed to rise to room temperature. After standing overnight, MnO_2 was filtered off and washed with 30—50 ml of water. The filtrate was evaporated at 40°C (10—15 mm Hg) to a thick syrup which was further dried over P_2O_5. The residue weighed 870 mg. 200 mg of this crude product was dissolved in 2 ml of water and the solution was applied to a 2.5×33 cm Sephadex G-10 column equilibrated with distilled water. The column was then eluted with water with a flow rate of 15 ml per hour. 0.5 ml fractions were collected and the fractions between the dashed lines in Fig. 3 were combined and evaporated as described above.

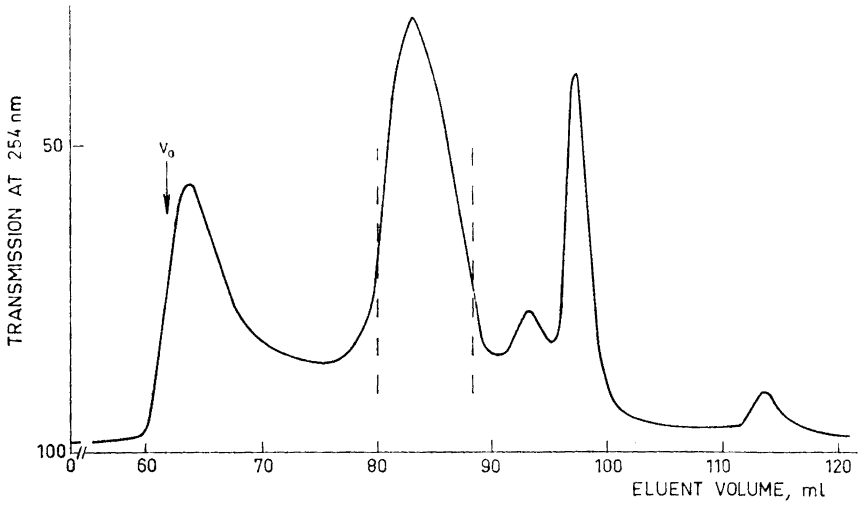

Fig. 3. Transmission at 254 nm of eluate from a Sephadex G-10 column charged with crude sodium bis(2,3-dihydroxy)propoxyacetate. Distilled water used as eluent.

Analysis of the eluate was made with a Uvicord using the 254 nm wavelength. The residue was dissolved in 4 ml of methanol and centrifuged. The clear supernatant was evaporated to dryness at 40°C (10—15 mm Hg). 135 mg of a white amorphous very hygroscopic solid was obtained. Yield: 587 mg (86.7 %). (Found: C 36.6; H 5.68. Calc. 36.7; H 5.78). d: 58—61°C (uncorr.). NMR: τ=5.07 ppm. Singlet. (CH) 1H. τ=5.72—6.42 ppm. Group of signals. (CH_2,CH) 10H, Hydrolysis with warm dilute HCl released glycerol.

Preparation of ion exchange mixture. 1 vol. of DEAE-Sephadex A-25 was allowed to swell in 20 vol. of deionized water for 24 h. Fines were decanted off after suspending the gel in water. The swollen gel was washed with 10 vol. of 0.5 M NaOH and then with 10 vol. of deionized water on a Büchner funnel. After this treatment, the gel was suspended in 2 vol. of deionized water. 1 vol. (equal to that of DEAE-Sephadex) of SE-Sephadex C-25 was allowed to swell in 20 vol. of deionized water for 24 h. Fines were decanted off and the gel was treated in the same way as DEAE-Sephadex A-25 using 0.5 M HCl instead of 0.5 M NaOH.

The DEAE-Sephadex suspension was poured into the SE-Sephadex suspension with vigorous stirring. The flocculent mixture was then homogenized for 2 min with an Ultra-Turrax and stored at 4°C.

Preparation of ion exchange columns. Glass tubes measuring 5×90 mm fitted with a piece of glass down were filled with the ion exchange mixture described above (Fig. 4).

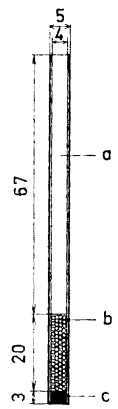

Fig. 4. Semi-micro ion exchange column for 0.25 ml of ion exchange mixture. Dimensions in mm.

a. Deionized water. b. Ion exchange mixture. c. Glass down.

The filling of the glass tubes with the ion exchange mixture was accomplished by placing the tubes in a semi-micro test tube with about 1 ml of water, adding the mixture with a Pasteur pipette and packing it with a glass rod. The ion exchange resin columns were then completely filled with deionized water and placed in a glass vessel with sufficient deionized water to cover the columns well. The columns were stored in this way at 4°C. The ion exchange columns were found to have unchanged capacity after several months of storing.

Method of incubation. The following incubation medium was used for the determination of the activity of neuraminidase: 40 mM Tris-HCl buffer 10 mM with respect to $CaCl_2$, pH 7.40, *N*-acetylneuraminyllactose 200 μg and enzyme in different amounts. The concentration of the substance to be tested for its effect on neuraminidase was maximized to 10 mM. The total volume was 100 μl, end pH adjusted to 7.4 and the incubation was made at 37°C with occasional shaking. Aliquots of 20 μl were removed from the incubation mixture at different intervals and transferred directly to the ion exchange columns.

Method of analysis. Ion exchange columns were placed one by one in test tubes with dimensions 16×110 mm. When the water above the ion exchange mixture had completely

run down into the test tubes the columns were taken up, wiped off and placed one by one in new test tubes with the above dimensions. An aliquot of 20 µl from the incubation medium was applied to the column with a constriction pipette. When the sample had run down into the column, 0.50 ml of deionized water was pipetted into the column. The water was allowed to run down into the test tube completely. Then two more 0.50 ml portions were added and allowed to run down in the same way as above. 5 min after the last portion of water had disappeared from the space above the ion exchange mixture the columns were discarded. The eluates in the test tubes were then analyzed directly. The concentration of lactose was estimated by the ferricyanide method developed by Park and Johnson.[23] To the test tubes containing 1.50 ml of eluate was added 1.0 ml of the ferricyanide reagent and 1.0 ml of the carbonate-cyanide reagent. After shaking the tubes were heated in a boiling water bath for 15 min. The tubes were then cooled with tap water to room temperature and 5.0 ml of the ammoniumferrisulfate reagent was added. After shaking, the extinction (blue colour) was measured within 15 min at 680 nm against a blank containing no lactose.

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