Preliminary Notes

Affinity of N-acetylneuraminic acid for influenza virus neuraminidase

It is well known[1,2] that neuraminidases from different sources split off NANA from several substrates. As observed in our laboratory[3] and confirmed by FAILLARD[4] and ROMANOWSKA[5], influenza-virus neuraminidase from several strains can be inhibited by NANA. Using our techniques[3], it can be shown that several sugars (including lactose) occurring in the substrates of this enzyme are without effect on the enzymic activity.

To obtain more quantitative data about the relationships involved, an accurate chemical determination of neuraminidase activity was necessary. Using WARREN's thiobarbituric acid assay[6] for free NANA such a method was worked out by JACOBS AND WALOP[7]. This method made it possible to study the interaction of neuraminidase and its substrates. However, in studies on the inhibition of neuraminidase by NANA, where amounts of NANA are added in excess of that released enzymically, the above-mentioned method does not permit a reliable interpretation. For this reason a method was developed in which instead of the NANA released by the enzyme, the NANA remaining in the substrate is determined. As HEIMER[8] observed, it is possible to reduce NANA by $NaBH_4$ to a compound, which—in contrast to NANA—does not act as a chromogen in the colour reaction with Ehrlich's *p*-dimethylaminobenzaldehyde reagent. Since ketosidically-bound NANA is not reduced by $NaBH_4$, a differentiation can be made in this way between free NANA—enzymically released or added—and ketosidically-bound NANA remaining in the substrate.

The substrate used in this study was N-acetylneuraminyl-lactose, prepared from cow colostrum by a procedure rather similar to the isolation of N-acetyl-O-acetyl-neuraminyl-lactose[9]. The determinations of neuraminidase activity were carried out in the following way: mixtures of equal volumes of 0.025 *M* phosphate buffer, substrate solution (N-acetylneuraminyl-lactose in concentrations varying from 100 to 400 μg/ml in phosphate buffer) and neuraminidase solution are incubated at 37°. The pH of all solutions is 6.8. In inhibition studies the volume of phosphate buffer is replaced by an appropriate buffered solution containing the inhibitor. At various intervals, starting at zero time, 1.50-ml aliquots of the incubation mixture are transferred to glass-stoppered tubes containing 0.20 ml of a solution of 2 mg ethylenediaminetetra-acetate (to stop the reaction) and 16 mg $NaBH_4$ in 0.1 *N* NaOH. The reduction of the free NANA is allowed to proceed for 30 min at room temperature, after which time 0.20 ml *N* HCl is added to decompose the excess $NaBH_4$. The remaining NANA is then determined with Ehrlich's reagent, using the procedure of WERNER AND ODIN[10], slightly modified.

This method was used to study the interaction of crystalline NANA and purified influenza-virus particles of the strain A_2 1 (1957 Singapore). Fig. 1 shows the results of a typical experiment, plotted according to DIXON[11] to determine both the type of inhibition and the inhibitor constant. From this figure, it can be inferred that:

Abbreviation: NANA, N-acetylneuraminic acid.

(a) the system influenza-virus and N-acetyl-neuraminyl-lactose follows Michaelis–Menten kinetics; (b) the inhibition by NANA is of the competitive type; (c) the inhibitor constant (K_i), representing the reciprocal of the affinity of NANA to influenza-virus neuraminidase, can be calculated. The mean value of K_i was determined as $5 \cdot 10^{-3}$ M.

With our method, based on the determination of NANA release[7], we determined the K_m of the system influenza-virus neuraminidase and N-acetylneuraminyl-lactose. Using the LINEWEAVER–BURK plot[11], as shown in Fig. 2, K_m can be estimated to be $6 \cdot 10^{-4}$ M. If we assume that K_m is a close approximation to the dissociation constant of the enzyme–substrate complex, comparison of this K_m for N-acetylneuraminyl-lactose with the K_i for NANA would indicate that the affinity of the NANA molecule for the enzyme surface is increased 8-fold by the ketosidic linkage of the NANA moiety to lactose.

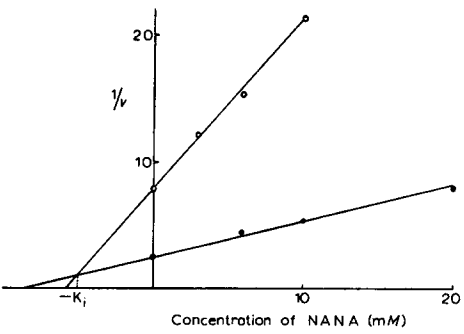

Fig. 1. Dixon plot for type of inhibition and determination of inhibitor constant, using influenza virus A_2 1 (1957 Singapore) as neuraminidase. N-acetylneuraminyl-lactose concentrations, 125 μg/ml (○—○) and 400 μg/ml (●—●). The units on the $1/v$ axis are arbitrary.

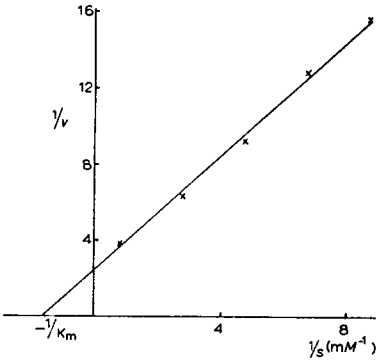

Fig. 2. Lineweaver–Burk plot for determination of Michaelis–Menten constant, using influenza virus A_2 1 (1957 Singapore) as neuraminidase. The units on the $1/s$ axis are the reciprocals of the mM concentrations of N-acetylneuraminyl-lactose. The units on the $1/v$ axis are arbitrary.

We acknowledge the technical assistance of Mr. J. ADMIRAAL and of Mr. N. DE LANGE.

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Received September 1st, 1960