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Induction and Regulation of Neuraminidase Synthesis in *Arthrobacter sialophilus*

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A variety of *N*-acetylneuraminic acid (AcNeu) derivatives and analogs were examined as inducers of the extracellular neuraminidase of *Arthrobacter sialophilus*. Neuraminidase inductions were primarily studied with tryptone-yeast extract-grown cells after washing and resuspension in a defined replacement medium. The addition of readily metabolizable carbon sources to the latter, such as 0.1% casein hydrolysate, glutamate, or glucose, enhanced enzyme synthesis. Enzyme appearance occurred after a lag in the uptake of inducers, suggesting the participation of a co-inducible transport system. Neuraminidase formation during exponential growth in the presence of AcNeu ceased after depletion of this end product from the medium. It was found, besides AcNeu, that its methyl ester, 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid and 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid methyl ester are each active inducers, whereas β-anomers of AcNeu-ketosides are not. These results, in comparison to known enzyme specificity, have revealed significant differences and parallels between the inductive and catalytic processes for neuraminidase. In particular, it would appear that the free carboxylate and oxygenation at C-2 of AcNeu, essential for enzyme catalysis with traditional AcNeu substrates, are not necessary for induction and, furthermore, that transition state analogs can specifically induce this enzyme. The failure to observe catabolite repression in this system is discussed in relation to the intermediary metabolism of the genus *Arthrobacter*.

Circumstantial evidence for the inducible nature of bacterial neuraminidase (EC 3.2.1.18) has been accumulating for several years. Thus, the earlier reports of neuraminidase formation by *Diplococcus pneumonia* (11), *Klebsiella aerogenes* (21), and *Clostridium perfringens* (19) each pointed to the conclusion that enzyme manifestation was correlated with the necessary presence of glycoproteins (or amino sugars) plus other complex nutrients in their respective growth media. We have recently described (5) the isolation of a soil bacterium, designated *Arthrobacter sialophilus* (25), which secretes this enzyme during growth on glycoproteins or, alternatively, after growth in glycoprotein-free medium after washing and resuspension in minimal medium containing potential inducers. The purification of this preparation to homogeneity has also been detailed (26). This report provides the results of an initial study of a number of *N*-acetylneuraminic acid (AcNeu) derivatives, analogs, and metabolically related compounds as enzyme potentiators with *A. sialophilus* under controlled experimental conditions. The pattern obtained clearly demonstrates the inducibility of *A. sialophilus* neuraminidase and, when compared to what is known about enzyme substrate specificity (2, 3), reveals certain significant differences and parallels between the inductive and catalytic processes.

MATERIALS AND METHODS

Chemicals. Glucosamine, *N*-acetylglucosamine, mannosamine, and *N*-acetylmannosamine were obtained from Sigma Chemical Co. Colominic acid was obtained from Calbiochem-Behring. Yeast extract, tryptone (Bacto), and casein hydrolysate were obtained from Difco Laboratories. All other chemicals were of reagent grade.

Growth and induction of neuraminidase. Growth of *A. sialophilus* (ATCC 31253) and induction of neuraminidase was carried out essentially as described previously (5). For most experiments described in this report, stationary-phase cells were utilized for induction of neuraminidase. These cultures were prepared as follows. The organism was grown at 30°C overnight on a rotary shaker in 500-ml Erlenmeyer flasks containing 50 ml of 1.0% (wt/vol) tryptone (Bacto) and 0.5% (wt/vol) yeast extract (TYE). The cells were harvested by centrifugation, washed with 0.9% NaCl, resuspended in 0.1 volume of M-9 medium containing 0.1% casein hydrolysate plus 1 mg of AcNeu per ml, and induction was carried out for 6 h at 30°C. To prepare cells for inductions under growing condi-

tions, overnight cultures after centrifugation and washing were diluted 40-fold with M-9 medium to overcome cell density inhibition. Neuraminidase activity was determined after cell removal and extensive dialysis against 0.010 M citrate-phosphate buffer, pH 6.0, as described below. Before dialysis, the concentrations of AcNeu in induction filtrates were determined by the Warren procedure (27).

Neuraminidase assay. The activity of neuraminidase preparations was assayed as described previously (26), with collocalia mucoid (9) as substrate. A unit of enzyme activity is defined as that amount releasing 1 μmol of AcNeu per min from the mucoid preparation under conditions of this standard assay. Protein was determined by the method of Lowry et al. (15) with crystalline bovine serum albumin as the standard.

AcNeu methyl ester conversion. Enzyme induction with AcNeu methyl ester was allowed to proceed until 50% of the inducer was taken up by the cells. After centrifugation, the induction filtrate was concentrated by freeze-drying and was redissolved in a minimum of water. Separations were carried out on cellulose plates (Eastman Kodak Co.) with butanol–*n*-propanol–0.1 M HCl (1:2:1) development; spots were located by spraying the plates with the Ehrlich reagent (6) followed by heating at ca. 110°C. Alternatively, thin-layer chromatography was performed on Silica Gel F 254 (Woelm, ICN Pharmaceuticals) plates developed with 10% H_2SO_4, and then heating at ca. 110°C.

Preparation of AcNeu derivatives. AcNeu was isolated from "edible bird's nest", as published previously (16). The α- and β-glycosides of AcNeu, 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid (dehydro-AcNeu) and its methyl ester, pentaacetyl AcNeu, and tetraacetyl AcNeu methyl ester were synthesized as described earlier (12, 17, 29). The physical constants for these compounds agreed with published values.

RESULTS

Dosage response to AcNeu. The effect of increasing concentrations of AcNeu on the induction of neuraminidase is shown in Fig. 1. Enzyme induction occurred at those concentrations indicated, reaching a plateau at about 2.0 mg/ml. In all subsequent experiments, inductions were carried out at a final AcNeu concentration of 1.0 mg/ml.

Other neuraminidase inducers. A variety of compounds were examined for their ability to induce neuraminidase synthesis. The results reported in Table 1 are presented relative to AcNeu as the control. As reported previously (5), all α-ketosides of AcNeu tested were effective enzyme inducers, with the homopolymer colominic acid being the most efficient. In contrast, neuraminidase induction was not stimulated by β-ketosides of AcNeu. Dehydro-AcNeu and its methyl ester, each proposed as transition state analogs for *A. sialophilus* neuraminidase (18), were also effective as enzyme inducers. As is well documented (2, 3) for enzyme catalysis, neuraminidase substrates must have a free carboxyl group because carboxylate derivatives are neither enzyme substrates nor inhibitors. As shown in Table 1, AcNeu methyl ester is 71% as effective an inducer as the parental compound.

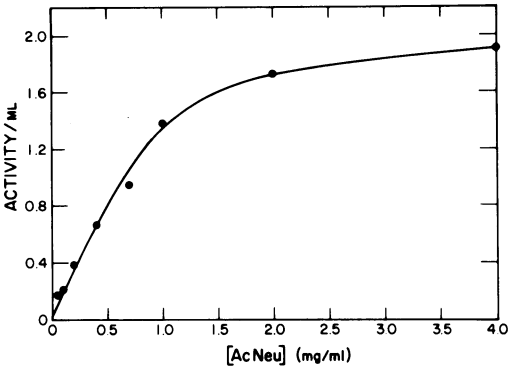

FIG. 1. *Induction of neuraminidase as a function of AcNeu concentration. Cells were grown for 18 h in TYE, washed with 0.9% NaCl, and were then suspended in minimal salts (M-9). Enzyme induction was carried out for 6 h at 30°C with 0.1% casein hydrolysate plus AcNeu at the indicated concentrations. The cells were centrifuged, and the supernatant was dialyzed against 0.01 M citrate-phosphate buffer, pH 6.0. The activity of neuraminidase was determined as described in the text.*

TABLE 1. *Stimulation of neuraminidase activity in replacement media*

Addition[a]	Concn (%)	Relative activity
AcNeu	0.10	1.00
N-acetylneuraminlactose	0.10	1.25
Colominic acid	0.10	1.95
N-acetylneuraminyl-α-methyl glycoside	0.10	0.98
N-acetylneuraminyl-β-methyl glycoside	0.10	0.009
Neuraminic acid-β-methyl glycoside	0.10	0.017
AcNeu methyl ester	0.10	0.71
N-acetylneuraminyl-α-methyl glycoside methyl ester	0.10	0.091
N-acetylneuraminyl-β-methyl glycoside methyl ester	0.10	0.00
Pentaacetyl AcNeu	0.10	0.00
Tetraacetyl AcNeu methyl ester	0.10	0.072
Dehydro-AcNeu	0.05	1.38
Dehydro-AcNeu methyl ester	0.05	0.33
Glucosamine	0.10	0.028
N-acetylglucosamine	0.10	0.032
Mannosamine	0.10	0.020
N-acetylmannosamine	0.10	0.019
Control		0.005

[a] Cells were grown and enzyme induction was carried out with the indicated inducer plus 0.1% casein hydrolysate as described in the legend to Fig. 1.

That this observation reflects causal enzyme induction was bolstered by the following experiment. Neuraminidase formation with AcNeu methyl ester was allowed to proceed until ca. 50% of the inducer was taken up by the cells (see above). The filtrate after analysis by thin-layer chromatography failed to reveal the presence of AcNeu. Therefore, conversion of this inducer to free AcNeu apparently did not precede enzyme formation. Also tested as potential inducers were a series of metabolically related monosaccharides, some of which, such as *N*-acetylmannosamine and *N*-acetylglucosamine, were reported as enzyme inducers for other bacterial neuraminidases (11). In each case, stimulated enzyme synthesis was not found.

Kinetics of induction. Figure 2 displays the time course of enzyme appearance and correlates its synthesis to the uptake of AcNeu and cell growth. In Fig. 2A, a typical growth curve of *A. sialophilus* is shown. After an initial lag period, logarithmic growth was observed, providing a generation time of 81 min. The uptake of AcNeu by *A. sialophilus* is also shown. Transport of AcNeu into the organism was characterized by a delay of about 3 h, followed by its rapid depletion from the medium. This observation suggests that AcNeu may not be freely permeable through the plasma membrane and that the observed delay reflects the time required for the synthesis of an AcNeu transport system. The rate of enzyme synthesis is also shown in Fig. 2A. Neuraminidase synthesis mirrored the uptake of AcNeu, as characterized by an initial slow phase followed by rapid enzyme synthesis which leveled off after depletion of the inducer from the medium.

In Fig. 2B a similar experiment is graphically depicted, except that stationary-phase cells were used to investigate the relationship between en-

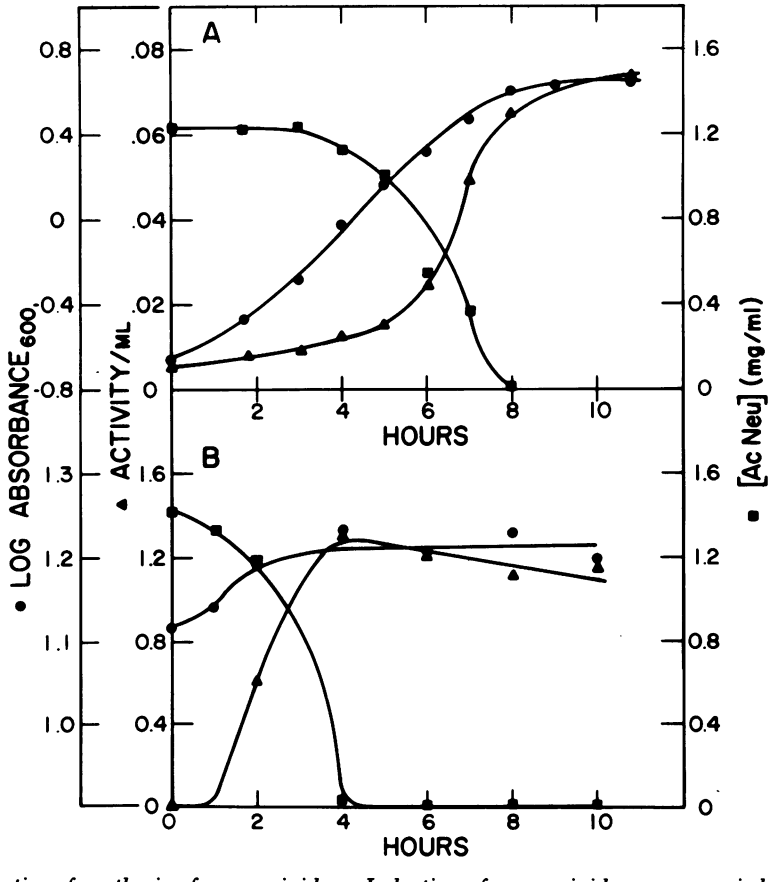

FIG. 2. *Kinetics of synthesis of neuraminidase. Induction of neuraminidase was carried out with either exponential-phase cells (A) or stationary-phase cells (B) prepared as described in the text. Cell densities were followed by measuring the absorbance of suitably diluted culture samples at 600 nm in a Varian model 635 spectrophotometer. The concentration of AcNeu and the activity of neuraminidase were determined as described in the text.*

zyme synthesis, cell growth, and AcNeu transport. Here again, the uptake of AcNeu was characterized by a slight lag followed by its rapid depletion from the induction medium at 4 h. The time course of enzyme synthesis also reflected the uptake of AcNeu, with an initial lag followed by rapid synthesis which terminated after inducer depletion.

Comparable experiments to the foregoing were carried out with AcNeu methyl ester and dehydro-AcNeu as inducers. With either exponential- or stationary-phase cells, the results found were similar to those described in Fig. 2A and B.

Regulation of neuraminidase by carbon sources. It was of interest to determine the effect of selected amplifying carbon sources on the induction of neuraminidase under the standard protocol. The compounds listed in Table 2 were each added along with AcNeu at the start of induction. In each case, enzyme synthesis occurred, with 0.1% casein hydrolysate giving a fourfold increase in activity. Further addition of 0.1% glucose, glutamate, or succinate to the casein hydrolysate–AcNeu mixture provided no significant changes in enzyme synthesis (data not shown).

DISCUSSION

Little work has been carried out to delineate the factors controlling the induction and regulation of neuraminidase synthesis by bacteria. The parameter probably limiting such prior physiological studies has been the rather fastidious nature of most neuraminidase-producing organisms. With the finding that washed cell suspensions of *A. sialophilus* readily induce enzyme synthesis in defined media, we were able to initiate such a study. As shown in Fig. 1, AcNeu itself is an inducer of neuraminidase, and thus this system is apparently under product induction, as defined by Ornston and Parke (20). By testing the efficacy of a variety of AcNeu analogs (Table 1), we have attempted to determine which chemical substituents are critical for neuraminidase induction and now can compare these results with its known substrate specificity. The major difference between induction and catalysis of *Arthrobacter* neuraminidase is the role played by the free carboxyl group of AcNeu. Whereas this functionality is absolutely essential for enzyme catalysis (2, 3), the methyl esters of AcNeu and AcNeu-α-methyl ketosides are effective or marginal inducers, respectively. Although hydrolysis by intracellular esterases cannot completely be ruled out, preliminary studies suggested that the AcNeu methyl ester was not altered during this process. The observation that dehydro-AcNeu methyl ester is an effective inducer provides additional evidence that the carboxyl group is not essential for the induction process. Several conclusions concerning the role that the 2-hydroxyl group of AcNeu plays in the overall induction process can also be made from the data presented in Table 1. At equilibrium, the stable anomeric form of AcNeu is $^2C_5$, with the C-2 hydroxyl group oriented in the axial, or β, position. Because the *N*-acetylneuraminyl-β-methyl ketoside is neither an inducer nor a substrate of neuraminidase and differs from AcNeu only in having a $-CH_3$ group instead of a $-H$, we can conclude that the size of this axial substituent is of great importance. Furthermore, because dehydro-AcNeu is an enzyme inducer and also lacks the C-2 oxygen, the presence of this atom is not essential for the induction process. We have recently provided evidence that dehydro-AcNeu and its methyl ester are transition-state analogs for *Arthrobacter* neuraminidase (18). To our knowledge, this may represent the first example that this class of competitive inhibitors can also act as enzyme inducers. Whether these compounds are transformed to AcNeu by basal levels of enzyme, or are recognized by the presumed repressor protein, is as yet not known.

TABLE 2. *Stimulation of neuraminidase activity by different carbon sources*

Addition	Activity (U/ml)
AcNeu	0.630
AcNeu + casein hydrolysate	2.51
AcNeu + glucose	1.40
AcNeu + glutamate	1.12
AcNeu + succinate	0.70

[a] Cells were grown and enzyme induction was carried out with 0.1% AcNeu and the added carbon source (0.1%) as described in the legend to Fig. 1.

The data given in Table 1 clearly establish that α-ketosides of AcNeu are inducers of bacterial neuraminidases, which fully substantiates earlier findings (5, 11, 19, 21). Our results suggest that the ability of these several compounds to potentiate enzyme synthesis is not necessarily due to the presence of the glycosidic linkage, but rather depends upon the ability of neuraminidase to catalyze the formation of free AcNeu. Because neuraminidase may generate the actual inducer, the overall process of enzyme induction in *A. sialophilus* is, in all probability, autocatalytic.

The relationship between enzyme synthesis and the transport of inducer by *A. sialophilus* was also investigated. These results primarily

reaffirm our previous conclusion (5) that *A. sialophilus* neuraminidase is a true exoenzyme (22), because secretion of enzyme occurs in the absence of cell lysis with either exponential- or stationary-phase cells. As shown in Fig. 2, there is a significant lag in the uptake of AcNeu, which strongly suggests the presence of an inducible AcNeu transport system. This latter observation is similar to those reported by Nees and Schauer (19) concerning the uptake of AcNeu by *C. perfringens*. Subsequent to transport, enzyme formation in *A. sialophilus* continues until AcNeu becomes depleted from the medium, at which time it stops. Our finding that enzyme synthesis occurs both during exponential- and stationary-phases of growth is markedly different from that seen with other bacterial extracellular hydrolytic enzymes. In these examples, synthesis was said to occur only during late-exponential and stationary phase of growth (23).

The synthesis of neuraminidase is modulated by various nutrients in the medium as shown in Table 2. The tricarboxylic acid cycle intermediates succinate and glutamate, glucose, and a mixture of amino acids (casein hydrolysate) each stimulate enzyme synthesis during induction. Krulwich and co-workers (14, 28) working with *A. pyridinolis*, have demonstrated that the addition of L-malate resulted in an increase in the transport of certain sugars as well as in the appearance of the inducible enzyme fructose kinase. Thus, it is conceivable that the increase in synthesis of neuraminidase that we observe in the presence of added carbon sources may reflect, in part, an increased rate of AcNeu transport. Because enzyme induction occurs in the presence of certain easily metabolizable carbon compounds, it would further appear that the regulation of *A. sialophilus* neuraminidase is not mediated by catabolic repression, as delineated for the lactose operon in *Escherichia coli* (24), for the proteases of *Pseudomonas maltophilia* (1) and of an *Arthrobacter* isolate (8), for the α-1,3-glucanase of a *Flavobacterium* sp. (4) and the dextranase of *Cytophaga johnsonii* (10), and for the cellulase from a *Clostridium* sp. (13). In this context we point out that Hamilton and Kolenbrander (7) have demonstrated transient fluctuations of cyclic AMP during the rod-sphere transformation in *A. crystallipoietes*. During inductions under either stationary or growth conditions *A. sialophilus* appeared microscopically exclusively in the sphere-shaped form. However, because cyclic AMP is a morphological regulator in *Arthrobacter* and plays a controlling role in many microbial inductions (24), additional studies to determine whether this effector is involved in the synthesis and control of neuraminidase by *A. sialophilus* are clearly indicated.

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