N-Acetylneuraminic acid uptake in *Pasteurella (Mannheimia) haemolytica* A2 occurs by an inducible and specific transport system

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Abstract The *N*-acetylneuraminic acid (NeuAc) transport system of *Pasteurella (Mannheimia) haemolytica* A2 was studied when this bacterium was grown in both complex and chemically defined media. Kinetic measurements were carried out at 37°C in 50 mM Tris–HCl buffer, pH 8.0, containing 50 μg/ml bovine serum albumin. Under these conditions, the uptake rate was linear for at least 3 min and the calculated K_m for NeuAc was 0.1 μM. The transport rate was increased by the addition of several cations and was inhibited by sodium arsenite (95%), *N*,*N*′-dicyclohexyl-carbodiimide (50%), and 2,4-dinitrophenol (40%) at final concentration of 1 mM (each). These results support the notion that NeuAc uptake is an active sugar cation symporter. Study of specificities showed that glucosamine, mannose and mannosamine inhibited the transport of NeuAc in this bacterium. Analysis of expression revealed that the NeuAc transport system was induced by NeuAc and by the simultaneous presence of glucose and galactose in the growth medium.

Key words: Sialic acid transport; Polysialic acid; *Pasteurella*

1. Introduction

Pasteurella haemolytica is a Gram-negative bacterium responsible for economically important diseases in ruminants, including pneumonic pasteurellosis and septicemia in sheep [1,2]. Traditionally, *P. haemolytica* has been subdivided into two biotypes, based on biochemical characteristics, and 17 serotypes, based on variations in capsular polysaccharides [3–6], whose presence has been demonstrated to be age-dependent [7] and is believed to play a role in the pathogenicity of the organism [8,9]. Nevertheless, new taxonomic studies have indicated that several of these serotypes belong to the genus *Mannheimia* [10].

P. (Mannheimia) haemolytica serotype A2, the strain studied here, is able to produce a capsular polysaccharide that consists of a (2–8)-α linked polymer of *N*-acetylneuraminic acid (NeuAc), namely colominic acid or polysialic acid [11,12]. This is identical to the capsular polysaccharides of *Neisseria meningitidis* group B and *Escherichia coli* K1. The production of colominic acid by *P. haemolytica* A2 and other bacterial organisms explains the difficulties found by workers attempting to develop vaccines from these organisms [13]. The exact mechanism(s) by which sialic acids imparts virulence to *P. haemolytica* A2 is obscure, although the mechanism for the synthesis and regulation of polysaccharide production in this organism has been recently defined [12,14].

Previous studies [15] have shown that different serotypes of *P. haemolytica* produce sialidase, with the capacity to cleave terminal NeuAc residues from different sources. However, until now nothing is known about the destination of this sugar after its cleavage. Here, we report a biochemical study of the NeuAc uptake system of *P. haemolytica* A2, confirming the existence of a specific and inducible transport mechanism for the uptake of this amino sugar.

2. Materials and methods

2.1. Strain, culture media and growth conditions

P. haemolytica A2 (ATCC 29694) was obtained from the 'Colección Española de Cultivos Tipo' (CECT 924). The strain was kept lyophilized and to avoid loss of the capacity to produce polysialic acid, the lyophilisates were incubated in a rotary shaker (250 rpm) at 37°C in 250 ml Erlenmeyer flasks containing 50 ml of BHIB (brain heart infusion broth). The cellular suspension was stored at −70°C in 50% (v/v) glycerol (preinoculum). Experimental cultures were grown by inoculating 25 ml of the required medium (see below) in 125 ml Erlenmeyer flasks with 25 μl of the preinoculum. The media used in this work were the BHIB medium (37 g l^{-1}) and a defined medium called Wessman medium [16]. In experiments addressing the expression of NeuAc transport, the carbon sources of Wessman medium (glucose and galactose) were replaced by other sugars.

2.2. NeuAc uptake in whole cells

Cells grown in the BHIB medium or in the required media were harvested at different times, washed twice with 50 mM Tris–HCl (pH 8.0), 50 μg ml^{-1} bovine serum albumin (BSA), dithiothreitol (DTT) 5 mM and 25 mM NaCl and resuspended in the same buffer. Cell concentrations were adjusted to an $A_{540nm} = 0.5$, placed in 25 ml Erlenmeyer flasks, and preincubated at 37°C for 2 min in a thermostatically controlled bath at 160 strokes per min. Then 1.5 nmol ml^{-1} of NeuAc (containing 0.16 nmol [^{14}C]NeuAc: 55 mCi $nmol^{-1}$) was added. Aliquots of 1 ml were taken from the uptake mixture after 2 min of incubation and the radioactivity incorporated was quantified as previously described [17] using Ecoscint A (National Diagnostic, UK) as scintillation fluid. Effectors were tested by adding these 2 min before the radiolabelled sugar. NeuAc uptake is given in units (pmol of NeuAc incorporated/min per ml of cellular suspension at $A_{540nm} = 0.5$).

2.3. Determinations of the half-life of the NeuAc transport system

Cells were grown in BHIB medium and after 4 h protein synthesis

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Abbreviations: NeuAc, *N*-acetylneuraminic acid; BSA, bovine serum albumin; DCCD, *N*,*N*′-dicyclohexyl-carbodiimide; BHIB, brain heart infusion broth; DTT, dithiothreitol; 2,4-DNP, 2,4-dinitrophenol

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was stopped by adding 50 μg of chloramphenicol per ml. From this time up to 6 h NeuAc transport was measured at different times.

2.4. Induction experiments on the NeuAc transport system

P. haemolytica A2 was grown in Wessman modified medium where the carbon sources, glucose and galactose, were replaced by glycerol up to $A_{540nm} = 0.2$. After this moment, different sugars were added and transport analysis was monitored at intervals. All procedures were carried out under sterile conditions.

3. Results and discussion

3.1. Time course of the appearance of the NeuAc uptake system

Time course analysis using cells grown in BHIB medium revealed that the enzyme (or enzymic system) appeared during the first hours of growth, maximum levels being attained at 4 h ($A_{540nm} = 1.1$). Thereafter, uptake decreased continuously and no transport was detected up to 6 h (Fig. 1). These results indicate that maximum uptake activity takes place during the early exponential phase of cellular growth, decreasing rapidly after this moment and disappearing at the end of this logarithmic phase. This suggests that the NeuAc transport system can be detected and assayed in a complex medium that is ideal both for growth and for colominic acid production [12]. Similar results have been described previously for NeuAc transport in *E. coli* K1, another bacterium that also produces colominic acid as a capsular polymer, also using defined media [17].

3.2. Characteristics of the NeuAc transport system

Analysis of the optimum physicochemical parameters of the *P. haemolytica* A2 NeuAc transport system showed that maximum uptake was attained at 37°C in 50 mM Tris–HCl buffer (pH 8.0) containing 50 μg ml^{-1} BSA, 5 mM DTT and 25 mM NaCl. NeuAc incorporation was linear for at least 3 min (Fig. 2). Under these conditions, and for 2 min, the initial rates of uptake were measured at different concentrations of NeuAc. Analysis of these results revealed that the K_m for sialic acid is 0.1 μM (Fig. 2d). This value is 300- and 210-fold lower than those reported for NeuAc transport in *E. coli* K1 [17] and *Streptococcus oralis* ARS [18] respectively, and is similar to that of 0.24 μM described for *Lactobacillus plantarum* [19]. These results suggest the existence of two NeuAc transporter types: one of low and the other of high affinity, corresponding to the system present in *P. haemolytica* A2.

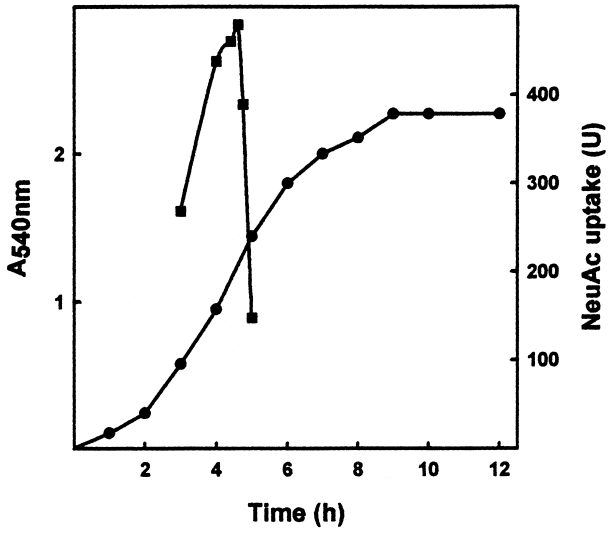

Fig. 1. Growth of *P. haemolytica* A2 at 37°C in BHIB medium (●) and time course of NeuAc transport (■).

Table 1
Effect of thiol reagents, ions and energy poisons on NeuAc transport in *P. haemolytica* A2

Effector	Concentration (mM)	% Uptake
None		100
Thiol reagents		
DTT	1	102
	5	130
2-Mercaptoethanol	1	100
	5	120
Iodoacetic acid	1	52
	5	46
N-Ethylmaleimide	1	9
	5	2.7
p-Hydroxymercuribenzoate	1	14
	5	5
5,5′-Dithio-bis(2-nitrobenzoic acid)	1	22
	5	10
Ions		
Na^+	10	190
	25	210
K^+	10	157
	25	168
Hg^{2+}	10	23
Co^{2+}	10	11
Cu^{2+}	10	8
Zn^{2+}	10	9
Energy poisons		
Cyanide	1	100
NaF	1	100
Valinomycin	2	86
Sodium azide	1	75
2,4-DNP	1	61
DCCD	1	50
Sodium arsenite	1	5

The half-life of permease was 40 min (data not shown). This brief time indicates that the transporter has a rapid turnover rate, suggesting that this bacterium has a good capacity to adapt rapidly to different nutritional and metabolic conditions.

The effect of exogenously added thiol-containing and thiol-modifying reagents on the uptake of sialic acid was tested. In the presence of mercaptoethanol or DTT at concentrations of 5 mM, activity increased (120% and 130% respectively) (Table 1). Analysis of the different concentrations of DTT revealed

Table 2
Effect of different sugars and sugar derivatives on NeuAc transport in *P. haemolytica* A2

Effector (5 mM)	% Uptake
None	100
Glucose	240
Arabinose	138
Fructose	400
Galactose	300
Xylose	158
Mannose	12
Mannosamine	10
Glucosamine	55
Galactosamine	119
N-Acetylmannosamine	285
N-Acetylglucosamine	257
N-Acetylgalactosamine	165
Saccharose	118

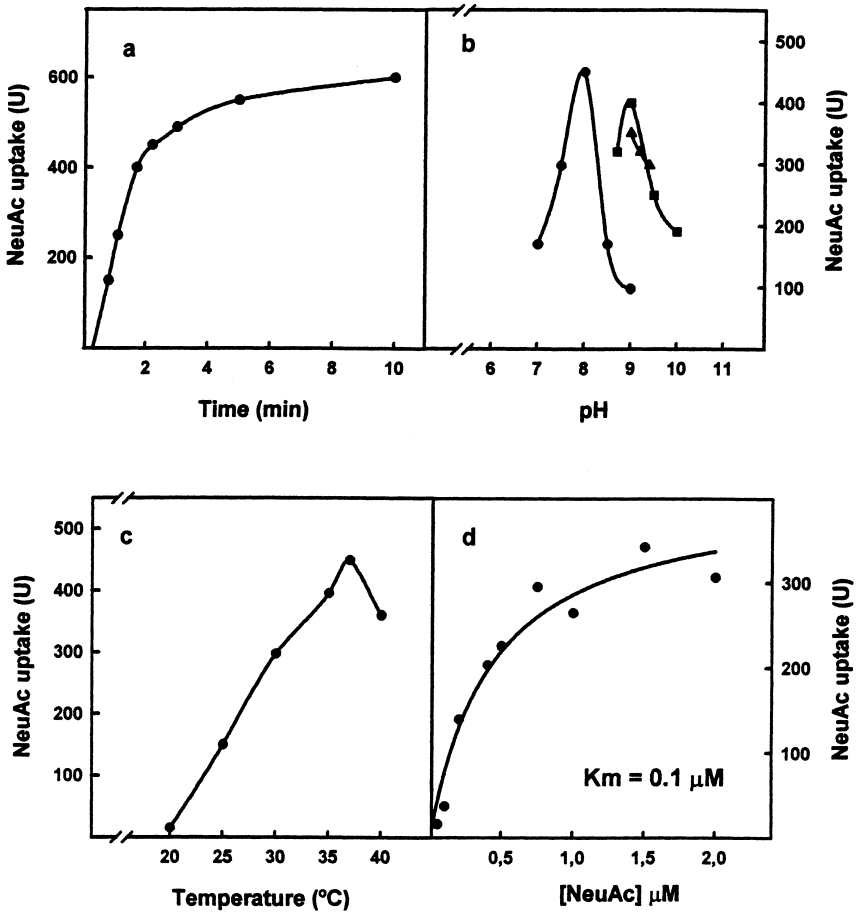

Fig. 2. Effect of (a) time of incubation, (b) pH, (c) temperature and (d) substrate concentration NeuAc transport system in *P. haemolytica* A2 (● Tris–HCl buffer, ■ CHES buffer, ▲ phosphate buffer).

that the maximum uptake level was achieved when 5 mM of this dithiol was added to the buffer of transport (data not shown). However, when thiol-modifying reagents (iodoacetate, *N*-ethylmaleimide, 4-hydroxymercuribenzoate or 5,5′-dithio-bis(2-nitrobenzoic acid)) were added to the uptake mixture NeuAc transport was strongly blocked (Table 1). These results suggest that an extracellular environment containing reduced thiols might be necessary for the transport of sialic acids [20].

The effect of several cations on NeuAc transport was studied. Whereas the addition of some heavy metal ions (10 mM) such as Zn^{2+}, Hg^{2+}, Co^{2+} or Cu^{2+} strongly inhibited uptake (91, 77, 89, and 92% respectively), corroborating the above mentioned requirement of SH groups for activity, the presence of other monovalent (Na^{+} and K^{+}) and divalent (Ca^{2+}) cations in the uptake mixture increased transport (Table 1). This stimulatory effect was reversed when 10 mM ethylenediaminetetraacetic acid was added to the uptake mixture. These observations suggest the necessary presence of cations around the cell for maximum NeuAc uptake to be achieved and hint that a sugar cotransporter might be involved in uptake of this sugar as previously described in other transport systems in *E. coli* [21,22].

Analysis of the energy requirements of the *P. haemolytica* A2 NeuAc transport revealed that the presence of metabolic inhibitors such as cyanide and NaF [23–25] caused any variations in transport efficiency (Table 1). By contrast, valinomycin, sodium azide, 2,4-dinitrophenol (2,4-DNP), *N*,*N*′-dicyclohexyl-carbodiimide (DCCD) and the high-energy intermediate synthesis inhibitor sodium arsenite were inhibitors of NeuAc transport (Table 1). These results clearly indicate that sialic acid uptake in live *P. haemolytica* A2 cells takes place via an active transport system dependent on an energized membrane or on a high-energy phosphate intermediate.

Another argument for the uptake of NeuAc as being active comes from the results obtained when the efflux of NeuAc was measured in the presence of the protonophore 2,4-DNP. Cells were incubated under standard conditions and, after 2 min of uptake, 2,4-DNP was added. No efflux of labelled NeuAc was detected, even after 15 min of incubation, suggesting that there is no passive NeuAc transport system in *P. haemolytica* A2.

All these results, together with the stimulatory effect observed following the addition of different cations, clearly support the possibility that in *P. haemolytica* A2 NeuAc transport occurs via an active sugar-cation symporter. Moreover, a symport system has also been reported in *S. oralis* [18] and *E. coli* K1 [17,26].

The specificity of the NeuAc uptake system was examined by adding different sugars and sugar derivatives to the uptake mixture. The presence of glucose, arabinose, fructose, galactose, xylose, galactosamine, *N*-acetylglucosamine, *N*-acetylgalactosamine, *N*-acetylmannosamine and the disaccharide su-

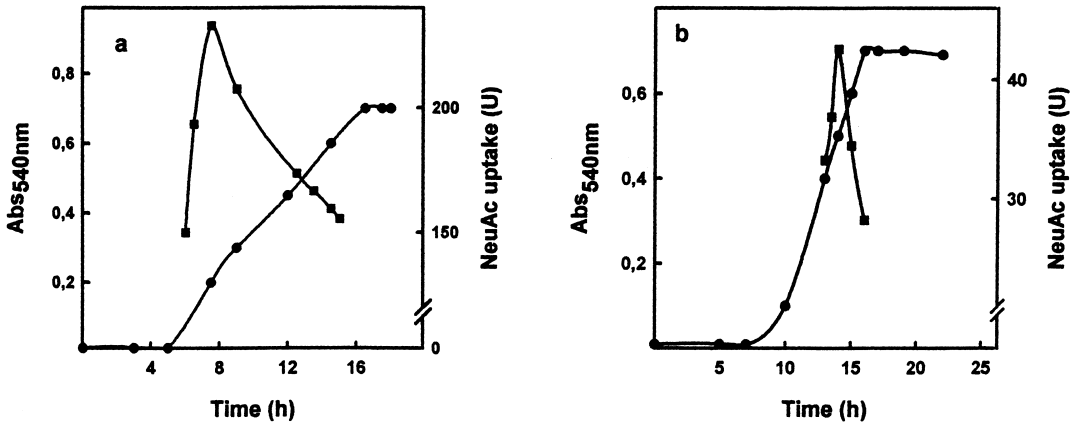

Fig. 3. Growth (●) and time course of NeuAc transport (■) of *P. haemolytica* A2 at (a) 37°C in Wessman medium and (b) Wessman medium when glucose and galactose were replaced by glycerol.

crose increased the rate of NeuAc transport (Table 2). The uptake of many of these sugars in bacteria occurs via a constitutive phosphoenolpyruvate-sugar phosphotransferase [27,28] and will enter energy-generating pathways in the starved cells, thus fueling NeuAc transport. These observations support our proposal that NeuAc transport in *P. haemolytica* is an active energy- requiring process. However, the presence of mannose, mannosamine and glucosamine caused a marked decrease in NeuAc uptake (Table 2). Kinetic study of the inhibition caused by glucosamine, mannose and mannosamine revealed K_i values of 1.6 mM, 0.87 μM and 2 mM respectively, suggesting that sialic acid and these three sugars are transported by a common carrier.

In Gram-negative bacteria mannose, mannosamine and glucosamine are taken up by a PTS-dependent mechanism (Man-PTS) [29,30]. The competitive effect of these sugars in *P. haemolytica* suggests that NeuAc can be transported by this mechanism and not by an active sugar cation symporter, as suggested above. However, a study with paper chromatography [17] of the labelled material accumulated inside the cells revealed that NeuAc is transported without chemical phosphorylation (results not shown). A similar kind of behavior has been described previously for NeuAc transport in *E. coli* K1 by Rodríguez-Aparicio et al. [17]. Moreover, if NeuAc used the specific Man-PTS, other sugars – such as glucose or fructose, described to be able to be used by the same transporter – might inhibit the uptake of NeuAc. However, these sugars increased the NeuAc transport rate (see before) (Table 2). These results clearly indicate that in *P. haemolytica* A2 the active mechanism involved in the NeuAc transport corresponds to a cation symport system.

Addition of *N*-glycolylneuraminic acid (a sialic acid with an *N*-glycolyl group replacing the *N*-acetyl group at the C-5 position of the neuraminic acid) to the reaction mixture revealed that this sugar acts as a competitive inhibitor ($K_i = 0.85$ μM). Furthermore, when NeuAc methyl ester (5 μM) and 2,3-dihydro-2-deoxy-NeuAc acid (5 μM) were tested as possible inhibitors, a decrease in NeuAc uptake was observed (53 and 38% respectively), suggesting that all sialic acids are taken up by a common transport system.

The lack of interference in the transport of sialic acid by other sugars indicates that, as occurs in *E. coli* and *S. oralis*

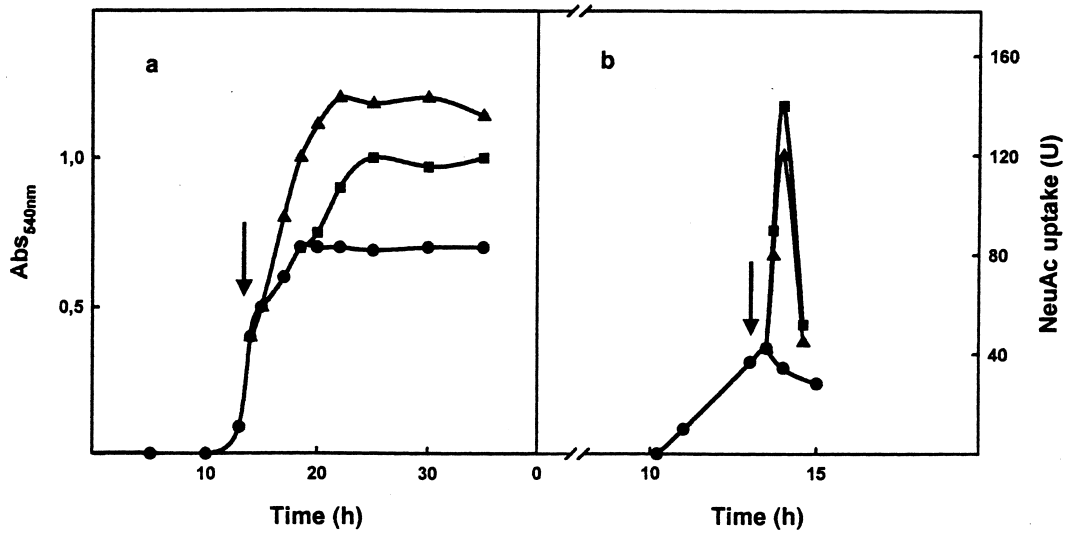

Fig. 4. Effect of NeuAc (■) and glucose plus galactose (▲) as carbon source on the cellular growth (a) and NeuAc transport rate (b). Cells were grown in the Wessman medium without glucose and galactose using glycerol as carbon source up to $A_{540nm} = 0.5$ and were supplemented with NeuAc (■), glucose plus galactose (▲) or glycerol (●). Arrows indicate the time of addition.

Table 3
Effect of the modification of carbon source of growth on the efficiency of NeuAc transport in *P. haemolytica* A2

Medium	Uptake (U)[a]
BHIB	500
Wessman	250
$Wessman_m$ [b] glycerol	30
$Wessman_m$ glucose	30
$Wessman_m$ glycerol+glucose	27
$Wessman_m$ glycerol+galactose	28

When $Wessman_m$ was supplemented by mannose, mannose plus galactose, mannosamine, *N*-acetylmannosamine, *N*-acetylmannosamine plus glycerol or without sugar no bacterial growth was detected.
[a]The uptake value corresponds at the maximal level along the growth of bacteria in each medium.
[b]$Wessman_m$: Wessman medium without sugar as carbon source (glucose and galactose).

[17,18], in *P. haemolytica* A2 the NeuAc permease is very specific. Moreover, the inhibition by mannose and mannosamine on NeuAc transport suggests that these sugars can modulate and modify its catalytic efficiency.

3.3. Expression of the NeuAc transport system

P. haemolytica A2 has special nutritional requirements. This microorganism is able to grow in different complex media [12], although traditionally BHIB medium has been employed for its culture. In this medium, the bacterium shows a good growth and a higher degree of polysialic acid production [12]. Moreover, the complex composition of this medium does not permit study of the complete expression or the possible induction of the NeuAc transport. Thus, for bacterium growth it is necessary to use a defined medium that will allow one to establish the molecules involved in the regulation of transporter expression. In this sense the defined medium (Wessmann medium) used for cultivation of *P. haemolytica*, containing 15 amino acids, citric acid, different salts, vitamins and glucose and galactose as carbon source [16], could be a good candidate for this study. Analysis of NeuAc uptake of cells grown in Wessman medium indeed revealed the presence of an efficient transport (Table 3 and Fig. 3a).

Study of this transport system revealed that it has identical physico-chemical, kinetic and modulatory characteristics to those observed in cells grown in the BHIB medium (data not shown), confirming that it is indeed the same transport system. However, when glucose, galactose or both sugars were eliminated from the growth medium and replaced by glycerol, a minimum uptake level was achieved (Table 3 and Fig. 3b). These results suggest that the process of NeuAc uptake occurs via an inducible system. To confirm this hypothesis, we studied the NeuAc transport when different sugars were added to cells grown in the Wessman medium in the absence of glucose and galactose containing glycerol as the carbon source ($A_{540nm} = 0.2$). None of the sugars added (mannose, glucose, galactose, mannosamine, glucosamine, galactosamine, *N*-acetylmannosamine, *N*-acetylglucosamine or *N*-acetylgalactosamine) modified the transport rate (data not shown). Only the addition of NeuAc or the simultaneous addition of glucose and galactose caused an induction of NeuAc uptake. As shown in Fig. 4, following the addition of these sugars we observed a rapid increase in NeuAc transport, which reached levels slightly lower than those of cells grown in the Wessman medium (Table 3). These results indicate that the sialic acid transport system in *P. haemolytica* A2 is induced by the action of NeuAc and suggest that the simultaneous addition of glucose and galactose is able to induce this transport system. It is possible that these hexoses act synergistically as gratuitous inducers of this system.

Here, the presence of a high-affinity and specific transport system for NeuAc in pathogenic *P. haemolytica* A2 has been identified. This is of relevance concerning the physiology of this microorganism, because it demonstrates that this bacterium is able to utilize free sialic acid found in fluids. Moreover, a specific sialidase present in this bacterium (manuscript in preparation) permits it to utilize bound NeuAc, which is a major component of glycoproteins present in host tissues. Finally, *P. haemolytica* A2 is the only bacterium described that is able to activate *N*-glycolylneuraminic acid [31], a sugar reported here that can be transported to inside the cell. These observations open new avenues for the study of the metabolism in bacteria of other sialic acids hitherto described only in eukaryotic cells. A better knowledge of the metabolism of these sugars is essential for the development of therapeutic approaches into this pathogenic bacterium. Further research on this topic is currently in progress.

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