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A protein–sialyl polymer complex involved in colominic acid biosynthesis

Effect of tunicamycin

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A protein–NeuAc complex involved in colominic acid biosynthesis has been identified in membrane preparations of *Escherichia coli* K-235. This compound had an M_r (estimated by SDS/polyacrylamide-gel electrophoresis and autoradiography) of about 100000 and played the role of an 'initiator' or 'primer' (endogenous acceptor) in the synthesis of the whole polymer. Incubations of *E. coli* membranes with CMP-[^{14}C]NeuAc (CMP-*N*-[^{14}C]acetylneuraminic acid) pointed to the existence of a protein fraction (primer acceptor) that linked residues of sialic acid (*N*-acetylneuraminic acid, NeuAc) up to a maximal size, later releasing them as low-M_r sialyl polymers (LM_rS, $M_r < 10000$). In the presence of colominic acid (final acceptor) the radioactivity linked to the protein quickly decreased, appearing stoichiometrically bound to the whole polysaccharide. When membrane preparations were previously digested with *Streptomyces* proteinase or de-activated by heating (80 °C, 10 min), no incorporation of labelled NeuAc into trichloroacetic acid-insoluble material was detected. These results suggested that colominic acid molecules are synthesized while they are bound to a proteinaceous acceptor that is subsequently excised in the presence of colominic acid, generating the native protein. The antibiotic tunicamycin inhibited the biosynthesis of colominic acid, affecting the synthesis of this protein–$(NeuAc)_n$ intermediate. All these results are described here for the first time.

INTRODUCTION

Colominic acid (CA) is a capsular homopolymer of *N*-acetylneuraminic acid (NeuAc) with α(2–8) ketosidic linkages (Barry & Goebel, 1957; Barry, 1958) or a mixture of α(2–8) and α(2–9) linkages (Liu *et al.*, 1977; Egan *et al.*, 1977). This sialyl polymer, which is produced by several strains of *Escherichia coli*, *Neisseria meningitidis* and species of *Salmonella* (Barry *et al.*, 1962; Barry, 1965) has been identified as a pathogenic determinant in *N. meningitidis* serogroups B, C, W-135 and Y (Liu *et al.*, 1971, 1977), in *E. coli* antigens (Troy, 1979; Rohr & Troy, 1980; Silver *et al.*, 1981) and in *Salmonella toucra* (Kedzierska, 1978), *S. arizona* and *Citrobacter freundii* (Barry *et al.*, 1963) serogroups. Synthesis of this polysaccharide starts with the formation of a molecule of NeuAc by condensation of *N*-acetylmannosamine 6-phosphate with pyruvic acid or phosphoenolpyruvic acid (Comb & Roseman, 1960; Warren & Felsenfeld, 1962). This NeuAc molecule is then activated by binding to CTP (Rohr & Troy, 1980), after which the residue of sialic acid is transferred from CMP-NeuAc to a lipidic acceptor (undecaprenyl phosphate) (Troy & McCloskey, 1979). The reaction is carried out by a membrane-associated sialyltransferase complex (Troy & McCloskey, 1979; Whitfield *et al.*, 1984), which, in a second step, also catalyses the incorporation of other molecules of NeuAc into an endogenous–exogenous-acceptor system, allowing the polymer to grow (Rohr & Troy, 1980). These last-named authors have found a sialyl polymer (166 NeuAc residues), linked to the membranes used as a source of enzyme, which seemed to be a true metabolic intermediate in the biosynthesis of the mature polymer. However, the lack of a terminal reducing group suggests that this compound is not free, but rather is linked to some other molecule whose structure was not clarified.

There is growing evidence that compounds previously thought to be homopolysaccharides belong to the family of proteoglycans, their biosynthetic origins being a protein on to which the polymers are built (Aon & Curtino, 1984, 1985; Blemenfeld & Krisman, 1985; Rodriguez & Whelan, 1985; Andaluz *et al.*, 1986). Thus it would not be surprising if, by analogy, the synthesis of CA involves an intermediate of similar nature.

Here we present the results of a systematic study to clarify some of these aspects, using a chemically defined medium that is ideal for CA production (Rodríguez-Aparicio *et al.*, 1988). In addition, the inhibition of CA biosynthesis by tunicamycin is described and discussed.

MATERIALS AND METHODS

Chemicals

Tunicamycin was obtained from Boehringer Mannheim G.m.b.H., (Mannheim, Germany). NeuAc, pyruvic acid, CA, D-xylose, resorcinol, bovine serum

Abbreviations used: CA, colominic acid; CMP-NeuAc, cytidine 5′-monophospho-*N*-acetylneuraminic acid; LM_rS, low-M_r sialyl polymers; $(NeuAc)_n$, sialyl polymer.

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albumin and L-proline were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). CMP-[4,5,6,7,8,9-^{14}C]NeuAc (351 mCi·mmol^{-1}) was from Amersham International (Amersham, Bucks., U.K.). Other reagents were of analytical quality.

Strain

Escherichia coli K-235 (A.T.C.C. 13027) was obtained from the American Type Culture Collection, and a culture was also kindly supplied by Professor F. A. Troy (Department of Biological Chemistry, University of California). Strains were kept freeze-dried or in liquid N_2 (gas phase).

Culture medium and growth conditions

E. coli K-235 was maintained on Trypticase Soy Agar (Difco) and grown as previously described (Rodríguez-Aparicio *et al.*, 1987).

CA determinations

The CA produced by *E. coli* K-235 was analysed by the Svennerholm (1958) methodology as previously described (Rodríguez-Aparicio *et al.*, 1988).

When the effect of tunicamycin was studied, different concentrations of antibiotic were added to medium E. Cells were collected at different times, centrifuged (20000 *g*, 10 min), and the CA present in the supernatant (fluid) was analysed as described previously (Rodríguez-Aparicio *et al.*, 1988). In transfer experiments, cells grown for different times in the incubation medium supplemented with tunicamycin (5.0 μg·ml^{-1}) were harvested by centrifugation (20000 *g*, 10 min), washed twice with sterile saline solution and resuspended in a medium in which *E. coli* K-235 had been grown for the same time under identical physical conditions but without tunicamycin. In order to ensure that no bacteria grown in the absence of tunicamycin remained in the broth, it was heated to 70 °C for 10 min.

Enzyme assay procedures

Cell-free extracts and enzymes were obtained as previously described (Troy *et al.*, 1982). Enzyme fractions were prepared fresh daily.

Sialyltransferase activity

The incorporation of NeuAc from CMP-[^{14}C]NeuAc into either sialyl polymer or the lipid fractions was determined by the procedure of Troy *et al.* (1975) as described by Rodríguez-Aparicio *et al.* (1988).

When the binding [^{14}C]NeuAc to proteins was analysed, reaction mixtures were precipitated with 10% (w/v) trichloroacetic acid as described by Luengo *et al.* (1980). The trichloroacetic acid solution was cooled to 0 °C for 10 min and centrifuged at 5000 *g* for 5 min. The pellet was immediately washed with a 10%-trichloroacetic acid solution (twice), resuspended in 100 μl of Protosol (du Pont) and kept at 37 °C for 6 h. Once dissolved, 1 ml of scintillation fluid {toluene (666 ml)/Triton X-100 (333 ml)/PPO (2,5-diphenyloxazole; 3.33 g)/POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene; 0.133 g]} was added, and the final clear solution was counted for radioactivity in a Beckman LS-1800 liquid-scintillation counter. A 10 μl portion of supernatant was subjected to paper (Whatman 3MM) chromatography and developed as described by Rodríguez-Aparicio *et al.* (1987*b*). Under these conditions the R_F values for NeuAc, CMP-NeuAc and sialyl polymer were 0.8, 0.5 and 0 respectively. The rest of the supernatant was dialysed (Medicell International dialysis tubing; M_r exclusion size < 10000) against 2500 vol. of distilled water (4 °C, 18 h) before measuring its radioactivity.

To improve the yields of precipitation, 200 μl of bovine serum albumin (2 mg/ml) were added.

Protein determinations were carried out by the method of Lowry *et al.* (1951). Bovine serum albumin was used for establishing the standard curves.

NeuAc aldolase (*N*-acetylneuraminate lyase, EC 4.1.3.3)

This enzyme was assayed by the method of Comb & Roseman (1960). One unit of enzyme was defined as the quantity of protein that yields 1 μmol of pyruvate under the assay conditions in 15 min.

SDS/polyacrylamide-gel-electrophoretic analysis of membrane proteins

Proteins from a sialyltransferase reaction mixture incubated for 2 h (under the conditions described above) were precipitated with $(NH_4)_2SO_4$ (80% satd.) (under these conditions free CA remained in the supernatant), dialysed (against 1000 vol. of water for 24 h) and freeze-dried. Portions were resuspended in Tris/HCl buffer and analysed by Coomassie Blue staining after their high-resolution separation in 0.1%-SDS(Laemmli, 1970)/10%-(w/v)-polyacrylamide gels. Samples were electrophoresed on a LKB vertical electrophoresis unit. Once dried, the gels were placed on MP-hyperfilm (Amersham International) and stored at −70 °C for 30 days. The radioactivity ([^{14}C]NeuAc$_n$) bound to the protein was revealed by autoradiography (Laskey & Mills, 1975).

Negative staining

The morphology of bacteria grown in the presence or absence of tunicamycin was studied by negative-contrast electron microscopy as described previously (Rodríguez-Aparicio *et al.*, 1988).

RESULTS AND DISCUSSION

Effect of tunicamycin on CA synthesis *in vivo*

CA biosynthesis implies the participation of a lipidic acceptor (undecaprenyl phosphate) (Kundig *et al.*, 1971; Troy & McCloskey, 1979) which receives the rest of its NeuAc from the donor nucleotide-sugar (CMP-NeuAc) (Warren & Blacklow, 1962; Troy *et al.*, 1982). For this reason we studied the effect that tunicamycin (an antibiotic that inhibits the glycosylation of lipid-carrier-dependent molecules in prokaryotic and eukaryotic cells; Heifetz *et al.*, 1979) exerts on CA synthesis when *E. coli* was grown in a chemically defined medium (Rodríguez-Aparicio *et al.*, 1988).

The addition of various amounts of tunicamycin to this medium at zero time caused different effects on the final production of CA (Fig. 1*a*). A concentration of 5.0 μg·ml^{-1} inhibited CA production by more than 50%, whereas cell growth, the rate of growth and morphology remained unaffected (Figs. 1 and 2). However, when larger amounts of tunicamycin (10, 20, 30 or 40 μg·ml^{-1}) were added to the broth, the inhibition of CA synthesis was not further increased; indeed, inhibition only took place at all if tunicamycin was added during the earliest stages (before 16 h) (Figs. 1*a* and 1*b*). When the antibiotic

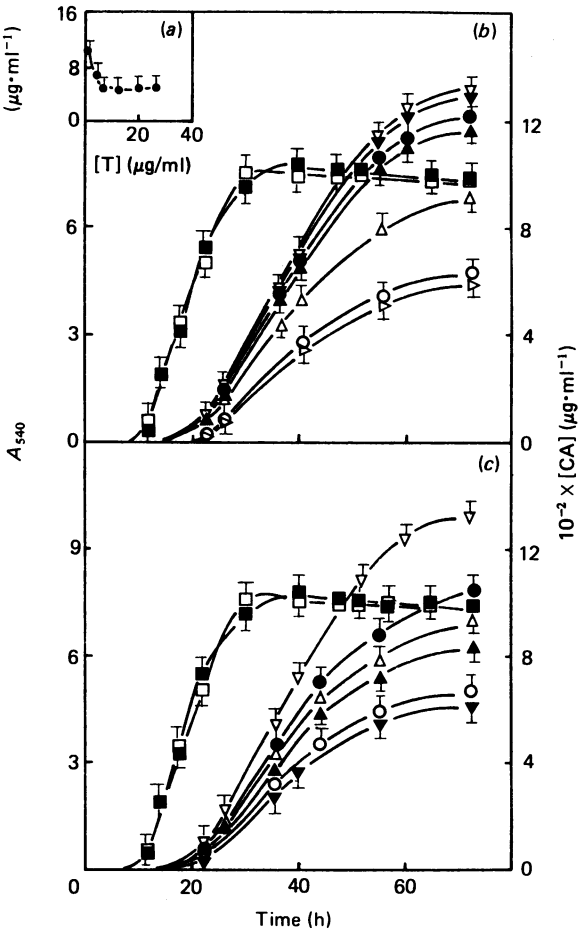

Fig. 1. Effect of tunicamycin on the production of CA *in vivo*

(*a*) Inhibition of CA production when *E. coli* K-235 was incubated for 72 h in the presence of different concentrations [T] of tunicamycin. (*b*) CA synthesis when the antibiotic was added at: zero time (○); 12 h (△); 16 h (▲); 23 h (●); 36 h (▼); when tunicamycin (5 μg·ml^{-1}) was added at 10 h intervals to the culture (▷) and without tunicamycin (▽). (*c*) CA production when *E. coli* was grown in the presence of tunicamycin (5 μg·ml^{-1}) for: 10 h (●), 12 h (△), 15 h (▲), 16 h (○) and 72 h (▼) or in its absence (▽). □, ■, cell growth when *E. coli* K-235 was incubated in medium E supplemented with (□) or without (■) tunicamycin (5.0 μg·ml^{-1}).

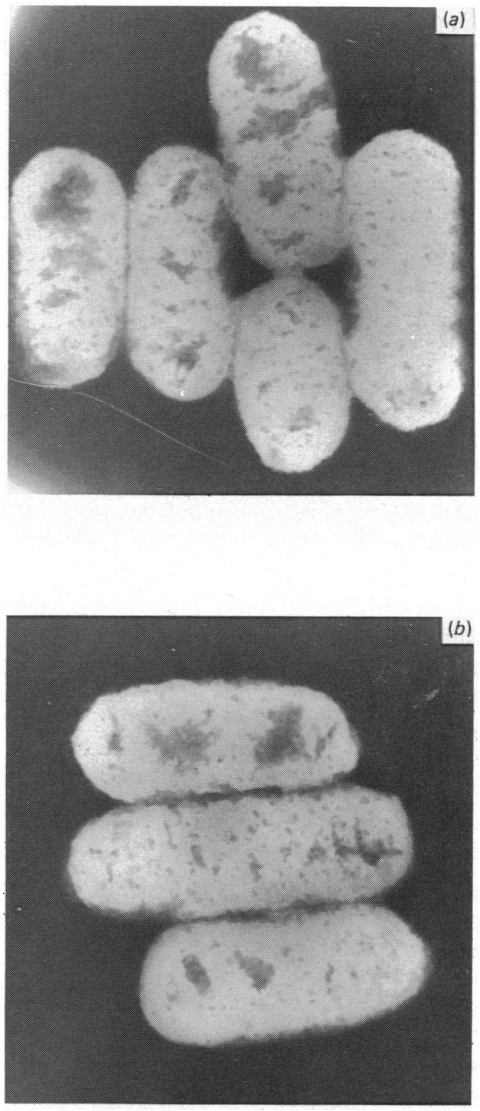

Fig. 2. Photomicrographs of *E. coli* K-235 grown for 19 h in the presence (*a*) and in the absence (*b*) of tunicamycin (magnification 23000 ×)

was added (even 50 μg·ml^{-1}) after 16 h of growth, no inhibition was detected, although at this time CA was being actively synthesized [see under 'Short incubation experiments' in Rodríguez-Aparicio *et al.* (1988)]. This effect suggests that tunicamycin affects the production of some intermediate(s) (undecaprenyl phosphate, endogenous acceptor or enzymes) involved in CA biosynthesis. The lack of inhibition *in vitro* by tunicamycin of both sialyltransferase and aldolase activities (Table 1) was in agreement with the above results.

Furthermore, when cells grown in the presence of tunicamycin were transferred to the same medium without antibiotic, the final titre of polymer accumulated in the broth was strictly dependent on the time of transference (Fig. 1*c*). Moreover, the inhibition caused by this antibiotic was only partially reversed if bacteria were washed and transferred to fresh medium (see the Materials and methods section) before 16 h. Regarding CA production, cells treated for more than 16 h behaved as though tunicamycin were still present, and the maximal inhibition of CA synthesis took place when the antibiotic was added to the medium before 12 h (Fig. 1*b*), precisely at a time when no CA synthesis was taking place (Rodríguez-Aparicio *et al.*, 1988). A schematic representation of all these findings is summarized in Fig. 3.

Studies *in vitro*

The determination of sialyltransferase in cell-free extracts of *E. coli* (grown in the presence of tunicamycin for 19 h) showed that the quantity of this enzyme was notably lower (46.2%) than when bacteria were grown in its absence (Table 1). This phenomenon could be due to the lack of either sialyltransferase or some CA intermediates (undecaprenyl phosphate, endogenous acceptor) that limit the efficiency of the biosynthetic

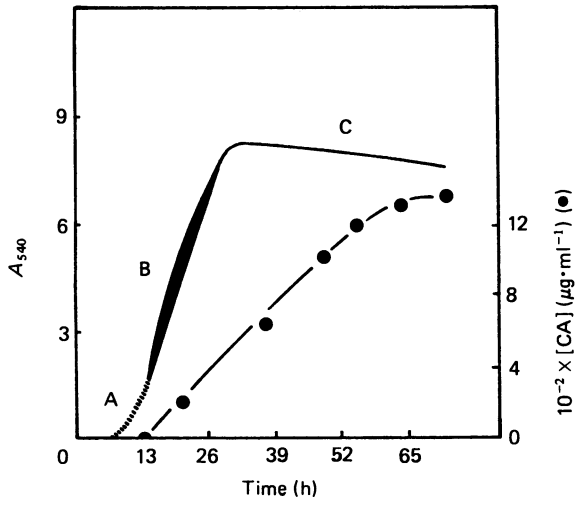

Fig. 3. Schematic representation of time course of CA formation and release into broth when *E. coli* K-235 was grown at 37 °C

A, Period during which inhibition is caused by tunicamycin; B, phase during which capsule is synthesized (thickness of line indicates variation in synthesis capacity); C, phase during which CA is released into broth (Rodríguez-Aparicio *et al.*, 1988).

process. A low level of undecaprenyl phosphate or endogenous acceptor is manifested as a decrease in sialyltransferase activity even when a large amount of enzyme was present in the membranes.

In order to clarify this point, several experiments were done (Table 2). A lipidic fraction of *E. coli* grown at 37 °C containing undecaprenyl phosphate (Troy *et al.*, 1975; Troy & McCloskey, 1979) was added to reaction mixtures containing cell-free extracts obtained from cells grown [medium E (Rodríguez-Aparicio *et al.*, 1988) 37 °C, 19 h, $A_{540} = 4.0$] either in the presence (T) or absence (C) of tunicamycin (5.0 $\mu g \cdot ml^{-1}$). When supplied to each reaction mixture, this lipid fraction (L) provided lipid (undecaprenyl phosphate) but neither endogenous acceptor (necessary for CA biosynthesis) (Troy & McCloskey, 1979), nor active sialyltransferase enzyme. Table 2 shows that when L was added to T and C, sialyltransferase activity did not increase, and the extracts containing enzyme from C always generated twice the quantity of product compared with those from T. This clearly does mean that the limiting molecule in the T extract was either the endogenous acceptor or the sialyltransferase enzyme.

With a view to establishing if the effect of tunicamycin was due to a low level of endogenous acceptor, CA (Troy & McCloskey, 1979) was added to reaction mixtures T

Table 1. Evaluation of the levels and activities of both aldolase and sialyltransferase (%) when *E. coli* K-235 was grown at 37 °C in medium E supplemented or not with tunicamycin

An aldolase activity of 100% corresponds to 0.435 unit/mg of protein; a sialyltransferase activity of 100% corresponds to 24 pmol of NeuAc/30 min per mg of protein. The results shown in this Table and in the following one are averages for at least four different determinations.

Reaction mixture...	Activity (%)			
	Control		Tunicamycin (5 $\mu g \cdot ml^{-1}$)*	
Source of enzyme	Aldolase	Sialyltransferase	Aldolase	Sialyltransferase
E. coli K-235 (A_{540}) = 4.0) grown in medium:				
E (control)	100.0±2.8*	100.0±0.8*	100.0±2.5	100.0±0.3
E+tunicamycin (5 $\mu g \cdot ml^{-1}$)	102.3±3.5	46.2±1.2	98.5±4.2	46.7±0.6

* When higher concentrations of tunicamycin (10, 20, 50, 100 $\mu g \cdot ml^{-1}$) were added to the reaction mixture, results were identical.

Table 2. Effect of different additions on the sialyltransferase activity (%) obtained from *E. coli* K-235 grown at 37 °C in the presence (T) or absence (C) of tunicamycin (5 $\mu g \cdot ml^{-1}$)

A sialyltransferase activity of 100% corresponds to 80 pmol of NeuAc/120 min per mg of protein.

Source of enzyme	Reaction mixture...	Activity (%)			
		Control (without additions)	Lipid+(100 μl)*	+CA (2 $mg \cdot ml^{-1}$) (exogenous acceptor)	+CA (2 $mg \cdot ml^{-1}$) and tunicamycin (200 $\mu g/ml^{-1}$)
C		100.0±0.8	101.0±1.1	212.5±2.9	210.6±1.4
T		45.6±1.6	46.8±2.3	206.8±2.2	209.3±3.2

* A lipid fraction (containing undecaprenyl phosphate) was obtained from a crude extract (50 mg of membrane protein $\cdot ml^{-1}$) of *E. coli* grown at 37 °C in medium E (Troy & McCloskey, 1979). The chloroform/methanol (2:1, v/v) phase was dried under an N_2 atmosphere, resuspended in buffer solution and added to reaction mixtures T and C.

and C (see above). As shown in Table 2, the negative effect caused by tunicamycin was completely re-established. This fact suggests that the inhibitory effect of tunicamycin is due to a limitation in the quantity of available endogenous acceptor. Furthermore, the addition of tunicamycin (200 $\mu g \cdot ml^{-1}$) to the reaction mixtures did not affect the efficiency of the reaction (see Table 2).

In order to establish the biosynthetic step at which tunicamycin acts, further studies on the synthesis of CA *in vitro* were performed.

[^{14}C]NeuAc-binding protein

Since membranes are very protein-rich fractions, sialyltransferase assays were carried out at different times (Fig. 4*a*) and, after stopping them with trichloroacetic acid, the radioactivity ([^{14}C]NeuAc) present in the precipitate and in the supernatant (previously dialysed) was measured. [^{14}C]NeuAc was linearly incorporated into trichloroacetic acid-insoluble material for 2 h, later remaining constant until 14 h (Fig. 4*a*). When the assays were carried out with membranes previously digested with proteinase from *Streptomyces griseus* (Sigma) or de-activated by heating (80 °C, 10 min), no incorporation of labelled NeuAc into trichloroacetic acid-precipitated proteins (Green & Hughes, 1955) was found (Fig. 4*a*). Furthermore, if the reaction mixtures were incubated for 2 h and proteinase (1 $mg \cdot ml^{-1}$) was supplied at this time, the radioactivity that had been found linked to protein was rapidly lost from the precipitate and appeared in the supernatant dialysed (Fig. 4*b*) ($M_r > 10000$). Moreover, when chloroform/methanol (2:1, v/v) extractions were carried out (four times) before trichloroacetic acid addition, no radioactivity was extracted into the organic phase (results not shown). This fact implies that the labelled NeuAc was not linked to a lipidic fraction. Furthermore, when the proteins present in the sialyltransferase reaction mixtures were precipitated with 80% $(NH_4)_2SO_4$ and later dialysed and freeze-dried (see the Materials and methods section), a large amount of radioactive material remained bound to the proteinaceous fraction, whereas free CA did not precipitate after $(NH_4)_2SO_4$ treatment (results not shown). Analysis by gel electrophoresis and autoradiography showed that [^{14}C]NeuAc polymer was bound to a protein (P), the P–NeuAc complex having an M_r of about 100000 (Fig. 5).

These results suggest that, in a early biosynthetic step, [^{14}C]NeuAc is linked to a protein that probably plays a role of 'initiator' or 'primer' in the synthesis of the whole endogenous acceptor. The existence of this protein–Neu5Ac acceptor is in good agreement with the results of Whitfield *et al.* (1984), who demonstrated that protein–NeuAc acceptor is in good agreement with the formation.

Synthesis of native sialypolymer *in vitro*

Analysis of the radioactivity present in the dialysed trichloroacetic acid supernatant of the sialyltransferase assays showed that non-dialysable sialyl polymers ($M_r > 10000$) were accumulated at a very low rate from 0 to 14 h (Fig. 4*c*). However, when CA was added exogenously, a rapid incorporation of labelled [^{14}C]NeuAc into non-diffusible sialyl polymer was detected (Fig. 4*c*). These findings suggest that sialyltransferase either catalyses the reaction more efficiently in the presence of

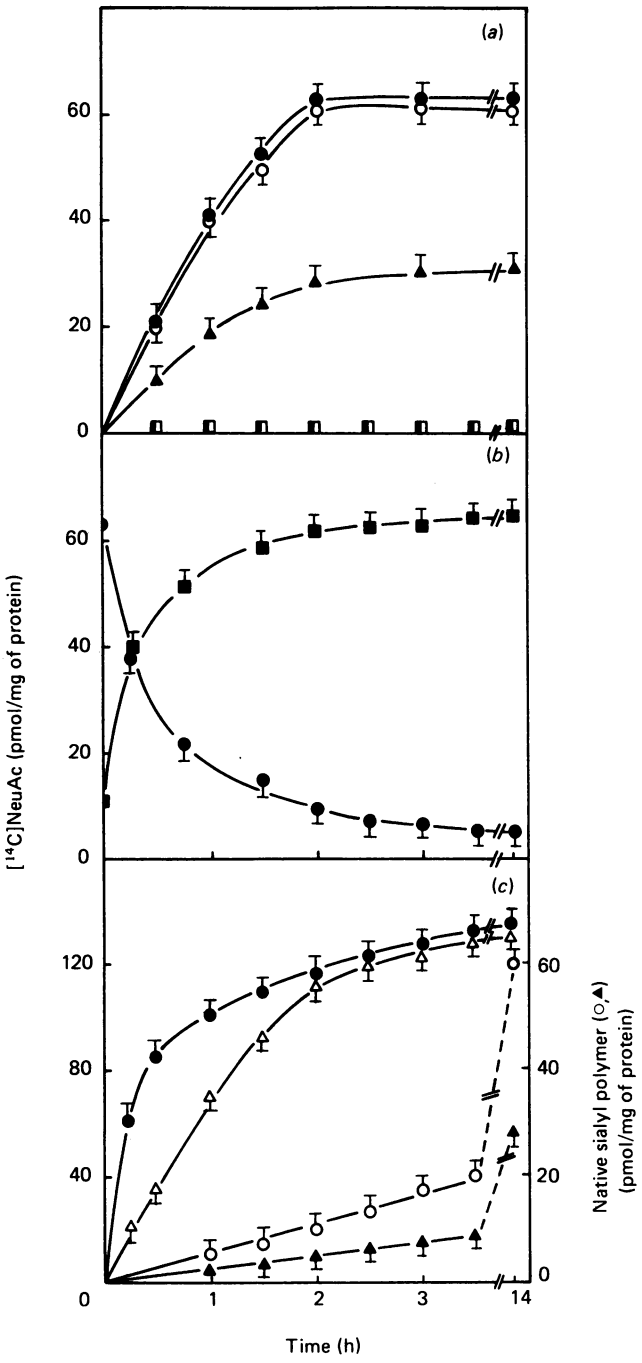

Fig. 4. Presence of [^{14}C]NeuAc polymers ligated to proteins

(*a*) Determination of [^{14}C]NeuAc levels linked to protein when membranes of cells grown in the absence (●) or presence (▲) of tunicamycin (5 $\mu g \cdot ml^{-1}$) were used; ○, □, ■, the same when tunicamycin was added to the reaction mixture (○), or when membranes were digested with *Streptomyces griseus* proteinase (□) or heated (80 °C, 10 min) (■). (*b*) Release of [^{14}C]NeuAc linked to proteins (●) and appearance in the trichloroacetic acid supernatant (■) when *Streptomyces griseus* proteinase was added to a sialyltransferase reaction previously incubated for 2 h. (*c*) Appearance of [^{14}C]NeuAc in the dialysed trichloroacetic acid supernatant when sialyltransferase reactions were carried out in the presence (●, △) or absence (○, ■) of CA. Membranes from cells grown in medium E supplied with (△, ▲) or without (○, ●) tunicamycin (5 $\mu g \cdot ml^{-1}$) were used as the source of enzyme.

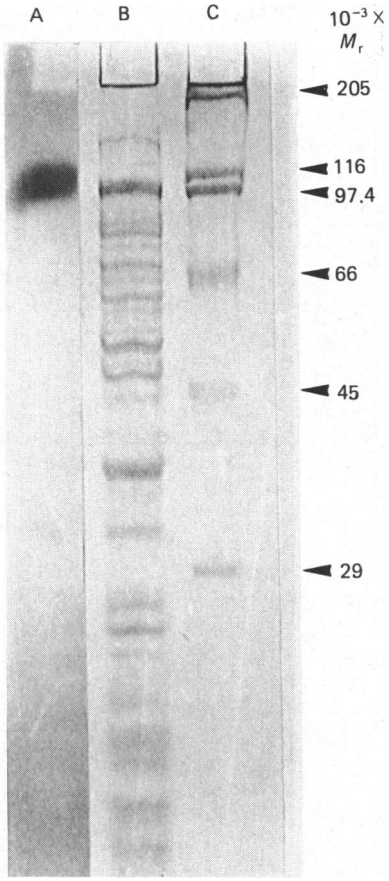

Fig. 5. SDS/10%-polyacrylamide-gel-electrophoresis profiles of proteins in a sialyltransferase reaction mixture

Proteins were stained with Coomassie Brilliant Blue R-250 (lane B). The location of the radioactivity [[^{14}C](NeuAc)$_n$] in the gel was done by autoradiography (lane A). The M_r standards were: myosin (205000), β-galactosidase (116000), phosphorylase *b* (97400), bovine serum albumin (66000), ovalbumin (45000) and carbonic anhydrase (29000) (lane C).

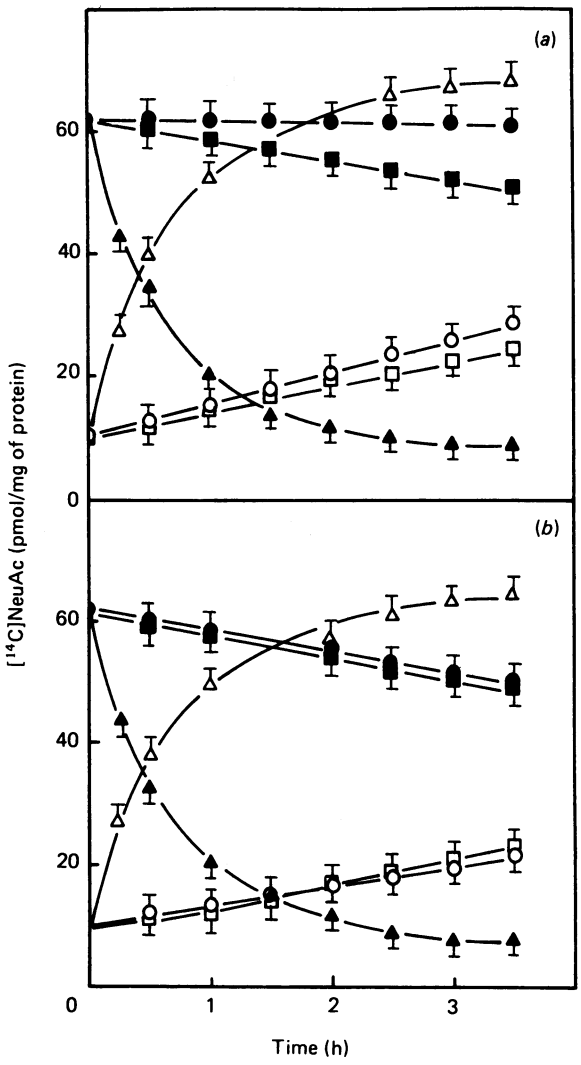

Fig. 6. Effect of CMP-NeuAc on the protein–NeuAc complex

(*a*) Release of [^{14}C]NeuAc linked to proteins (●, ■, ▲) and its appearance in dialysed trichloroacetic acid supernatant (○, □, △) when 1 mM unlabelled CMP-NeuAc (■, □), 1 mM unlabelled CMP-NeuAc and CA (2 mg·ml^{-1}) (▲, △) and water (●, ○) were added to a sialyltransferase reaction mixture that had been preincubated for 2 h. (*b*) As (*a*), but when a similar sialyltransferase reaction mixture was incubated for 2 h, dialysed against 2500 vol. of buffer for 2 h and incubated for 3½ h more.

final acceptor or that this enzyme is also able to incorporate [^{14}C]NeuAc residues directly from the undecaprenyl phosphate intermediate (Vijay & Troy, 1975; Troy *et al.*, 1975; Troy & McCloskey, 1979) without participation of an endogenous-acceptor molecule.

Until now no evidence has been given here concerning the role of this protein–(NeuAc)$_n$ complex as a CA-biosynthetic intermediate. In order to test this hypothesis, experiments using unlabelled CMP-NeuAc were performed. Reaction mixtures were incubated for 2 h and, after this time, CMP-NeuAc (1 mM) or CMP-NeuAc (1 mM)+CA (2 mg·ml^{-1}) were added. Fig. 6(*a*) shows that, in both cases (after adding CMP-NeuAc and CMP-NeuAc+CA) the radioactivity linked to the protein (■, ▲) decreases at the same rate as it appears in the supernatant (□, △), but not stoichiometrically (the quantity of [^{14}C]NeuAc accumulated in the supernatant was greater than that corresponding to the residues released from the protein) (Fig. 6*a*). However, this difference disappeared when the reaction mixtures were dialysed before adding unlabelled CMP-NeuAc or CMP-NeuAc+CA (Fig. 6*b*).

These results strongly support the existence of a protein–(NeuAc)$_n$ complex as a biosynthetic intermediate, and the lack of stoichiometry suggests the presence of labelled molecules which have been accumulated during the preincubation time (2 h) and that continue to supply [^{14}C]NeuAc residues, even when the radioactivity (CMP-[^{14}C]NeuAc) was many times (431-fold) diluted. These molecules are not proteins (they are not precipitated by trichloroacetic acid) or lipids [not extractable with chloroform/methanol (2:1, v/v)] and have an M_r of less than 10000 [they were lost during dialysis (Fig. 6*b*)]. Analysis of these diffusable molecules by paper chromatography after hydrolysis with *Clostridium perfringens* neuraminidase (Sigma) (0.5 unit, 40 h, 37 °C) indicated that they are short sialyl polymers similar to those described by Rohr & Troy (1980). The

untreated molecules had an R_F of 0.0 (sialyl polymers), whereas after hydrolysis all the radioactivity was found at 0.8 (NeuAc) (results not shown). Moreover, when CA was added to the reaction mixture, these short polymers were not found.

All these findings suggest that CA receives NeuAc residues: (*a*) from the protein–$(NeuAc)_n$ complex (when sialyltransferase reactions were carried out in the presence of CA) and (*b*) from the low-M_r sialyl polymers previously synthesized (when the reactions were performed 2 h before the addition of CA) (Figs. 4*c*, 6*a* and 6*b*).

Effect of tunicamycin on the synthesis of the protein–$(NeuAc)_n$ complex

When membranes from cells grown at 37 °C in the presence of tunicamycin ($5\ \mu g \cdot ml^{-1}$) were used as an enzyme source, the results obtained were in complete agreement with those described in Table 1. As shown in Figs. 4(*a*) and 4(*c*), the radioactivity linked to protein and that present in the dialysed supernatant (newly synthesized polymer) was about 50% of the control (enzyme from cells grown at 37 °C without tunicamycin) value. However, tunicamycin did not affect the efficiency of the '*in vitro*' reaction even when antibiotic was present at $100\ \mu g \cdot ml^{-1}$ (Table 1). The results shown in Fig. 4(*a*) support the '*in vivo*' effect (Fig. 1) and strongly suggest that tunicamycin prevents the formation of the protein–$(NeuAc)_n$ complex, of sialyltransferase, or of both. Prolonged incubation experiments (14 h) demonstrated that the radioactivity linked to the protein (Fig. 4*a*) or to the native sialyl polymers (Fig. 4*c*) was 50% of the control value if membranes of tunicamycin-grown cells were used. However, when exogenous CA was added, the radioactivity accumulated in the supernatant was re-established within 2 h (Fig. 4*c*). According to the assumption that the same enzyme or enzyme system links new residues of NeuAc to the native polymer and to CA (Fig. 6*a*) (Troy & McCloskey, 1979; Whitfield *et al.*, 1984), it is clear that, in the tunicamycin-grown cells, there is no limitation of sialyltransferase but rather of the protein–$(NeuAc)_n$ complex. A lower level of this 'primer' molecule takes into account lower quantities of native sialylpolymer and, therefore, a lower level of CA. However, when the titre of native sialylpolymer was re-established (by adding exogenous CA), the radioactivity accumulated in the supernatant was also re-established, albeit slowly (Fig. 4*c*).

The results reported here provide the first evidence concerning the inhibition of CA synthesis in *E. coli* K-235 by tunicamycin and the existence of a protein–$(NeuAc)_n$ complex directly involved in the biosynthesis of CA. They also strongly suggest that the inhibition caused by the antibiotic is due to the interference of this molecule with the synthesis of the protein–$(NeuAc)_n$ intermediate.

Although a considerable body of information is available concerning the biosynthesis of CA (Comb & Roseman, 1960; Kundig *et al.*, 1971; Troy *et al.*, 1975, 1982; Troy & McCloskey, 1979; Rohr & Troy, 1980; Whitfield *et al.*, 1984), many points remain to be elucidated. Thus we still do not know how many proteins are involved in the binding of NeuAc residues, what the nature of this linkage is, how the native polymer is translated across the cell membrane and how it is assembled to the external cellular surface.

A number of important questions obviously remain to be answered and should be the object of further research in this field.

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