A Factor in Animal Tissues that Causes Sialyl Residues of Mucoproteins to React as Free Sialic Acid in the Warren Assay

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1. The mucin of the Cowper's gland of the boar is a sialomucoprotein similar to submaxillary-gland mucin. When a solution of either mucin has been incubated for 5 min or less with a particulate fraction from homogenized uterine endometrium-plus-myometrium of the rabbit, 10–20% of sialyl residues (*N*-acetylneuraminic acid) give a positive Warren reaction for free *N*-acetylneuraminic acid. The particulate fraction is devoid of neuraminidase and no free (diffusible) *N*-acetylneuraminic acid appears during incubation. The factor that catalyses the formation of direct-reading non-diffusible *N*-acetylneuraminic acid occurs also in liver, kidney and intestinal mucosa of the rabbit. The factor is present in very small ('microsomal') particles and has not yet been solubilized. Homogenates of boar Cowper's gland contain both factor and mucin; thus direct-reading non-diffusible *N*-acetylneuraminic acid appears when such homogenates are stored. 2. Under optimum conditions 1 mg of uterine protein catalyses the formation of 0.05–0.1 μ mol of direct-reading non-diffusible *N*-acetylneuraminic acid/min. This activity is considerably higher than the neuraminidase activities of comparable homogenates of animal tissues or of liver lysosomes. The factor is thermostable and its activity shows little variation within (i) the pH range 3–10, (ii) the temperature range 20–37°C. Activity is inhibited strongly by 2,2'-bipyridyl and by ammonium pyrrolidine dithiocarbamate but is unaffected by EDTA. Its action can be simulated by low concentrations of Fe^{2+} . From this it may be inferred that the factor is a protein-bound form of bivalent iron. A number of pure iron-containing proteins and haemoproteins were completely inactive. The following substrates were not sources of direct-reading non-diffusible *N*-acetylneuraminic acid: methoxyneuraminic acid, sialyl-lactose, brain gangliosides, and sialoproteins in which *N*-acetylneuraminic acid is linked to galactose residues. 3. It is proposed that the factor (or Fe^{2+}) reacts with the mucin in a manner that renders the C-4–C-5 bond of sialyl residues susceptible to periodate oxidation.

Detection of bound sialic acid in acrosomes (head-caps) of ram spermatozoa (Hartree & Srivastava, 1965) prompted us to examine rabbit and sheep uteri for neuraminidase (mucopolysaccharide *N*-acetylneuraminylhydrolase, EC 3.2.1.18). A report that this enzyme is present (Srivastava, Adams & Hartree, 1965) was based on tests in which the substrate was the mucous secretion from the bulbo-urethral gland of the boar (Cowper's-gland mucin, CGM†). This mucin is a sialoprotein containing 21–25% of NANA, all of which is liberated by bacterial neuraminidase (Hartree, 1962).

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† Abbreviations: CGM, Cowper's-gland mucin (boar); NANA, *N*-acetylneuraminic acid; ND-NANA, non-diffusible direct-reading NANA residues.

CGM gives a very weak reaction for free NANA in the Warren (1959) assay. However, when CGM was incubated with a homogenate of uterine endometrium there was rapid formation of material that reacted in the Warren assay as free NANA. We later found that this Warren-positive material is not diffusible through cellophan. A similar non-diffusible but direct-reading form of NANA (ND-NANA) has been observed in stored homogenates of ovine submaxillary glands (Eichberg & Karnovsky, 1966). Carubelli, Bhavanandan & Gottschalk (1965) observed that when a solution of ovine submaxillary-gland mucin is incubated at pH 8 about 25% of the bound NANA is slowly converted into ND-NANA.

The terminal NANA residues of ovine submaxillary-gland mucin are part of disaccharide prosthetic groups (I) (see Scheme 1) which are linked to serine

Scheme 1. Periodate oxidation of *N*-acetylneuraminic acid (I, II) (after Paerels & Schut, 1965): R = H, Ac = $\cdot\text{COCH}_3$. The prosthetic group of ovine submaxillary-gland mucin is (I) where R = (2 $\rightarrow$ 6)-*N*-acetylgalactosamine.

and threonine residues in the protein (Graham & Gottschalk, 1960; Harbon, Herman, Rossignol, Jollès & Clauser, 1964; Carubelli *et al.* 1965). CGM is similar to ovine submaxillary-gland mucin in terms of (a) the terminal position of NANA residues, (b) sugar and amino acid analyses, and (c) the linkage of carbohydrate to serine and threonine (Boursnell, Hartree & Briggs, 1970). The ketosidic links in both mucins stabilize the pyranose ring of NANA (I); consequently the C-6-C-7 bond is resistant to the periodate reagent of the Warren assay. In free NANA the open-chain form (II) is oxidized by periodate to a C_6 intermediate (III) (Paerels & Schut, 1965). The latter reacts as formylpyruvic acid when heated in the Warren assay with 2-thiobarbituric acid and a pink pigment is formed.

In the present paper we summarize work on the nature of the uterus factor that converts Warren-negative NANA residues to ND-NANA and we discuss different views on the nature of the transformation.

MATERIALS

NANA was prepared from boar seminal gel (Srivastava *et al.* 1965). It was free from other neuraminic acids (Boursnell *et al.* 1970). Methoxynuraminic acid and *N*-acetylmannosamine were gifts from Professor R. Kuhn and Mr L. E. A. Rowson respectively. Sialyl-lactose was obtained from bovine colostrum (Schneir & Rafelson, 1966) as a mixture of (2 $\rightarrow$ 3)- and (2 $\rightarrow$ 6)-isomers, with the former predominating. 2-Thiobarbituric acid, Triton X-100 and ammonium pyrrolidine dithiocarbamate were from BDH Chemicals Ltd., Poole, Dorset, U.K. Mixed brain gangliosides were supplied by Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K., and *Vibrio cholerae* neuraminidase by Hoechst Pharmaceuticals, Brentford, Middx., U.K.

Crude ovine submaxillary-gland mucin was obtained by repeated extraction of minced glands (Hashimoto, Hashimoto & Pigman, 1964) and the purified mucin was provided by Dr Ward Pigman. CGM was removed from boar Cowper's glands as described by Boursnell *et al.* (1970) and stored at -20°C . Stock CGM solutions were prepared by stirring the mucin, cut into small pieces, with 20 times its weight of 0.15 M-NaCl for 6–12 h. Merthiolate (0.1 mg/ml) (Eli Lilly and Co. Ltd., Basingstoke, Hants., U.K.) was added and solutions were stored at 5°C . Ovomucin was prepared by the method of Gottschalk & Lind (1949). Two samples of fetuin were examined. One, the pure protein, was provided by Dr R. G. Spiro: the other was partially purified material from foetal calf serum. The serum (from Flow Laboratories Ltd., Irvine, Ayrshire, U.K.) was treated with zinc acetate and ethanol and centrifuged, as described by Spiro (1960). The supernatant was exhaustively dialysed against water and freeze-dried. The product contained 12.7% of sialic acid (as NANA). Horse serum α_1 -glycoprotein was a gift from Dr R. Got.

The following haemoproteins and iron-containing proteins were obtained by the methods cited: horse heart cytochrome *c* (Margoliash, 1954); horseradish peroxidase (Keilin & Hartree, 1951); ox spleen ferritin (Granick, 1942); egg-white conalbumin (Feeney & Komatsu, 1960); oxyhaemoglobin (washed horse erythrocytes diluted with water). Human plasma transferrin and parsley ferredoxin were gifts from Dr D. J. Perkins and Dr D. S. Bendall respectively.

Phosphate buffers consisted of KH_2PO_4 and Na_2HPO_4 . Concentrations of tris-maleate buffer are expressed as the sum of the concentrations of tris and maleic acid. Hydroxylamine solution (pH 3.5) was freed from iron by shaking it repeatedly with chloroform plus 4,7-diphenyl-1,10-phenanthroline and then four times with chloroform.

METHODS

Determination of NANA

Method of Warren (1959). NANA that gives a positive reaction is referred to here as direct-reacting or as Warren-

positive. Such NANA may be either free or still bound to a macromolecule (ND-NANA).

Method of Mahadevan, Nduaguba & Tappel (1967). In this procedure proteins are precipitated with phosphotungstic acid and the clear fluid is analysed by the Warren method.

Method of Svennerholm (1957). This assays total NANA.

Other analytical methods

Tests for deacylated sialyl residues in CGM were made as described by Sato, Yosizawa, Masubuchi & Yamauchi (1967). The method is similar to the Warren assay but periodate oxidation takes place in neutral solution. To analyse methoxynuraminic acid by the periodate-thiobarbituric acid method oxidation was carried out (in 0.3 ml) with neutral periodate for 0.5 h under the conditions used by Paerels & Schut (1965) for 'C₇-methoxynuraminic acid'. The reaction was stopped by adding 0.1 ml of 9 M-H₃PO₄ followed by arsenite and thiobarbituric acid as in the Warren procedure. Protein was assayed by the method of Lowry, Rosebrough, Farr & Randall (1951) with ovalbumin as standard.

Acetate in mixtures of uterus fraction and CGM was determined as follows. The sample (>10 ml) was adjusted to pH 3.5 with H₂SO₄ and placed in a 50 ml flask fitted with a B19/26 standard joint. This flask and a similar empty one were attached to two limbs of a Y-piece the third limb of which carried a stopcock. The sample was frozen in a solid-CO₂ bath at -80°C, the apparatus was evacuated to 0.1 mmHg and the stopcock was closed. The cooling bath was then transferred to the empty flask. After 1-2 h all water plus acetic acid had distilled into the second flask. The distillate, plus phenolphthalein, was titrated with ethanolic 0.1 M-KOH in a stream of CO₂-free N₂.

Enzymic methods

Complete removal of NANA residues from CGM by means of *V. cholerae* neuraminidase was effected at 5°C (Boursnell *et al.* 1970). NANAlyase (*N*-acetylneuraminic pyruvate-lyase, EC 4.1.3.3) was assayed in terms of release of *N*-acetylmannosamine (Brunetti, Jourdain & Roseman, 1962). Activity units (U) are expressed as μmol of substrate cleaved/min and specific activities as U/mg of protein. Activities of tissue preparations catalysing the formation of ND-NANA are similarly expressed as μmol of ND-NANA formed/min from a Warren-negative sialoprotein.

Fractionation of rabbit uterus

Uterine horns were split lengthwise, and the endometrium and myometrium were scraped off with a microscope slide and suspended in 0.15 M-phosphate buffer, pH 6 (3-5 ml according to size of uterus). All subsequent operations were at 5°C. The mixture was centrifuged at 10000g for 15 min and the supernatant was discarded. The pellet was suspended in 4-8 ml of hypo-osmotic buffer, pH 6, containing Triton X-100 (0.1%) and homogenized for 5 min in a Teflon-glass homogenizer. The composition of the buffer is not critical: we have normally used 0.05 M-phosphate or 0.05 M-tris-maleate. The homogenate was

frozen (-80°C) and thawed three times, rehomogenized for 2 min and centrifuged at 30000g for 30 min. The opalescent supernatant is the standard preparation *F* of the uterus factor catalysing the transformation of NANA residues into ND-NANA. The pellet contains more of the factor, which can be extracted by further homogenizations in the presence of Triton X-100. A purer fraction was obtained by centrifuging preparation *F* for 30 min at 100000g. The inactive supernatant was discarded and the almost white pellet was resuspended in a little water (preparation *Fp*).

Attempts were made to remove endometrium and myometrium separately. Although this proved not to be possible *F* fractions were prepared from scrapings that consisted predominantly of one or the other tissue. Combined endometrium-plus-myometrium was also fractionated into 'mitochondrial', 'microsomal' and soluble fractions by using the Hübscher, West & Brindley (1965) procedure for intestinal mucosa.

Fractionation of other tissues

The following tissues were also subjected to the standard procedure to yield *F*-type fractions: chicken egg-gland, boar Cowper's gland, sow uterus, and lung, small intestine, liver, kidney and brain of the rabbit.

RESULTS

In the experiments described below volumes of incubation mixtures were 0.75-1.50 ml unless otherwise stated. When incubation temperature is not stated it was 20-22°C. Concentrations of CGM

Fig. 1. Formation of ND-NANA by incubating rabbit uterus preparations (*F*) with different concentrations of CGM at 37°C. Concentrations in incubation mixtures were: preparation *F*, 0.93 mg of protein/ml; phosphate buffer, pH 5.8, 33 mM. Concentrations of CGM (mg wet wt./ml) were: Δ , 1; $\square$, 5; $\bullet$, 10; $\circ$, 15; $\blacksquare$, 20. Samples (0.2 ml) were taken for Warren assay.

Fig. 2. Formation of ND-NANA by incubation of different concentrations of a preparation *F* with CGM for 1 h at 37°C. Concentrations were: CGM, 10 mg wet wt./ml; phosphate buffer, pH 5.8, 40 mM.

Fig. 3. Early changes in the concentration of ND-NANA in mixtures of a preparation *F* and CGM. Concentrations of reactants were: CGM, 10 mg/ml; phosphate buffer, pH 5.8, 0.1 M. Concentrations of preparation *F* (mg of protein/ml) were: ○, 0.19; ●, 0.37; □, 0.75. —, Time-scale 0–20 min; ----, time-scale 0–4 min.

are given on a fresh-weight basis. The mucin normally contains 84–90% of water and stock solutions were 4–6 mM with respect to bound NANA. To follow the production of ND-NANA samples (>0.2 ml) of incubation mixtures were transferred at intervals to 0.1 ml of Warren's (1959) periodate reagent and the assay was completed in the normal way.

Fig. 4. Formation of ND-NANA from CGM: comparison of fractions from endometrium and myometrium of a rabbit uterus. Concentrations of reactants were: CGM, 10 mg/ml; tris-maleate buffer, pH 6.1, 30 mM; endometrial protein (○), 0.23 mg/ml or myometrial protein (□), 0.25 mg/ml, or combined endometrial-plus-myometrial protein (●), 0.47 mg/ml.

Formation of ND-NANA with different concentrations of CGM and of uterus fractions. Figs. 1 and 2 illustrate the effects of independently varying the concentrations of CGM and factor *F* on the production of Warren-positive NANA. Tests described below established that this was not free NANA. The considerable inhibition of the reaction at high CGM concentration is no doubt due, at least in part, to the high viscosity of the mucin. On the basis of the first hour of incubation a concentration of about 10 mg of CGM/ml ensures maximum rate of formation and also maximum extent of formation of ND-NANA. Excess of preparation *F* had only a small depressing effect on the rate.

Attempts to determine accurately initial rates of formation of ND-NANA yielded unexpected results (Fig. 3). A very rapid initial formation of ND-NANA was followed by a fall in ND-NANA concentration. This second phase was most marked at low concentrations of *F* factor, i.e. those corresponding to the steeply rising part of the curve in Fig. 2. The second phase became less marked as the concentration of *F* factor increased and it eventually disappeared. (The second fall shown by the 20 min run in Fig. 3 is atypical.)

There was a marked difference between fresh endometrial and myometrial uterus preparations (Fig. 4). With the latter there was a rapid but transient appearance of ND-NANA. With the former the initial rate was lower and the second phase no longer apparent. The endometrial-plus-myometrial fraction combined the initial high rate with a transient fall in ND-NANA concentration, a sequence that was commonly obtained with

Table 1. *Activities of F and Fp preparations, and of centrifugation pellets, isolated from rabbit uterus scrapings*

Scrapings were homogenized in 50mm-phosphate buffer, pH 5.8, containing Triton X-100 (0.1%). Pellets from the last stage in isolation of factor *F* were resuspended in the same buffer. Incubations were for 5 min under the conditions given in Fig. 3.

Preparation type (and number)	Treatment	$10^3 \times$ Specific activity (U/mg of protein)	$10^3 \times$ Total activity (U/preparation)	
			<i>F</i>	Resuspended pellet
<i>F</i> (1)}	Untreated, fresh		23	12
<i>F</i> (2)}			6.6	17
<i>F</i> (1)}	-20°C for 24 h		55	65
<i>F</i> (2)}			65	
<i>F</i> (3-8)	Repeated freezing and thawing to obtain maximum activity	8.0-16.6	239-330	
<i>Fp</i>		(mean 12.2)	(mean 265)	
(3 samples)}		45-101 (mean 67)		

standard *F* preparations. *Fp* preparations behaved like endometrial preparations, the second phase being absent.

Effects of different extraction procedures on the activity of preparation F. It must be emphasized that curves obtained under similar conditions with different *F* preparations, or with the same preparation tested at intervals, vary considerably in form. With freshly prepared *F* factor at an appropriate concentration the first and second phases are clearly defined. Fresh preparations varied widely in activity, but if they were frozen and thawed several times, or stored for different periods at -20°C, they became uniformly highly active (Table 1). Another effect of freezing is to decrease the magnitude of the second phase. Thus there may be two factors in uterus extracts: one that induces formation of ND-NANA and another less stable one that brings about disappearance of ND-NANA. Because the reaction is not linear calculated activities depend on choice of incubation time. Results obtained with 0.19mg of uterus protein/ml (Fig. 3) correspond to a specific activity of 0.075 U/mg on the basis of the second minute of the reaction. It is likely that specific activities of preparations 3-8 (Table 1) would have been higher had calculations been based on selected shorter incubation periods. On the other hand the high specific activities of *Fp* preparations were based on the period 0-5 min. Hypo-osmotic homogenization media yielded the most active *F* preparations and the presence of Triton X-100 always increased activity.

When uterus scrapings were fractionated by the method of Hübscher *et al.* (1965) all three fractions ('mitochondrial', 'microsomal' and cytoplasmic) were inactive even though the tests were carried out in hypo-osmotic buffer (50mm-tris-maleate, pH 6.1). After the first two fractions had been homogenized

for 5 min in the presence of 0.1% Triton X-100 both were active. Distribution of activity was 30% in 'mitochondria' and 70% in 'microsomes'.

The maximum extent of formation of ND-NANA by fully active (repeatedly frozen and thawed) *F* fractions is usually 10-20% of the total bound NANA in CGM. Further additions of preparation *F* do not give rise to additional ND-NANA, but a second addition of CGM to an incubation mixture gives rise rapidly to more ND-NANA.

Prolonged dialysis of uterus fractions against water did not diminish their activities.

Evidence against an enzymic mechanism for the formation of ND-NANA. Samples of preparation *F* in 50mm-phosphate buffer, pH 5.8, were heated for 10 min at 60, 80, 90 and 100°C. A fifth sample was vigorously boiled for 10 min in a bath at 130°C. Samples were in long narrow tubes to prevent evaporational losses. The amount of ND-NANA formed from CGM in 10 min was unaffected by heating preparation *F* at temperatures up to 100°C. The boiled preparation showed only a 10% decrease in activity. A similar experiment in tris-maleate buffer also demonstrated complete thermostability. A series of tests at different pH values (in acetate, phosphate and glycine-NaOH buffers) showed only slight variations in activity: a progressive fall of about 30% on passing from pH 3 to pH 10. There was no consistent difference between reaction rates at 37 and 20°C.

Tests for neuraminidase and NANA lyase in preparation F. The effects of preparation *F* and of bacterial neuraminidase on CGM are compared in Table 2. After dialysis the total NANA in Expt. A was unchanged but 12.6% was now ND-NANA. In Expt. B most of the originally bound NANA had passed, as free NANA, into the diffusate but a trace was present as ND-NANA. The latter weak reaction is often given by both dialysed and undialysed

Table 2. *Effects of preparation F and of V. cholerae neuraminidase on the bound NANA of CGM*

Mixtures were made up to include 50 mM-tris-maleate buffer, pH 6.1, and dialysed for 24 h against two 500 ml lots of the same buffer containing 0.1 mg of merthiolate/ml. NANA determinations on the non-diffusible material were made by methods 1, 2 and 3 with 0.2, 0.4 and 0.4 ml respectively. Results are expressed as percentages of the total NANA (Svennerholm, 1957) originally present in the CGM.

Incubation mixture	ND-NANA after dialysis assayed by the method of		
	(1) Warren (1959)	(2) Mahadevan <i>et al.</i> (1967)	(3) Svennerholm (1957)
Expt. A: 1 ml of prep. <i>F</i> ; 40 mg of CGM; final vol. 4 ml	12.6	Not detectable	[100]
Expt. B: 1 ml of neuraminidase; 40 mg of CGM; final vol. 4 ml	0.5*	Not detectable	39

* See Table 3.

CGM solutions. However, since tests by the method of Mahadevan *et al.* were negative the Warren-positive material in Expt. B (and in untreated CGM) is not free NANA.

Other tests for neuraminidase included (i) dialysing mixtures as used in Expt. A (Table 2) against small volumes of water, and (ii) incubating preparation *F* with sialyl-lactose under different conditions. The diffusates in the former experiments were consistently negative in the Warren assay as also were the mixtures in the latter experiments.

Assays for *N*-acetylmannosamine were carried out on incubated mixtures of (a) preparation *F* and CGM (pH 4–8) and (b) preparation *F* and NANA (pH 6.8). Only the second experiment yielded *N*-acetylmannosamine but the specific activity of NANA lyase was $>7 \times 10^{-4}$ U/mg. This would be far too low to account for the rapid disappearance of 'apparent' NANA that was observed, e.g. in the experiments of Fig. 3.

Effects of chelating agents, heavy-metal cations, iron-containing proteins, oxidizing agents and enzyme inhibitors on the formation of ND-NANA. The association of uterus activity with very small particles suggested that the factor is a protein or is associated with a protein. For this reason we examined the effects of a range of compounds with protein-binding or protein-modifying properties.

For the experiments in this section the composition of incubation mixtures for the 'standard test' was: CGM, 7.5 mg/ml; 0.04 M-tris-maleate buffer, pH 6.1; preparation *F*, approx. 0.8 mg of protein/ml. The use of tris-maleate buffer avoided precipitation of heavy metals.

Addition to the standard test of salts of Ca^{2+} , Mg^{2+} , Mn^{2+} , Zn^{2+} , Cu^{2+} , Fe^{2+} , Fe^{3+} , Co^{2+} or Ni^{2+} (all at 10 mM) had no appreciable effects on the rates and extents of formation of ND-NANA. In control experiments without the uterus preparation the Warren test was negative except with Cu^{2+} ,

Fig. 5. Formation of ND-NANA from CGM in the presence of Fe^{2+} . Incubation mixtures contained: CGM, 10 mg/ml; tris-maleate buffer, pH 6.1, 30 mM; ferrous ammonium sulphate as indicated. Warren assays were performed on 0.2 ml samples removed after incubation for 5 min (○) and 15 min (●).

which caused a weak reaction, and Fe^{2+} , which gave rise to a strong reaction. The formation of ND-NANA catalysed by different concentrations of Fe^{2+} is shown in Fig. 5. The maximum yield of ND-NANA (formed with $70 \mu\text{M-Fe}^{2+}$) corresponded to about 30% of the bound NANA present.

The catalytic actions on CGM of both Fe^{2+} and factor *F* are inhibited by metal-chelating agents (Table 3). These experiments suggest that the uterus factor is a bivalent iron derivative. In the experiments with Fe^{2+} more than stoichiometric amounts of EDTA and ammonium pyrrolidine dithiocarbamate were present but only 84% of the stoichiometric amount of 2,2'-bipyridyl. It seems therefore that Fe^{2+} retains some catalytic activity when chelated with EDTA.

If the uterus factor is a bivalent iron derivative oxidizing agents are likely inhibitors and reducing

Table 3. *Metal-chelating agents as inhibitors of the formation of ND-NANA from CGM*

The order of addition to incubation mixtures for the 'standard test' (see the text) was: CGM, buffer, catalyst, chelating agent. After 5 min 0.2 ml samples were taken for analysis (Warren, 1959). Controls were set up (a) minus chelating agent, (b) minus inhibitor and catalyst, and the necessary small corrections were applied. APD, Ammonium pyrrolidine dithiocarbamate.

Catalyst for ND-NANA formation	Inhibitor (167 μ M)	Inhibition of ND-NANA formation (%)
66 μ M-Fe ²⁺	EDTA	50
	2,2'-Bipyridyl	89
	APD	>100*
Preparation <i>F</i> (0.37 mg of protein)	EDTA	0†
	2,2'-Bipyridyl	47
	APD	100

* Samples from mixtures Fe²⁺+CGM+APD and CGM+APD gave lower NANA values than did a sample of CGM. Thus APD eliminates the small content of ND-NANA present in untreated CGM (see Table 2).

† Also zero at 667 μ M-EDTA.

agents might activate the reaction. Samples of preparation *F* were incubated with H₂O₂, H₂O₂+azide, H₂O₂+peroxidase, potassium ferricyanide and potassium ferrocyanide. The samples were subsequently dialysed and subjected to the standard test. The activities of all samples were close to those of untreated preparation *F*. Activity was also unchanged if the incubation was carried out in an evacuated Thunberg tube.

Prolonged dialysis of CGM solution against 10 mM-disodium EDTA followed by dialysis against water (plus merthiolate) did not influence the rate of formation of ND-NANA in the standard test. A similar dialysis of preparation *F* did not affect its activity. Although the activity of preparation *F* diminished after it had been dialysed against sodium acetate buffer, pH 4.5, containing ammonium pyrrolidinedithiocarbamate, activity was restored by subsequent dialysis against the buffer. Dialysis of preparation *F* against solutions of ammonium pyrrolidinedithiocarbamate or 2,2'-bipyridyl under reducing conditions, i.e. in iron-free hydroxylamine hydrochloride-sodium hydroxide buffer, pH 3.5, and subsequent dialysis against the hydroxylamine buffer also failed appreciably to diminish the activity of preparation *F*.

Azide, fluoride and semicarbazide (0.05–10 μ M) did not inhibit the activity of factor *F*. Cyanide, within the same concentration range, had a marked activating effect. However, cyanide also catalysed the formation of ND-NANA in the absence of either the uterus fraction or Fe²⁺, and when the concentration of uterus protein was low a distinct synergistic effect of cyanide was observed (Fig. 6). Dialysis of cyanide-CGM mixtures led to disappearance of ND-NANA.

The following were catalytically inactive when

Fig. 6. Comparison of cyanide and factor *F* as catalysts for the formation of ND-NANA. All incubation mixtures contained: CGM, 13.3 mg/ml; phosphate buffer, pH 5.8, 30 mM. Other components were: preparation *F* (○), or 5 mM-HCN (□), or preparation *F*+HCN (●).

tested, at iron concentrations of 50 μ M, as replacements for preparation *F*: peroxidase, haemoglobin, transferrin, conalbumin, ferritin and ferredoxin. Cytochrome *c* that had been stored as an aqueous solution for several months at 5°C showed activity, but a freshly prepared sample was inactive in both the oxidized and reduced states.

Is the appearance of ND-NANA due to deacetylation of NANA residues in CGM? Using the test of

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Fig. 7. Reaction of periodate-treated neuraminic acids with thiobarbituric acid: effect of time of heating on colour formation. ----, E_{551} of cyclohexanone extract in a Warren assay of $0.04\mu\text{mol}$ of NANA (taken from Warren, 1959); —, comparable experiment with methoxyneuraminic acid except that treatment with periodate was at pH 7 (see the Methods section).

Sato *et al.* (1967) we could not detect deacetylated NANA residues in CGM or in CGM that had been incubated with preparation *F* or with Fe^{2+} . Methoxyneuraminic acid gives a positive response under these conditions (Paerels & Schut, 1965), the product of periodate oxidation being compound (IVa). Fig. 7 shows the effect of time of heating of the product with thiobarbituric acid on the extinction of the subsequent cyclohexanone extract. Maximum colour is developed as rapidly as in the assay of NANA, where the oxidation product is compound (III). The colour yield, however, is smaller.

Determinations of ND-NANA and acetate in CGM and in CGM that had been incubated with preparation *F* (Table 4) showed that formation of ND-NANA was not accompanied by liberation of a significant quantity of acetate. Recovery of acetic acid ($0.25\text{--}2.5\mu\text{mol}$) added to mixtures of CGM and preparation *F* was good, the errors being $\pm 2\%$.

Other sialic acid derivatives as sources of ND-NANA. Crude ovine submaxillary-gland mucin reacted with preparation *F* and with Fe^{2+} in the same way as did CGM, and with selected concentrations of the preparation it was possible to demonstrate rapid appearance followed by a decrease of ND-NANA. Purified bovine submaxillary-gland mucin, how-

ever, formed only very small amounts of ND-NANA when treated with preparation *F* and none in the presence of Fe^{2+} . Fractionation of CGM with ethanol yielded mucin fractions all of which reacted more weakly with preparation *F* than did the original mucin (in terms of percentage transformation of NANA residues into ND-NANA). This was apparently not due to separation of components. When a solution of CGM in 0.05M -sodium chloride was treated at 4°C with 4vol. of ethanol both protein and carbohydrate were completely precipitated. However, solutions of this precipitate, either in water or in the ethanolic supernatant that had been freed from ethanol by evaporation, again reacted more weakly with preparation *F* than did the original mucin.

No ND-NANA could be obtained by the usual methods from methoxyneuraminic acid, sialyl-lactose, either sample of fetuin, horse serum α_1 -glycoprotein, ovomucin or brain gangliosides.

Occurrence in other tissues of the factor that gives rise to ND-NANA. *F*-type fractions from other tissues were incubated with CGM for periods of up to 40 min. Both sow uterus and rabbit intestinal mucosa yielded active preparations. Homogenized boar Cowper's gland contained ND-NANA, a result similar to those obtained with submaxillary gland by Eichberg & Karnovsky (1966). In both instances the homogenates contained factor and sialoprotein substrate. Rabbit liver and kidney were good sources of the factor. Table 5 shows that there was a sixfold decrease in protein concentration when *Fp* fractions were obtained from *F* fractions, but the *Fp/F* specific-activity ratios were much higher than 6. Thus some inhibitory agent was present in *F* fractions. When *Fp* fractions of liver and kidney were fresh they gave no Warren reaction after having been incubated in the absence of CGM. During storage at -20°C they became Warren-positive, but the pink pigment had the

Table 4. *Acetate ion in relation to ND-NANA in CGM and in CGM treated with preparation F*

Incubation mixtures contained: CGM, 150mg; preparation *F*, 4ml; 50mm-tris-maleate buffer, pH6.1; final vol. 10ml. After 10min incubation 0.2ml was taken for Warren assay and the remainder for acetate determination.

Components of incubation mixture	Acetate found (μ mol)	ND-NANA found (μ mol)
CGM, buffer	1.18	0.16
CGM, buffer, 2.5 μ mol of acetic acid	3.72	
CGM, buffer, preparation <i>F</i>	1.22	3.28
CGM, buffer, heated preparation <i>F</i> *	1.80	3.73

* Previously held at 100°C for 10 min.

Table 5. *Formation of ND-NANA by F and Fp preparations from rabbit kidney and rabbit liver*

To prepare fraction *Fp*, 3ml of preparation *F* was diluted to 9ml with 50mm-tris-maleate buffer, pH6, and centrifuged at 100000g. The pellet was washed in the same buffer and resuspended in buffer to 3ml. Conditions for the test were as given in Fig. 4. Incubation with CGM was for 5 min.

Source of factor	10 ³ × Specific activity		Concn. of protein (mg/ml)	
	<i>F</i>	<i>Fp</i>	<i>F</i>	<i>Fp</i>
Kidney	0.09	29	58	8.8
Liver	0.30	26	59	9.6

absorption characteristics of the pigment produced by Warren assay of deoxyribose (Warren, 1959). The positive reaction was thus due to breakdown products of DNA. The factor appears to be present also in acrosomes of rabbit spermatozoa (Srivastava, Zaneveld & Williams 1970). Preparations from rabbit lung, rabbit brain and chicken egg-gland did not convert CGM into ND-NANA.

In other species the neuraminidases of liver and kidney have been characterized as lysosomal constituents (Mahadevan *et al.* 1967; Horvat & Touster, 1968). Under our experimental conditions and with 5 min incubations no appreciable amounts of free NANA were formed from liver and kidney *F* fractions. This is consistent with the low activities of lysosomal neuraminidases.

DISCUSSION

The factor *F* shows some unusual properties that at present we cannot explain. Although it appears to be a non-diffusible derivative of bivalent iron it shows striking differences from well-characterized iron-containing proteins and haemoproteins. Dialysis against chelating agents and subsequent removal of these agents fails to inactivate the factor. Not only do inhibitors of haemoprotein catalysis

fail to inactivate the factor, but cyanide, like Fe^{2+} , can replace it. This does not appear to represent an activation of traces of iron, since ferrocyanide and ferricyanide are inactive. The thermostability of factor *F* cannot be interpreted unequivocally because Fe^{2+} can replace it. Because the factor has always been obtained in particulate form further purification has not been possible. However, preliminary experiments have indicated that the specific activity on the total iron in *F* preparations is higher than that of Fe^{2+} .

The implications of our experiments can be considered under two headings: (1) the nature of the reaction leading to ND-NANA and the mechanism of action of factor *F* and Fe^{2+} ions on sialoproteins; (2) the extent to which the presence of the factor may interfere with the assay of free NANA in biological material and with measurement of neuraminidase activity.

A possibility that has not been explored in this paper is that agents which give rise to ND-NANA may be exerting their effect not during the incubation of agent with CGM but during the subsequent Warren assay. This question has been studied and we have found that ferrous salts, at concentrations as low as 30 μM in samples for analysis, may markedly decrease the colour yield in Warren assays of pure NANA (Hartree & Brown, 1970). Although the decrease is less when protein is present the possibility remains that concentrations of ND-NANA quoted here may be up to 20% below true values.

Some previous views on the nature of the reaction giving rise to ND-NANA. Carubelli *et al.* (1965) proposed that formation of ND-NANA in solutions of ovine submaxillary-gland mucin incubated at pH 8 (see the introduction) arose from deacetylation of bound NANA. When a prosthetic group of this mucin (I) is treated with periodate only the *N*-acetylglactosamine residue is oxidized (at C-3-C-4). If, however, the NANA residue is deacetylated periodate oxidation of its C-4-C-5 bond is possible. Carubelli *et al.* (1965) assigned the structure IVb to the oxidation product of a deacetylated prosthetic group and deduced that it would readily

yield formylpyruvic acid at the heating step of the Warren assay.

On the basis of their own work, and on that of Carubelli *et al.* (1965), Eichberg & Karnovsky (1966) listed four possible explanations for the presence of ND-NANA in submaxillary-gland homogenates. These are: (a) some of the NANA residues may be unacetylated; (b) the presence of a deacetylase could give rise to deacetylated NANA residues during storage; (c) there may be present a precursor of formylpyruvic acid other than NANA; (d) NANA residues might be attached to the macromolecule by linkages other than the ketosidic linkage at C-2. Another possibility, suggested by the work of Paerels & Schut (1965), is (e) that ND-NANA could arise through aldol splitting of the C-4-C-5 bond of NANA residues.

It is clear that (a) and (b) are not valid for our systems. Tests for deacetylated NANA on CGM and on CGM treated so as to contain ND-NANA were negative. In addition, the appearance of ND-NANA was not accompanied by the production of significant quantities of acetic acid. It is improbable that a deacetylase would be as thermostable as the uterus factor or that Fe^{2+} would catalyse deacetylation. We have found that omission of periodate abolishes the Warren reaction given by ND-NANA; thus (e) can be dismissed.

In fact, one cannot expect deacetylated NANA residues to be Warren-positive. Methoxyneuraminic acid does not react in the Warren assay (Warren, 1959) because the free amino group is protonated (Paerels & Schut, 1965). For the same reason deacetylated glycoprotein-bound NANA residues are not Warren-positive. By using neutral periodate solutions Paerels & Schut (1965) and Sato *et al.* (1967) obtained positive Warren-type reactions with unacetylated neuraminyl derivatives. A related situation occurs in determinations of NANA and ND-NANA in the presence of tris buffer. In neutral solution tris is oxidized by periodate to formaldehyde and ammonia. In acid solution protonation renders tris resistant to periodate and it does not interfere in the Warren assay. Against (d) is the finding that all NANA residues are split from CGM by bacterial neuraminidase (Hartree, 1962). We have been unable to obtain evidence in favour of (c). None of the known constituents of CGM gives the Warren reaction; in addition CGM from which all NANA residues have been removed enzymically is Warren-negative. The absorption spectra of the Warren pigments from NANA and ND-NANA are not significantly different. One might suppose that the effect of factor *F*, or Fe^{2+} , is to change protein conformation so exposing previously hidden Warren-positive groups. However, viscometry has provided no evidence for conformational changes.

Free haemin reacts in the Warren assay to yield a pigment with λ_{max} near 550 nm (Cowlshaw, 1961). However, the iron-containing proteins that we have tested cannot replace haemin. We conclude therefore that the Warren-positive non-diffusible material in CGM is bound NANA and that no extraneous precursor of formylpyruvic acid is present in CGM or in uterus fractions.

Possible mechanism for the formation of ND-NANA. If in CGM or ovine submaxillary-gland mucin the C-4-C-5 bonds of NANA were to be split by Warren's periodate reagent, structure (IVb) would arise. The ketosidic link in structure (IVb) will be at least as labile as that in structure (IVa) and, since the latter is a precursor of the Warren pigment (Fig. 7), we deduce, as did Carubelli *et al.* (1965), that structure (IVb) will give rise to a positive Warren reaction. We therefore suggest that Fe^{2+} , or an as yet uncharacterized iron-containing protein, labilizes the C-4-C-5 bond of NANA so that it becomes susceptible to periodate. NANA chelates Fe^{2+} weakly (Hartree & Brown, 1970) and NANA residues could have similar properties. The fall in percentage of NANA residues convertible into ND-NANA that occurs when CGM is precipitated with ethanol suggests that binding of Fe^{2+} (or the factor) is influenced by the tertiary structure of the mucin.

Two lines of evidence suggest that under certain circumstances the C-4-C-5 bond of NANA may be split by periodate. In the procedure of Aminoff (1961) for assay of NANA periodate oxidation leads to release of 6–16% of NANA nitrogen as ammonia. Spiro (1964) established that fetuin contains terminal NANA residues linked (2→3) to galactose. During that work he treated fetuin briefly with periodate, then reduced with borohydride and hydrolysed with acid. The only sugar residues to be degraded were NANA and among the products was about 0.5 mol of glycerol/mol of NANA destroyed. Spiro (1964) argued that since the sequence from C-1 to C-4 provides the chromogen for the Warren reaction the glycerol must have originated from C-5-C-6-C-7. From this it follows that about 50% of the C-4-C-5 bonds of NANA residues were split by periodate, and therefore that fetuin should be Warren-positive. However, we have found it to be Warren-negative both before and after treatment with preparation *F* or Fe^{2+} . This paradox calls for further investigation. From our limited information it appears that ND-NANA is formed only from NANA residues linked to *N*-acetylgalactosamine that is part of a macromolecule.

Interference by preparation F with the assay of free NANA in tissue extracts and with measurement of neuraminidase activity. Uncertainties inherent in the Warren assay of free NANA in sialoprotein-

containing material can be resolved in three ways: (1) by carrying out the assay before and after dialysis of the sample; (2) by inhibiting the reaction due to ND-NANA by means of ammonium pyrrolidinedithiocarbamate; (3) by using the modified assay method of Mahadevan *et al.* (1967). The Warren assay of free NANA is unaffected by concentrations of ammonium pyrrolidinedithiocarbamate up to 2.5mM in the mixture of sample and periodate (Hartree & Brown, 1970).

The tissue factor (*F*) is distinguishable from animal neuraminidases by simple tests. (a) It is thermostable, it can be stored frozen for long periods and it is unaffected by prolonged dialysis. (b) It is associated with very small ('microsome') particles and is not solubilized by addition of Triton X-100 or by repeated freezing and thawing. Neuraminidase is either soluble (e.g. Ada, 1963) or it is lysosomal. In the latter case it can be partially solubilized with Triton X-100 and it is very unstable (Taha & Carubelli, 1967; Horvat & Touster, 1968). (c) It shows no pH optimum of activity. (d) It yields maximum Warren-positive material within 15min and usually within 5min. (e) It does not convert sialyl-lactose into a Warren-positive product.

Preparations of the factor show much higher specific activities than do comparable crude neuraminidase preparations from animal tissues. Thus we reproducibly obtain uterus fractions (*Fp*) with specific activities of <0.05 . A 30–50-fold purification of neuraminidase in Cohn fraction IV of human serum led to a specific activity of 3.8×10^{-4} (Warren & Spearing, 1960). Even after 50 000-fold purification neuraminidase from chorio-allantoic membranes of chick embryos showed a specific activity of only 1.2 (Ada, 1963). Corresponding values for liver lysosomes are about 10^{-3} (Mahadevan *et al.* 1967; Horvat & Touster, 1968).

The widespread use of sialyl-lactose as substrate by investigators of liver lysosome neuraminidase may explain why the factor has remained undetected until now. Warren & Spearing (1960) tested bovine submaxillary-gland secretion as substrate but their enzyme was in soluble form. It is noteworthy that Ghosh, Kotowitz & Fishman (1968), using sialyl-lactose, detected neuraminidase in human intestinal mucosa but not in a range of other human tissues. We found the factor, but no neuraminidase, in intestinal mucosa of the rabbit.

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REFERENCES

- Ada, G. L. (1963). *Biochim. biophys. Acta*, **73**, 276.
 Aminoff, G. (1961). *Biochem. J.* **81**, 384.
 Bournsnel, J. C., Hartree, E. F. & Briggs, P. A. (1970). *Biochem. J.* **117**, 981.
 Brunetti, P., Jourdan, G. W. & Roseman, S. (1962). *J. biol. Chem.* **237**, 2447.
 Carubelli, R., Bhavanandan, V. P. & Gottschalk, A. (1965). *Biochim. biophys. Acta*, **101**, 67.
 Cowlishaw, B. (1961). *Biochim. biophys. Acta*, **49**, 583.
 Eichberg, J. & Karnovsky, M. L. (1966). *Biochim. biophys. Acta*, **124**, 118.
 Feeney, R. E. & Komatsu, St. K. (1960). In *Structure and Bonding* vol. 1, p. 149. Ed. by Jørgensen, C. K., Neilands, J. B., Nyholm, R. S., Reinen, D. & Williams, R. J. P. Berlin: Springer-Verlag.
 Ghosh, N. K., Kotowitz, L. & Fishman, W. H. (1968). *Biochim. biophys. Acta*, **167**, 201.
 Gottschalk, A. & Lind, P. E. (1949). *Br. J. exp. Path.* **30**, 85.
 Graham, E. R. B. & Gottschalk, A. (1960). *Biochim. biophys. Acta*, **38**, 513.
 Granick, S. (1942). *J. biol. Chem.* **146**, 451.
 Harbon, S., Herman, G., Rossignol, B., Jollès, P. & Clauser, H. (1964). *Biochem. biophys. Res. Commun.* **17**, 57.
 Hartree, E. F. (1962). *Nature, Lond.*, **196**, 483.
 Hartree, E. F. & Brown, C. R. (1970). *Analyt. Biochem.* (in the Press).
 Hartree, E. F. & Srivastava, P. N. (1965). *J. Reprod. Fert.* **9**, 47.
 Hashimoto, Y., Hashimoto, S. & Pigman, W. (1964). *Archs Biochem. Biophys.* **104**, 282.
 Horvat, A. & Touster, O. (1968). *J. biol. Chem.* **243**, 4380.
 Hübscher, G., West, G. R. & Brindley, D. N. (1965). *Biochem. J.* **97**, 629.
 Keilin, D. & Hartree, E. F. (1951). *Biochem. J.* **49**, 88.
 Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
 Mahadevan, S., Nduaguba, J. C. & Tappel, A. L. (1967). *J. biol. Chem.* **242**, 4409.
 Margoliash, E. (1954). *Biochem. J.* **56**, 529.
 Paerels, G. B. & Schut, J. (1965). *Biochem. J.* **96**, 787.
 Sato, T., Yosizawa, Z., Masubuchi, M. & Yamauchi, F. (1967). *Carbohydr. Res.* **5**, 387.
 Schneir, M. L. & Rafelson, M. E. (1966). *Biochim. biophys. Acta*, **130**, 1.
 Spiro, R. G. (1960). *J. biol. Chem.* **235**, 2860.
 Spiro, R. G. (1964). *J. biol. Chem.* **239**, 567.
 Srivastava, P. N., Adams, C. E. & Hartree, E. F. (1965). *J. Reprod. Fert.* **10**, 61.
 Srivastava, P. N., Zaneveld, L. J. D. & Williams, W. L. (1970). *Biochem. biophys. Res. Commun.* (in the Press).
 Svennerholm, L. (1957). *Biochim. biophys. Acta*, **24**, 604.
 Taha, B. H. & Carubelli, R. (1967). *Archs Biochem. Biophys.* **119**, 55.
 Warren, L. (1959). *J. biol. Chem.* **234**, 1971.
 Warren, L. & Spearing, C. W. (1960). *Biochem. biophys. Res. Commun.* **3**, 489.