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Comparative Effect and Fate of Non-Entrapped and Liposome-Entrapped Neuraminidase Injected into Rats

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Non-entrapped and liposome-entrapped *Clostridium perfringens* neuraminidase (0.5–0.6 unit) was injected into rats and its fate as well as its effect on plasma and erythrocyte *N*-acetylneuraminic acid was investigated. The following observations were made. (1) Although removal of both non-entrapped and liposome-entrapped neuraminidase from the circulation was completed within 5h after injection, their recovery in tissues was distinctly different; 7–10% of the injected non-entrapped enzyme was found in the liver and none in the liver lysosomal fraction or the spleen. In contrast, 20–26% of the liposome-entrapped enzyme was found in the liver of which 60–69% was in the lysosomal fraction. Spleen contained 3.6–5.0% of the enzyme. (2) The presence of the non-entrapped neuraminidase in blood led to the extensive desialylation of plasma and to a decrease in the concentration or total removal from the circulation of some of the plasma glycoproteins. (3) Injection of non-entrapped neuraminidase also led to the partial desialylation of erythrocytes the life span of which was diminished and their uptake by the liver and spleen augmented. (4) Entrapment of neuraminidase in liposomes before its injection prevented the enzyme from acting on its substrate in plasma or on the erythrocyte surface, and values obtained for plasma glycoproteins and erythrocyte survival were similar to those observed in control rats. (5) Entrapment in liposomes of therapeutic hydrolases intended for the degradation of substances stored within the tissue lysosomes of patients with storage diseases could prevent the potentially hazardous enzymic action of hydrolases in blood and at the same time direct the enzymes to the intracellular sites where they are needed.

The increasing use of enzymes in the treatment of enzyme deficiencies and other disorders in man has made apparent the necessity for controlled conditions under which a therapeutic enzyme, usually foreign, is introduced into target sites of the body. Direct administration of an enzyme gives rise to a number of problems among which the presence of the free enzyme in blood is prominent. Apart from its possible immunological effect or inactivation by other enzymes in blood and its non-specific uptake by the reticulo-endothelial system, free enzyme in blood can interact with its substrate(s) occurring in the plasma or on the cell surfaces of blood elements or tissues. This possibility becomes important in the treament of some storage diseases in which, because of a hydrolase deficiency in the lysosomes, hydrolysis of relevant substrates terminally located on complex molecules does not occur. This leads to the arrest of the sequential action of other hydrolases and to the accumulation of undegraded or partially degraded substances in the lysosomes of a variety of tissues. Administration of an exogenous hydrolase designed to replace the missing one could result in the interaction of the enzyme en route to its destination with its substrate conceivably occurring in blood plasma or on cell surfaces in direct contact with blood. Thus injection of, for example, fucosidase or arylsulphatase A in the hope of degrading stored fucose or sulphate-containing substances in fucosidosis and metachromatic leucodystrophy respectively, will almost certainly result in the interaction of the injected enzymes, before their uptake by tissues, with their respective substrates terminally located on plasma or cell-surface constituents. The metabolic implications of such enzyme action are unknown.

Avoidance of undesirable reactions of this kind in the course of enzyme chemotherapy could be achieved by the use of a carrier that would transport therapeutic enzymes in isolation from the environment to the diseased areas in need of the enzyme. The proposition that liposomes, which are concentric lipid bilayers alternating with aqueous compartments (Bangham, 1968), may be a convenient vehicle for the carriage of enzymes (Gregoriadis *et al.*, 1971), or drugs (Gregoriadis, 1973) to diseased areas of the body has been supported by experiments in which it was shown that liposomes can transport enzymes into the lysosomes of the liver and the spleen

(Gregoriadis & Ryman, 1972*a*,*b*). In a model for storage disease we have recently shown that liposomal enzymes can function within the lysosomes towards stored substrates (Gregoriadis & Buckland, 1973).

In the present experiments we have investigated the protection offered *in vivo* by the lipid bilayers of liposomes containing *Clostridium perfringens* neuraminidase (EC 3.2.1.18) to its substrate *N*-acetylneuraminic acid terminally located in plasma glycoproteins and on cell surfaces. The choice of the enzyme was based on the following factors. (*a*) The abundance of *N*-acetylneuraminic acid in blood plasma and cell surfaces and its importance as part of the protein molecule in the survival of some plasma proteins (Morell *et al.*, 1971). (*b*) The possibility of the use of neuraminidase in conjunction with *Cer-Glc-Gal(AcNeu)-GalNAc hexosaminidase in the treatment of Tay–Sachs disease. In this lysosomal storage disease, despite the normal amounts of neuraminidase activity in tissues, there is accumulation of the ganglioside Cer-Glc-Gal(AcNeu)-GalNAc (Brady, 1973). As there is an exceptionally high activity of hexosaminidase B in the conventional Tay–Sachs patient this might catalyse the hydrolysis of desialylated Cer-Glc-Gal(AcNeu)-GalNAc formed by action of additional neuraminidase administered via liposomes (R. O. Brady, personal communication). (*c*) The commercial availability of the enzyme. It was found that non-entrapped neuraminidase effected profound alterations in the biological properties of some blood constituents. These were prevented by the entrapment of the enzyme in liposomes.

Materials and Methods

Chemicals

Clostridium perfringens neuraminidase, chromatographically pure, was purchased from Worthington Biochemical Corp., Freehold, N.J., U.S.A. (1.25 units/mg) and from Sigma (London) Chemical Co., London S.W.6, U.K. (1.10 units/mg); bovine fetuin was from Grand Island Biological Co., Grand Island, N.Y., U.S.A. Sources and grades of phosphatidylcholine, cholesterol and phosphatidic acid and the sources of Triton X-100 and Triton WR-1339 have been described elsewhere (Gregoriadis & Ryman, 1972*b*). *N*-Acetylneuraminic acid was from General Biochemicals, Chagrin Falls, Ohio, U.S.A., and D(+)-galactose and D(+)-mannose were from Fisher Scientific Co., Springfield, N.J., U.S.A. $Na_2{}^{51}CrO_4$ (0.25μCi/ml) for the labelling of rat erythrocytes was from Squibb, Princeton, N.J., U.S.A. and rabbit anti-(whole rat serum) (Dakopatts) from Mercia, Watford, Herts., U.K.

* Abbreviation: Cer (in sequences), ceramide.

Preparation of neuraminidase-containing liposomes

The procedure used for the entrapment of neuraminidase in liposomes was basically the same as that applied for the entrapment of other enzymes (Gregoriadis & Ryman, 1972*a*,*b*). However, separation of the entrapped from the non-entrapped enzyme was effected by centrifugation instead of column chromatography. Briefly, 40μmol of phosphatidylcholine, 11.4μmol of cholesterol and 5.7μmol of phosphatidic acid (molar ratio 7:2:1) were dissolved in chloroform. The thin film formed on the walls of the flask after rotary evaporation was dispersed with 2ml of 3mM-sodium phosphate buffer, pH7.2, containing 15.4–44.0 units (14–40mg) of neuraminidase. The suspension was then sonicated for 30s at 4°C with a 1.8cm titanium probe at 1.5A in an MSE 60W sonicator. Liposomes containing neuraminidase were separated from the non-entrapped enzyme by centrifugation at 100000*g* for 90min. The liposomal pellet was resuspended in 3mM-phosphate buffer and centrifuged again at 100000*g* for 60min. The pellet containing the entrapped neuraminidase (5.4–6.1% of the enzyme used) was suspended in 1.0ml of 10mM-sodium phosphate buffer, pH7.2, containing 1% NaCl (buffered saline). The supernatants containing the non-entrapped neuraminidase were pooled and utilized for further preparation of neuraminidase-containing liposomes.

Liver lysosomal fraction

The liver of rats injected with Triton WR-1339 and 3.5 days later with non-entrapped or liposome-entrapped neuraminidase was fractionated in 0.3M-sucrose (unbuffered) (Gregoriadis *et al.*, 1970) and the lysosome-rich fraction obtained was then subjected to subfractionation on a discontinuous sucrose gradient (Trouet, 1964) to obtain the purified lysosomal fraction.

Neuraminidase assay

Neuraminidase activity in the plasma of rats injected with the non-entrapped or liposome-entrapped enzyme was measured as follows. Two 0.01ml samples of rat plasma were added to two tubes each containing 0.4ml of 0.1M-sodium acetate buffer, pH5.5, 1.0% NaCl, 0.5% fetuin and 0.01% Triton X-100. To one of the tubes, which served as control, 0.1ml of 0.25M-H_2SO_4 was added. After 60min incubation at 37°C, the reaction in the second tube ('experimental') was stopped with 0.1ml of 0.25M-H_2SO_4. Free *N*-acetylneuraminic acid was determined and the value of the control was subtracted from that of the 'experimental' to give *N*-acetylneuraminic acid liberated by the action of neuraminidase during

the incubation period. For the assay of liposomal, liver (homogenate and lysosomal fraction) and spleen neuraminidase 0.02–0.1ml of sample was used under conditions similar to those for neuraminidase assay in plasma. Neuraminidase activity (units/ml of plasma, ml of liposomal suspension or whole tissue) was computed from a standard curve made up under the same conditions, by using neuraminidase-free liposomes and pooled plasma, tissue homogenates or liver lysosomal fractions from six untreated rats and into which increasing amounts of neuraminidase (0–0.33munit) were added.

Other assays

Free and bound plasma *N*-acetylneuraminic acid was determined by the method of Warren (1959). Erythrocyte *N*-acetylneuraminic acid was measured as follows: duplicate samples of 0.1ml of heparinized rat blood was pipetted into 1.0ml of 1% NaCl and centrifuged at 3000rev./min for 5min. The pellet was washed 10 times with 2ml of 1% NaCl, vigorously mixed with 0.2ml of 0.25M-H_2SO_4 and then made up to 1.0ml with water. After incubation at 80°C for 60min proteins were precipitated with 0.2ml of 5% phosphotungstic acid in 2M-HCl. The colourless supernatant after centrifugation at 3000rev./min for 10min was analysed for free *N*-acetylneuraminic acid. Occasional yellowish supernatants were discarded. Blank and controls with added *N*-acetylneuraminic acid were run under the same conditions. Plasma-bound hexoses were assayed by the method of Dubois *et al.* (1956) adjusted to measure 5–20μg of hexoses. A D(+)-galactose–D(+)-mannose, 1:1 (w/w), solution was used as a standard. Blood haemoglobin was determined by the method of Clegg & King (1942) and liposomal lipid as previously described (Gregoriadis *et al.*, 1971).

Seromucoid and desialylated seromucoid fraction

The seromucoid fraction (plasma proteins soluble in 0.6M-$HClO_4$) was isolated (Winzler, 1955) and measured (Lowry *et al.*, 1951) before and after complete desialylation of plasma with neuraminidase (0.02 unit/ml of plasma), at 37°C for 6–8h.

Radioactivity

Measurement of ^{51}Cr radioactivity in blood and various tissues was carried out in a Nuclear–Chicago DS-202 well-type scintillation detector.

Antigen–antibody crossed electrophoresis

This was performed on samples of rat plasma by using sheep antiserum to whole rat serum, by the technique described by Clarke & Freeman (1968) modified for the use of small-sized plates and superimposed second-dimension run (Davies *et al.*, 1971). Identification of some of the proteins in the plates was carried out by comparison with proteins of established identity in plates of human serum (Clarke & Freeman, 1968).

Animal experiments

In the principal experiments of this study 0.5–0.6 unit of neuraminidase dissolved in 1.0ml of buffered saline or entrapped in liposomes (1.0ml suspension) was rapidly injected into the tail vein of male albino rats (Wistar) weighing 180–220g. At 1–2h before injection (zero time) and at time-intervals thereafter, 0.1–0.2ml of blood was collected from the tail in heparinized microcapillaries and immediately centrifuged in the cold in an International microcapillary centrifuge (model MB) to separate the plasma. Rats injected with 1.0ml of buffered saline served as controls. In one experiment four rats previously treated with Triton WR-1339 were injected with non-entrapped (two rats) or liposome-entrapped neuraminidase, killed after 5h and their livers fractionated for the isolation of lysosomes. In another experiment erythrocytes from blood obtained from the tail vein of four rats were labelled with $Na_2{}^{51}CrO_4$ (Nour-Eldin, 1972) and returned to the donors, which were then injected with 0.6 unit of non-entrapped neuraminidase (two rats) or buffered saline. Blood from these rats was subsequently injected into other rats (recipients) which were killed 5h later.

Results and Discussion

Fate of injected neuraminidase

Injection of non-entrapped neuraminidase (0.5–0.6 unit) into rats resulted in the removal of most of the enzyme from the circulation within 5h (Fig. 1). A similar period of time was needed for the removal of neuraminidase entrapped in liposomes (0.5–0.6 unit and 17mg of lipid) (Fig. 1). In one other experiment, however, the same amount of neuraminidase entrapped in liposomes composed of about half the quantity of lipids (8mg) disappeared from the circulation in 3h (see the legend to Fig. 1). This is consistent with earlier findings that the rate of elimination from the plasma of substances entrapped in liposomes is directed by their carrier (liposomes) rather than the entrapped substances (Gregoriadis & Ryman, 1972*a*; Gregoriadis, 1973). Despite the similarity in their rate of disappearance from the plasma, tissue recovery of non-entrapped neuraminidase was distinctly different from that of liposome-entrapped neuraminidase. Of the injected liposomal neuraminidase, 20–26% was found in the liver 5h after treatment mostly in the lysosomal fraction (60–69%

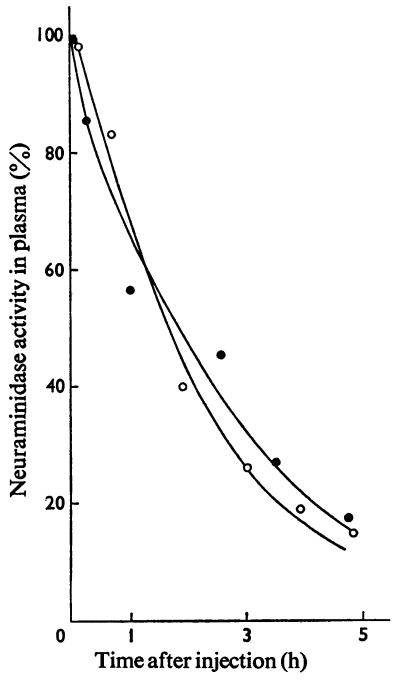


Fig. 1. *Elimination from the blood of neuraminidase injected into rats*

Rats were injected with non-entrapped neuraminidase (0.5–0.6 unit) or with liposome-entrapped neuraminidase (0.5–0.6 unit and 17mg of lipids). At time-intervals blood samples were obtained from the tail vein of the animals and neuraminidase was determined in the plasma (see the Materials and Methods section) and expressed as percentage of the injected enzyme activity in total plasma. In a separate similar experiment the same amount of neuraminidase (0.5 unit) entrapped in liposomes of lower lipid content (8mg of lipid) was removed from the plasma in about 3h. The volume of plasma was taken as 9.8ml/200g rat (Gregoriadis *et al.*, 1970). Each point is the average value from three rats. ○, Non-entrapped neuraminidase; ●, liposome-entrapped neuraminidase.

of the total activity in the liver) and 3.6–5.0% in the spleen (Table 1). Considerably less enzyme (7–11% of the dose) was recovered in the liver of rats injected with non-entrapped neuraminidase and none in the liver lysosomal fraction or spleen (Table 1).

The lysosomal localization of liposomal neuraminidase confirms earlier findings of a similar fate of injected liposome-entrapped invertase (Gregoriadis & Ryman, 1972*b*). The low recovery of injected non-entrapped neuraminidase in liver and spleen could result from either a rapid degradation of the enzyme in the cells that form the reticuloendothelial system (e.g. Kupffer cells and spleen macrophages) and to which neuraminidase, a foreign enzyme, would be preferentially directed, or from a widespread binding of the enzyme on various cell surfaces (McQuiddy & Lilien, 1973), which would limit its uptake by the reticuloendothelial system. Both possibilities are unlikely to apply in the liposome-entrapped enzyme; the first because of the almost certain localization of the liposomal enzyme mainly in the parenchymal cells of the liver (Gregoriadis & Ryman, 1972*a*), which are less active than cells of the reticuloendothelial system in degrading endocytozed materials (Gordon, 1973) and the second because of the abolition of neuraminidase-cell membrane contact as carriage of the enzyme is effected via liposomes.

Plasma N-acetylneuraminic acid

The concentration of bound *N*-acetylneuraminic acid in rat plasma before and after injection of non-entrapped neuraminidase is shown in Fig. 2. There was a marked decline of bound *N*-acetylneuraminic acid reaching a value of 19–44% of the concentration at zero time (0.74–1.41mg/ml of plasma) 3–5h after injection of neuraminidase. Thereafter, bound *N*-acetylneuraminic acid concentrations increased to reach normal values in about 48h. Despite the decline in bound *N*-acetylneuraminic acid concentration, free *N*-acetylneuraminic acid in plasma was

Table 1. *Fate of neuraminidase injected into rats*

Four rats were injected with Triton WR-1339 (Gregoriadis *et al.*, 1970) and 3.5 days later with 0.6 unit of non-entrapped or liposome-entrapped neuraminidase (see legend to Fig. 1). All rats were killed 5h later and neuraminidase was measured in the liver, the liver lysosomal fraction and the spleen (see the Materials and Methods section). N.D., Not detected.

Rat	Treatment	Liver		Spleen
		Homogenate	Lysosomal fractions	
1	Non-entrapped neuraminidase	0.040*	N.D.	N.D.
2	Non-entrapped neuraminidase	0.070	N.D.	N.D.
3	Liposome-entrapped neuraminidase	0.120	0.072*	0.030*
4	Liposome-entrapped neuraminidase	0.155	0.108	0.022

* Neuraminidase activity is expressed as units/total tissue homogenate or lysosomal fraction from total liver.

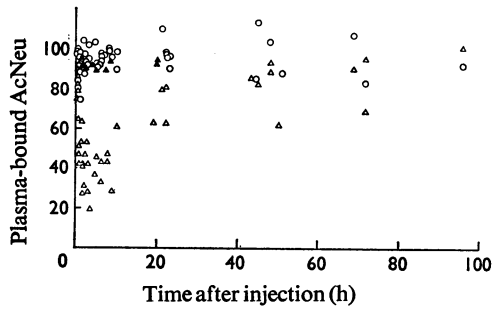


Fig. 2. *Effect of injected neuraminidase on plasma bound N-acetylneuraminic acid*

Rats were injected with buffered saline (6), non-entrapped (6) and liposome entrapped (2) neuraminidase (see legend to Fig. 1). At 1–2h before injection (zero time) and at time-intervals thereafter blood samples were obtained from the tail vein of the animals and bound and free *N*-acetylneuraminic acid determined in the plasma (see the Materials and Methods section). All values of bound *N*-acetylneuraminic acid from individual rats are presented here and expressed as percentage of the value in the corresponding rat, measured at zero-time (0.74–1.41 mg/ml of plasma). Free *N*-acetylneuraminic acid in plasma was absent from the rats given buffered saline or liposomal neuraminidase and very low in the rats given non-entrapped neuraminidase (0.02–0.03 mg/ml of plasma at 10–60 min after injection of neuraminidase). △, Non-entrapped neuraminidase; ▲, liposome-entrapped neuraminidase; ○, buffered saline.

very low, at most 0.02–0.03 mg/ml 10–60 min after injection of neuraminidase (see the legend to Fig. 2).

In contrast with the action of the non-entrapped enzyme, surrounding of neuraminidase by the lipid bilayers of liposomes prevented the enzymic hydrolysis of *N*-acetylneuraminic acid from plasma glycoproteins *in vivo* (Fig. 2) during the 5h period needed for the removal of most of the injected neuraminidase from the circulation (Fig. 1). A small decrease of bound *N*-acetylneuraminic acid, also observed in rats injected with buffered saline, was most probably due to blood sampling (Fig. 2).

Plasma-bound hexoses

Bound hexoses in plasma, reflecting the concentration of plasma glycoproteins, decreased to 70–73% of the zero-time values 5–8h after treatment with non-entrapped neuraminidase (Fig. 3). In rats treated with buffered saline or liposome-entrapped neuraminidase, concentrations of bound hexoses varied between 90 and 120% of the zero-time values (Fig. 3).

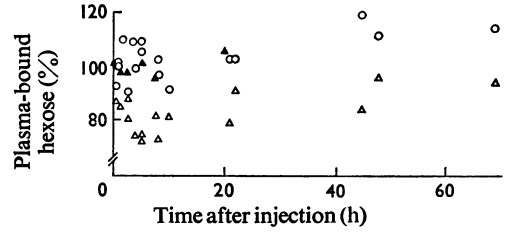


Fig. 3. *Effect of injected neuraminidase on plasma-bound hexoses*

Rats were injected with buffered saline (2), non-entrapped (2) and liposome-entrapped (2) neuraminidase (see legend to Fig. 1), bled at time-intervals (see legend to Fig. 2) and total bound hexoses measured in plasma (see the Materials and Methods section). All values from individual rats are presented here and expressed as percentage of the value in the corresponding rat measured at zero time (1.30–1.74 mg/ml of plasma). △, Non-entrapped neuraminidase; ▲, liposome-entrapped neuraminidase; ○, buffered saline.

Plasma seromucoid-fraction proteins

It was observed during these studies that partial or total desialylation of plasma proteins *in vitro* decreased the amount of protein soluble in 0.6M-$HClO_4$ (seromucoid fraction). Determination of the seromucoid fraction in the partially desialylated plasma (Fig. 2) of rats injected with non-entrapped neuraminidase could not therefore reveal whether a decline of seromucoid protein concentration in plasma was due to a decrease in solubility of some seromucoid proteins in 0.6M-$HClO_4$ or to the disappearance of these proteins from the circulation as a result of their desialylation (Morell *et al.*, 1971). To overcome this difficulty, in addition to the seromucoid fraction the desialylated seromucoid-fraction proteins, i.e. plasma proteins soluble in 0.6M-$HClO_4$ after their complete desialylation, were determined. In a typical experiment, after the assay of the seromucoid fraction plasma from rats injected with buffered saline, non-entrapped or liposome-entrapped neuraminidase was completely desialylated *in vitro* (see the Materials and Methods section) and processed for the isolation and determination of the desialylated seromucoid fraction. In rats treated with non-entrapped neuraminidase (Fig. 4) there was a gradual decrease in the concentration of desialylated seromucoid-fraction proteins, reaching a minimum of 44–67% of the zero-time values. Only small changes were observed in rats treated with buffered saline or liposome-entrapped neuraminidase (Fig. 4).

Antigen–antibody crossed electrophoresis

Typical electrophoretic patterns obtained from the plasma of rats before and after injection with non-entrapped neuraminidase are shown in Plates 1 and 2

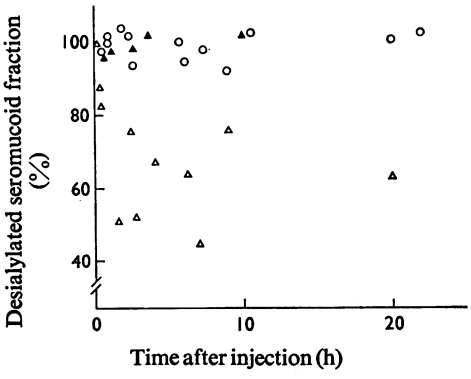


Fig. 4. *Effect of injected neuraminidase on the desialylated seromucoid fraction*

Rats were injected with buffered saline (2), non-entrapped (2) and liposome-entrapped (2) neuraminidase (see legend to Fig. 1) and bled at time-intervals (see legend to Fig. 2). $HClO_4$-soluble proteins were measured in plasma from all rats before and after complete desialylation with neuraminidase *in vitro* (see the Materials and Methods section). Values from individual rats obtained after complete desialylation of plasma *in vitro* are shown here (desialylated seromucoid fractions) and expressed as percentage of the value in the corresponding rat measured at zero time (3.8–4.6mg of protein/ml of plasma). Seromucoid-fraction values (before desialylation *in vitro*) for all rats at zero time were 5.9–6.5mg of protein/ml of plasma. △, Non-entrapped neuraminidase; ▲, liposome-entrapped neuraminidase; ○, buffered saline.

respectively. Desialylation of plasma proteins resulted in a decrease in the net negative charge and modification of the electrophoretic mobility of several of the plasma proteins (e.g. α_1-acid glycoprotein, α_1-antitrypsin, caeruloplasmin) and in a decrease in the concentration of others (e.g. α_1-antitrypsin, haemopexin, α_1-acid glycoprotein) (Plate 2). Although at this stage it was not possible to identify all the proteins in the electrophoretogram of the untreated rat or to recognize all of those proteins with altered mobility after neuraminidase treatment, we were nevertheless able to identify several of them and tentatively name a few others (see the legend to Plates 1 and 2). Further comparison of the two patterns (Plates 1 and 2) revealed a decrease in the actual number of proteins after neuraminidase treatment: of 28 detected in the plasma before treatment (Plate 1) 22 could be seen after neuraminidase treatment (Plate 2). Although this could be partly due to a possible loss of the antigenic determinant for some desialylated proteins, the observed decrease in the plasma total bound hexose (Fig. 3) and in the desialylated seromucoid-fraction protein (Fig. 4) strongly implies an actual loss of desialylated protein from the circulation. Indeed, recent work has shown that *in vitro* partial or total enzymic desialylation of several plasma glycoproteins and subsequent injection into rats results in their prompt clearance from the circulation (Morell *et al.*, 1971) and recovery in the lysosomes of the hepatic parenchymal cells where they are catabolized (Gregoriadis *et al.*, 1970).

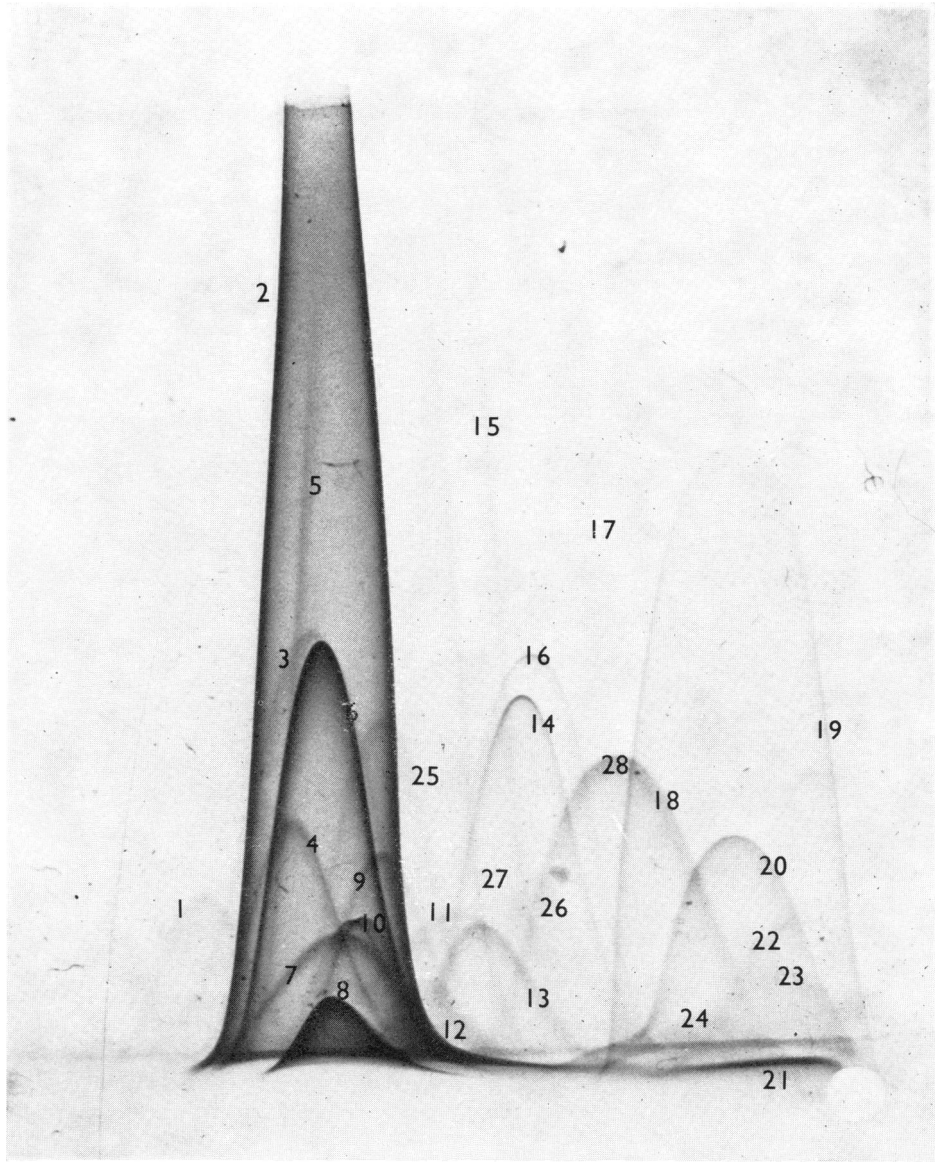
Erythrocytes

Injection of non-entrapped neuraminidase removed a considerable proportion of *N*-acetylneuraminic acid from the membrane of blood erythrocytes; the average value of 38.1μg of *N*-acetylneuraminic acid per erythrocytes derived from 1ml of blood (two rats) decreased to 12.9μg 21h after treatment of the animals with the enzyme. There was a concurrent decline in haemoglobin content from 13.6 to 10.3g/100ml of blood reflecting a decrease in the erythrocyte content, but this could account only for part of the lost *N*-acetylneuraminic acid; a small decrease in erythrocyte *N*-acetylneuraminic acid with an almost parallel loss of haemoglobin (attributed to blood sampling and similar to that seen in the neuraminidase-treated rats) was observed in the control and the liposomal neuraminidase-treated rats (Table 2). Results from experiments in which rat erythrocytes

Table 2. *Erythrocyte N-acetylneuraminic acid in rats treated with non-entrapped and liposome-entrapped neuraminidase*

Rats were injected with buffered saline (two rats) or with non-entrapped (two rats) or liposome-entrapped (two rats) neuraminidase as described in the legend to Fig. 1. Blood haemoglobin (g/100ml of blood) and erythrocyte *N*-acetylneuraminic acid (μg/erythrocytes derived from 1ml of blood) were measured 1.5h before treatment (zero time) and at time-intervals thereafter. All values are the average obtained from two rats.

Time after injection (h)	Buffered saline		Liposomal neuraminidase		Non-entrapped neuraminidase	
	Haemoglobin	AcNeu	Haemoglobin	AcNeu	Haemoglobin	AcNeu
0.0	12.6	36.7	13.5	43.3	13.6	38.1
1.3	11.5	29.2	12.1	40.1	13.3	17.2
5.0	11.2	31.8	11.5	40.2	10.6	13.4
8.5	12.0	33.2	12.0	39.4	11.2	14.2
21.0	10.9	30.0	12.1	38.0	10.3	12.9



EXPLANATION OF PLATES 1 AND 2

Antigen–antibody crossed electrophoresis of rat plasma before and after injection of neuraminidase

Antigen–antibody crossed electrophoresis was carried out in the plasma of rats 1.5 h before (Plate 1) and 5 h after (Plate 2) injection with 0.6 unit of non-entrapped neuraminidase (see the Materials and Methods section). In a typical pattern shown here obtained from one rat, 28 proteins were counted in plasma before neuraminidase and 22 proteins after neuraminidase. Identification of some of the proteins was carried out in Plate 1 by comparison with proteins of established identity in electrophoretograms of human serum. Proposed position of proteins in Plate 2 is shown by the corresponding numbers. The following proteins were identified, some tentatively: (1) prealbumin, (2) albumin, (5) α_1-acid glycoprotein, (6) α_1-antitrypsin, (7) α_1-lipoprotein?, (8) α_2-macroglobulin?. (9) Inter (α_1–α_2)-α trypsin inhibitor?, (4) caeruloplasmin, (16) Gc (group component) globulin?, (17) α_2 H.S. (Heremans and Schültze) glycoprotein?, (18) haptoglobin?, (19) haemopexin, (20) transferrin, (21) immunoglobulin, (24) β-lipoprotein?, (25) α_1 anti-chymotrypsin. Question marks signify tentative identification.

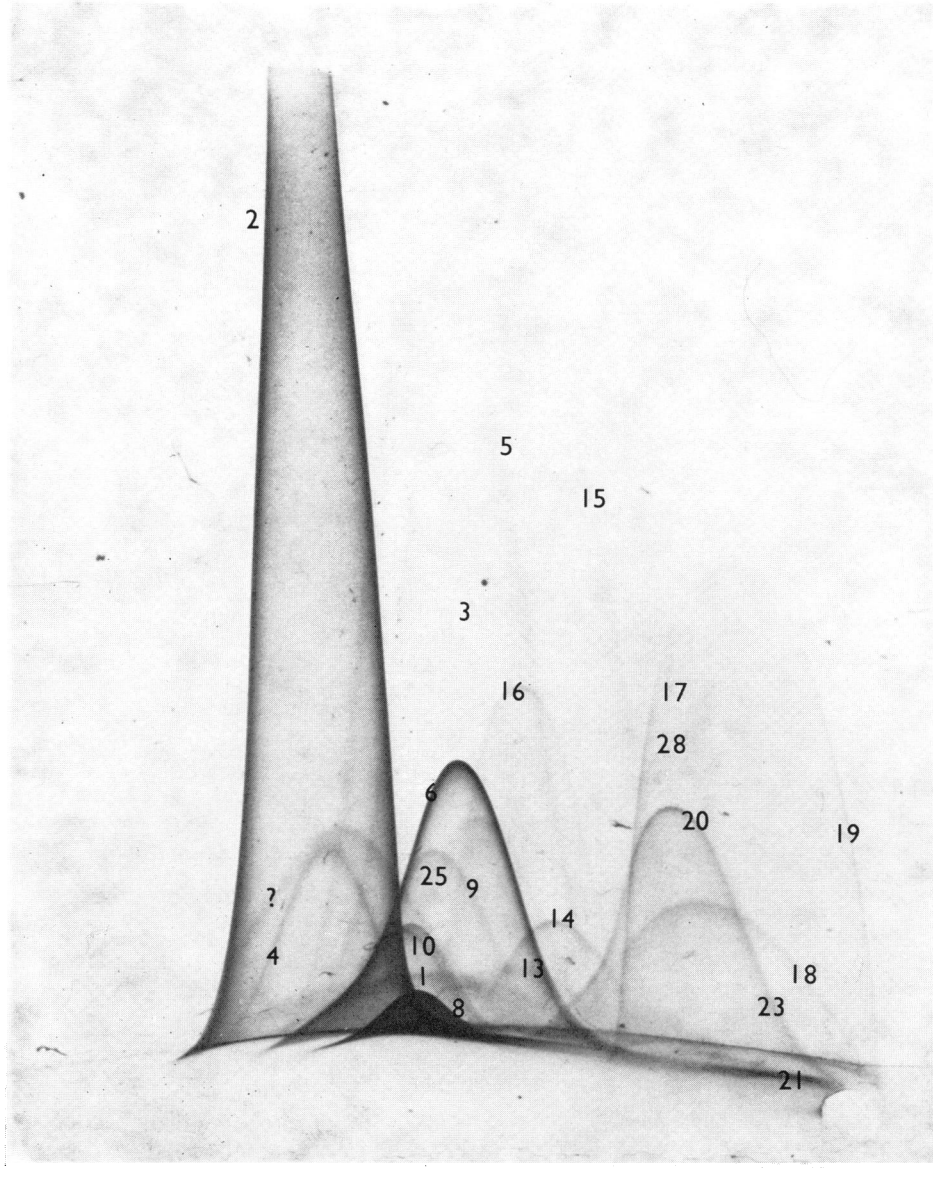


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Table 3. *Effect of injected neuraminidase on erythrocyte survival in the rat*

Blood samples from rats A, B, C and D were labelled with ^{51}Cr and reinjected into the donors (1.8×10^5 c.p.m./rat). At 12h later rats A and B were injected with buffered saline and rats C and D with neuraminidase (see the Materials and Methods section). Rats A and C were killed 7 days and rats B and D 9 days after treatment. Radioactivity was measured in blood and tissues, corrected for blood contamination and expressed as percentage of the injected radioactivity. No detectable radioactivity was found in blood plasma, lungs and kidney. No haemolysis was observed in any of the blood samples obtained; haemoglobin content was within the normal values (12–14g/100ml of blood) and microscopic examination of blood from all rats showed absence of agglutination. A similar experiment with rats treated with liposome-entrapped neuraminidase gave results similar to those obtained with rats A and B.

Rat	Neuraminidase	Blood (%)	Liver (%)	Spleen (%)
A	−	52.0	1.3	2.0
B	−	43.7	0.5	2.6
C	+	36.3	13.2	4.9
D	+	18.0	15.4	7.2

Table 4. *Fate of desialylated rat erythrocytes injected into rats*

Erythrocytes from 0.2ml of ^{51}Cr-labelled blood obtained from rats A, B, C and D 6h after their treatment with buffered saline or neuraminidase (see legend to Table 3) were injected into recipient rats a, b, c and d respectively (5×10^3–7×10^3 c.p.m.) which were killed 5h later (see the Materials and Methods section). Radioactivity was measured in blood and tissues, corrected for blood contamination, and expressed as percentage of the injected radioactivity. No radioactivity was detected in blood plasma, lungs and kidney.

Rat	Treatment	Blood (%)	Liver (%)	Spleen (%)
a	Erythrocytes from rat A	98.2	0.0	0.0
b	Erythrocytes from rat B	102.5	0.0	0.0
c	Erythrocytes from rat C	26.8	62.7	4.4
d	Erythrocytes from rat D	32.1	54.5	7.2

were labelled with ^{51}Cr and reinjected into the donors, which were subsequently treated with buffered saline or non-entrapped neuraminidase are shown in Table 3. At 7 and 9 days after treatment with neuraminidase liver contained 13.2 and 15.4% and spleen 4.9 and 7.2% of the injected radioactivity respectively. Very little radioactivity was found in the liver and spleen of control rats and again, entrapment of neuraminidase in liposomes, which prevented the action of the enzyme on its substrate on the surface of erythrocytes, also prevented the enzyme from altering the life span of these cells (see the legend to Table 3). The pronounced hepatic and splenic uptake of radioactivity in the neuraminidase-treated rats was reflected in the blood radioactivity (18 and 36.3% compared with the values of 43.7 and 52.0% of the injection observed in control rats) (Table 3).

To ascertain that ^{51}Cr uptake by the liver of the neuraminidase-treated rats was a consequence of erythrocyte desialylation rather than that of some other indirect effect of neuraminidase, erythrocytes from control and neuraminidase-treated rats (donors) were washed with 1% NaCl five or six times and injected into the tail vein of other rats (recipients). Results from such experiments are shown in Table 4. By 5h after injection of the recipient rats with erythrocytes from control donors, almost all the radioactivity was recovered in their blood. In contrast, two recipient rats given erythrocytes from neuraminidase-treated donors had 54 and 63% of the injected radioactivity in their liver, 4 and 6% in their spleen and the remainder in their blood.

Direct injection of neuraminidase into rats appears to have caused profound alterations in some of the properties, biological and otherwise, of plasma proteins, erythrocytes and, undoubtedly, of other cells. The decrease in the seromucoid-fraction proteins, reflecting a partial to total removal of some plasma proteins from the circulation (Plates 1 and 2 and Figs. 3 and 4), and the decrease in erythrocyte survival (Tables 3 and 4) as a result of a single neuraminidase injection have stimulated further research in this laboratory on the role of *N*-acetylneuraminic acid on the survival of glycoproteins and cells and have pointed to the possibility of using neuraminidase for the production of experimental specific protein or cell deficiencies.

Our studies indicate that as an approach to the therapy of lysosomal storage diseases, introduction of a hydrolase into specific intracellular targets by its

direct administration is for neuraminidase, and could be for other hydrolases, inefficient (Table 1) as well as being potentially hazardous. On the other hand, entrapment of a hydrolase in liposomes seems to protect the environment from undesirable reactions and directs the enzyme into the intracellular compartment (lysosomes) where the enzyme action is needed.

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