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TUBULAR STRUCTURES FORMED BY PIG ERYTHROCYTE GLOBOSIDE IN AQUEOUS SYSTEMS

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SUMMARY

1. Pig erythrocyte globoside (2-acetamido-2-deoxy-galactopyranosyl-β-(1→3)-galactopyranosyl-α-(1→4)galactopyranosyl-β-(1→4)glucopyranosyl-(1→1)ceramide) was dispersed in water or 0.02 M NaCl in concentrations of 1–50% lipid (w/w), and the ultrastructure of the mixtures examined by electron microscopy of samples treated in three ways: (a) negatively stained with sodium silicotungstate and dried; (b) fixed with RuO_4 vapour, dehydrated, embedded in epoxy resin and sectioned; or (c) fracturing, etching and replicating a frozen sample.

2. All methods showed the lipid to exist in long, unbranched, filamentous tubes having a diameter of about 100 Å, which is close to twice the long dimension of the globoside molecule. The distribution of tube lengths was consonant with their formation by linear polymerisation, and tube lengths increased in a dilute sol on aging.

INTRODUCTION

Complex amphiphilic lipids from biological sources form a variety of structures when equilibrated in aqueous systems[1,2]. All of the structures are formed from aggregates of lipid molecules in which minimal contact between the aqueous solvent and the hydrocarbon part of the lipid molecule is maintained, but subject to this restraint a variety of arrangements are possible, for example spherical micelles, lamellar and hexagonal liquid crystalline phases, and so on. It is still difficult to predict the structures and phases that a given lipid–water system will form under prescribed conditions of temperature, concentration, pH, ionic strength *etc.*, and even more difficult to relate the behaviour of a pure lipid in aqueous systems to its structural or functional role in biological membranes. Nevertheless as detailed studies of the structural properties of defined lipid–water systems accrue, it should become possible to obtain a predictive understanding of the forces determining such properties, and to apply this knowledge to more complex cases. To this end several structural studies on pure lipids have been carried out in this laboratory[3–5].

Globoside, the major glycolipid of pig erythrocytes (as well as other species, including human) has the structure 2-acetamido-2-deoxy galactopyranosyl-β-(1→3)-

galactopyranosyl-α-(1→4)-galacto-pyranosyl-β-(1→4)-glucopyranosyl-(1→1)-ceramide. It is one of an important class of biologically occurring lipids whose structural properties in aqueous systems have not been thoroughly investigated previously. Globoside constitutes almost 10% of the total lipid of the pig erythrocyte by weight[6], and two types have been distinguished by Hanahan *et al.*[6], differing only in the types of fatty acids in the ceramide moiety. The major globoside, designated "Glycolipid D" by these authors contains 2-hydroxy fatty acids (principally the 22h:0, 24h:0 and 24h:1 species), while a species designated "Glycolipid C", occurring in lesser amounts, contains normal fatty acids. In the present report, an electron microscopic investigation of the structures formed by "Glycolipid D" in aqueous systems is described.

MATERIALS AND METHODS

Analytical techniques as well as the general procedures for isolation of lipids have been described in previous reports[3,4]. Globosides were isolated in the course of the preparation of phosphatidyl ethanolamine already described[3]: after the latter lipid was eluted from the column of silicic acid treated with NH_4OH, the column was further eluted with methanol. All the globosides of the original lipid mixture, together with phosphatidylserine and phosphatidylinositol were found in this eluate. The latter was chromatographed on a column of DEAE-cellulose, (Whatman DE-52), acetate form, packed in chloroform–methanol (7:1, v/v) using a loading factor of 1 g resin per mg lipid phosphorus in the mixture. Glycolipids, free of phosphorus-containing lipids, were eluted from the column using chloroform–methanol (7:3, v/v). Separation of Glycolipids C and D was attempted according to the procedure of Hanahan *et al.*[6] except that silicic acid (Mallinckrodt, 100 mesh) was used in place of SilicAR-CC7. However although Glycolipid D could be obtained in pure form by this procedure, Glycolipid C (which was present in markedly lesser amount in the mixture) was contaminated with another, as yet unidentified glycolipid in each of several runs, and it has not been further studied. Glycolipid D was precipitated as a white amorphous solid from methanol by the addition of diethyl ether.

Glycolipid fractions were examined by thin-layer chromatography on Silica Gel G using the solvent chloroform–methanol–water, (95:35:6,v/v/v); spots were visualised by charring with H_2SO_4 and heat, or by means of diphenylamine spray reagent (prepared by mixing 2.5 ml of a 10% (w/v) solution of diphenylamine in acetone with 10 ml acetone, 0.125 ml aniline and 1.25 ml concentrated H_3PO_4). Glycolipids C and D were well separated in this system and easily identified by their high concentration relative to other diphenylamine-positive spots and by their mobilities, which were similar to those found by Hanahan *et al.*[6]. The pure Glycolipid D gave one diphenylamine-positive spot, which was negative to ninhydrin or phosphorus-specific[7] sprays. Elemental analysis of Glycolipid D (Microanalysis Laboratories Ltd, 329 St. George St., Toronto, Canada) gave the following results: Found: C, 57.65; H, 9.39; N, 2.07%. Calculated for globoside containing 24h:0 fatty acid: C, 60.24; H, 9.37; N, 2.07%. The infrared spectrum of the material was identical to that reported by Miyatake *et al.*[8]. for pig erythrocyte globoside. The lipid dispersed easily in distilled water or 0.02 M NaCl to give free-flowing,

turbid sols when the lipid was present at a concentration of 1–2% (w/w), and a viscous gel when 30–50% lipid (w/w) was present.

Electron microscopy methods

Negative staining. 1–2% (w/w) of glycolipid was dispersed in distilled water or 0.02 M NaCl by mechanical agitation. About 0.5 ml of a 3% solution of sodium silicotungstate was placed on a piece of wax film to make a large drop, and 1 μl of glycolipid dispersion was placed on top of this drop. The surface of the drop was then touched with a grid covered with a 10-nm thick carbon film, rendered hydrophilic by exposure to ultraviolet light. Most of the liquid was removed from the grid with a piece of filter paper, and the grid was placed immediately into the sample port of a Philips EM 200 electron microscope equipped with an anti-contamination device.

Preparation of specimens for thin sectioning. 20 mg of glycolipid was stirred into 40 mg of distilled water. A sample of the resulting gel was placed into one end of a plastic tube which contained in the other end a drop of RuO_4 (ref. 9). This operation was carried out at 4 °C to prevent evaporation of the RuO_4. The tube was sealed and left at room temperature for several days, during which time the sample was fixed by the RuO_4 vapour. The sample was subsequently washed with distilled water, dehydrated with a series of concentrations of ethanol, and embedded in low viscosity epoxy resin[10]. Sections between 20–30 nm in thickness were cut with a diamond knife and some were stained with uranyl acetate and/or lead citrate[11].

Freeze-etching. Small samples of glycolipid gel at a concentration of 30% were placed on collared gold specimen holders and frozen in liquid Freon 22 at about −150 °C. Fracturing and etching was performed according to the method of Moor[12], using a Balzer BA 300 freeze-etching unit. The fractured specimen was etched at −100 °C for 1 min and shadowed with platinum using an electronic evaporation unit. A carbon replica of the surface was prepared and cleaned by floating on the surface of concentrated NaOCl solution. Mounted replicas were examined in a Philips EM 200 electron microscope.

RESULTS

Fig. 1 is an electron micrograph of a freshly prepared 1% (w/w) sol of globoside (Glycolipid D) in distilled water, obtained using the negative staining technique. The lipid is seen to exist entirely in unbranched, flexible tubular structures of fairly uniform diameter; in fact the micrograph is very reminiscent of the structure of a plastic formed from a linear polymer such as polyethylene: most of the linear tubes are randomly distributed in space, but there are "microcrystalline" regions where several tubes form bundles of parallel strands. There is however no tendency of the tubes to form an ordered hexagonal liquid crystalline phase as was found in the case of phosphatidylethanolamine[3]. A remarkable feature of these tubular structures is that the ends are very hydrophobic: the white blobs at the ends of the tubes are areas not wet by the hydrophilic negative stain. Measurements of the diameters of 54 of the tubes yielded a mean value ($\pm$ standard deviation) of 100 $\pm$10 Å. Measurements of the lengths of 359 tubes were performed on a micrograph with a magnification of $1.657 \cdot 10^5$ by applying lengths of thread along all tubes

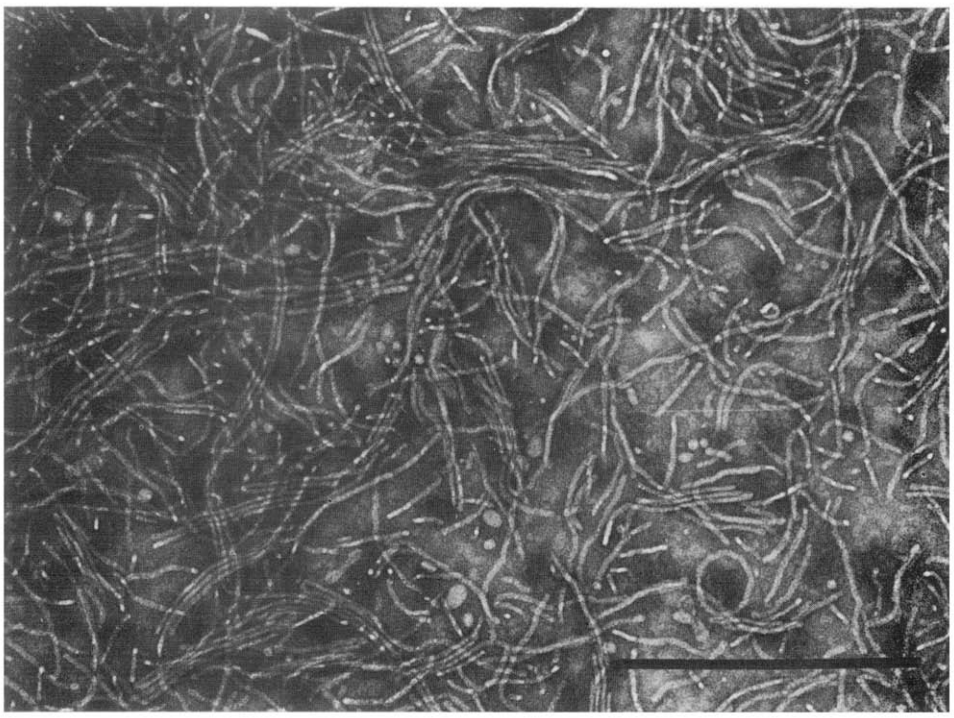

Fig. 1. Micrograph of a negatively stained 1% globoside sol, showing tubular structures of about 100 Å diameter. Stained with 3% sodium silicotungstate, pH 7.0. Marker is 0.5 μm.

which could be distinguished throughout their entire length, and then measuring the stretched threads to the nearest millimeter. The mean length ($\pm$ standard deviation) corresponded to 156 ± 101 nanometers; the histogram of observed lengths is given in Fig. 4. The curve in Fig. 4 is that expected for a linear polymerisation mechanism of tube growth as outlined in the Appendix.

When the sol used to obtain Fig. 1 was allowed to stand at room temperature without disturbance for 3 days, a large increase in the lengths of the tubes was visible, but no change was seen in the diameters on examination by negative staining and electron microscopy. The mean length was not measured since relatively few tubes could be traced throughout their entire length. After standing 3 days, globoside was recovered from the sol by freeze-drying, and examined by thin-layer chromatography. No chemical degradation of the lipid had occurred. Dispersions of globoside were also prepared at a concentration of 30% (w/w) in water and examined by electron microscopy after negative staining. The micrographs showed the same tubular structures as in Fig. 1, except that most of the tubes were aligned in bundles.

In order to confirm that the negative staining technique introduced no artifacts into the structures, electron microscopy of thin sections of fixed and embedded lipid dispersions, and of replicas of etched fractured surfaces of frozen samples of lipid dispersions was carried out. Because of the nature of these techniques, fairly concentrated (30–50%, w/w) lipid dispersions had to be used. The

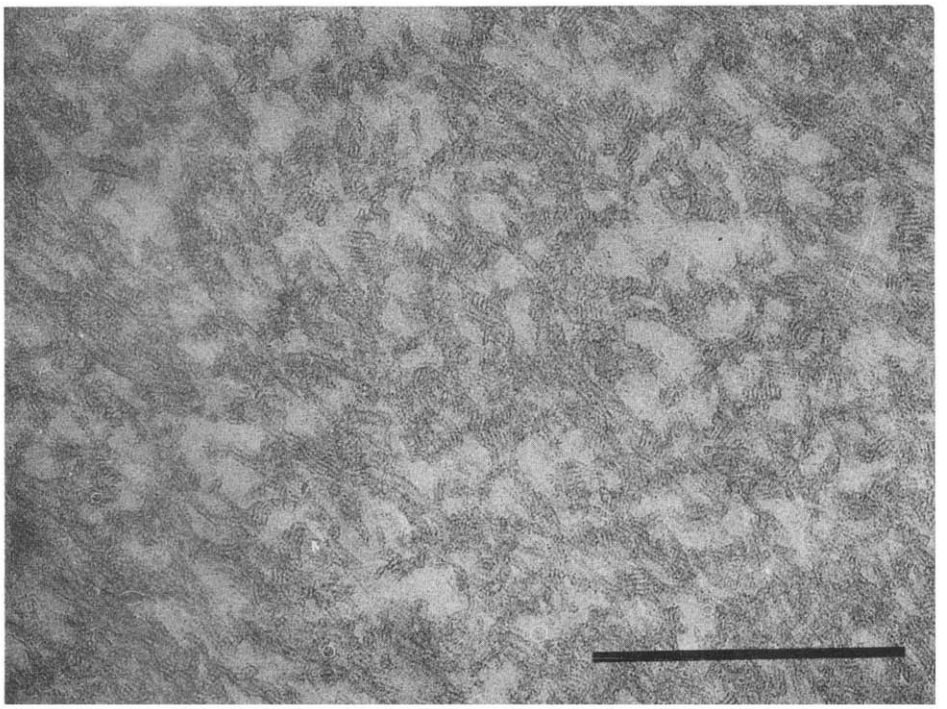

Fig. 2. Thin section of a 50% (w/w) globoside gel, fixed with RuO_4 vapour. Tendency of the tubes to align is more apparent than in Fig. 1. Sections that were also stained with uranyl acetate and /or lead citrate did not differ in appearance. Marker is 0.5 μm.

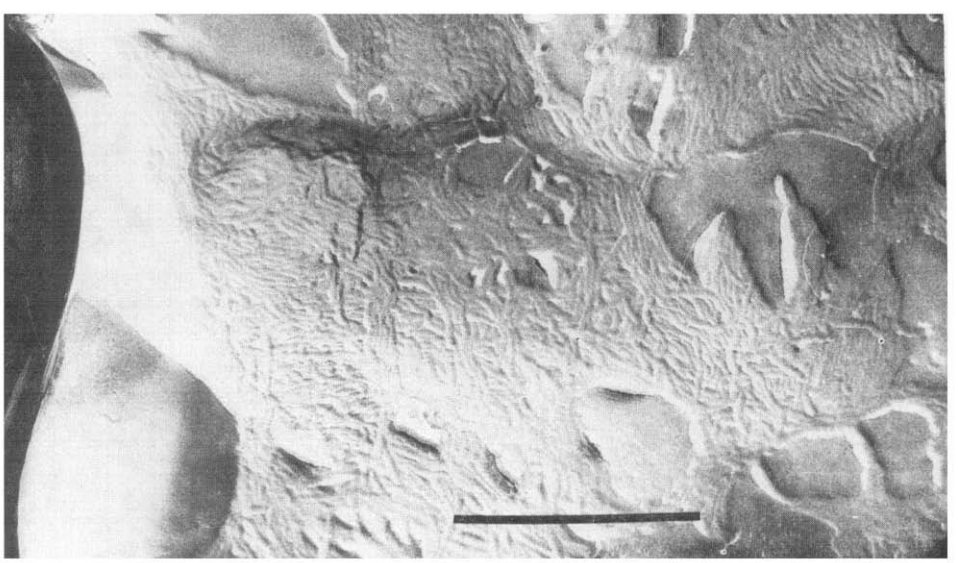

Fig. 3. "Freeze-etched" specimen of a 30% (w/w) globoside gel showing randomly distributed filaments interwoven in three dimensions. Tubes appear to be thickest in the direction of the shadowing by platinum. Marker is 0.5 μm.

results are shown in Fig. 2 and 3. In both cases the tubular nature of the lipid aggregates is clearly apparent. The diameter of the tubes seen in the sectioned material is 83±11 Å (79 measurements), while the tubes seen in the "freeze–etched" sample have a diameter of 132±25 Å (41 measurements).

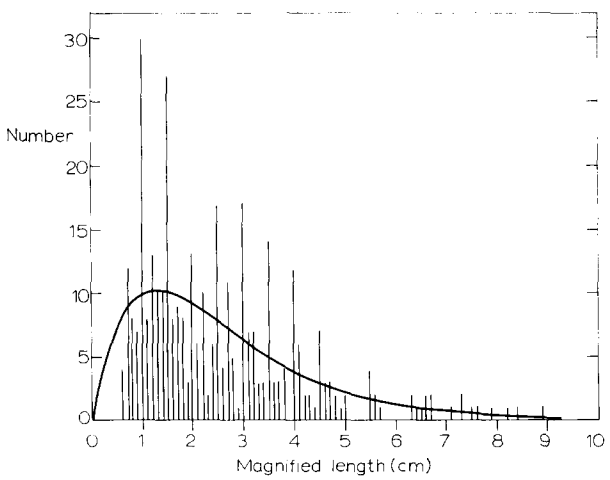

Fig. 4. Distribution of magnified lengths among 359 tubes in a micrograph similar to Fig. 1 with a magnification of $1.657 \cdot 10^5$. One tube with a length of 12.5 cm was observed but is not shown on the histogram. The curve was calculated as described in the appendix.

DISCUSSION

The evidence presented clearly establishes that globoside forms tubular aggregates when dispersed in water. Though not shown here, the same structures were formed in 0.02 M NaCl solution. The diameter of the tubes varied depending on the method of preparation of the samples for electron microscopy; however it would be expected that the tubes in fixed and embedded samples would shrink due to dehydration if anything, while the diameters of the tubes seen in the freeze–etch replicas would certainly be larger than the true diameters since the tubes are coated with a layer of platinum in the shadow-casting procedure. Hence the diameter measured in the negatively stained samples (100 Å) is probably a good estimate of the true diameter. A space-filling model of a globoside molecule containing 24h:0 fatty acid was constructed (Fig. 5), which indicated that the molecular length is about 50 Å (this would vary with changes in the conformation of the model), hence it is reasonable to suppose that the glycolipid molecules are arranged in a cross-section of the tubes like the spokes of a wheel, with the polar polysaccharide on the exterior of the tubes.

While other lipids are known to form tubular structures[1,3] the tubes found here are unique in that they are quite straight (hence probably rather stiff) and non-branched. This could be due to strong interactions of the polar groups of adjacent molecules in a direction parallel to the axis of the tube, and certainly there are many possibilities for hydrogen bonding between adjacent polysaccharide chains. Interactions between polysaccharide chains on the surfaces of separate

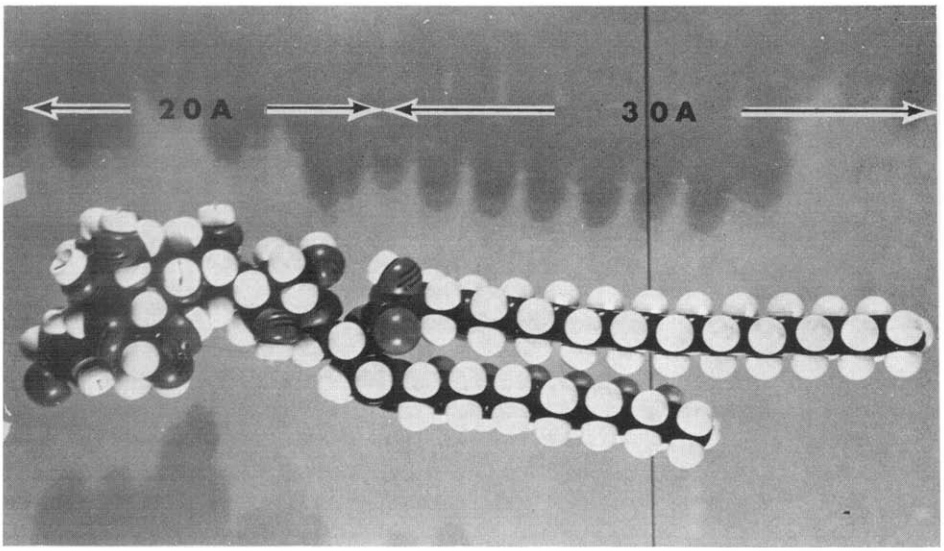

Fig. 5. Corey–Pauling space-filling molecular model of globoside with 24h:0 fatty acid.

tubes are fairly weak however, as shown by the lack of packing of the tubes into an ordered hexagonal phase.

It is an interesting feature of the tubes seen in negatively stained sols that the ends appear to be extremely hydrophobic. It is considered likely that the ends of the tubes are exposed hydrocarbon surfaces. If so, the tubes would be expected to fuse end-to-end as the dispersion ages in order to minimise hydrocarbon–water contact, and an increase in tube length on aging has been observed to occur. The process of tube growth is analogous to formation of a condensation polymer[13]. The distribution of tube lengths formed by such a process can be calculated as outlined in the appendix, and the calculated distribution agrees in its general features with the observed distribution (Fig. 4).

The behaviour of pure globoside in water can at the present time be rationalised only in general terms. Thus inspection of the space-filling model shows that the bulky polar group imparts an overall wedge shape to the molecule, so that on packing into aggregates the latter would tend to have curved rather than flat surfaces; it is not clear however why cylindrical rather than spherical aggregates are formed. The significance of the present observation in terms of the biological role of globoside is also not clear. Experiments are currently in progress to determine the morphology and other properties of hydrated mixtures of globoside with other lipids, and these may cast further light on the structural role of these glycolipids in the erythrocyte membrane.

APPENDIX

Formation of linear lipid aggregates

We assume that longer tubes are formed by end-to-end fusion of shorter tubes. The process of tube growth is analogous therefore to formation of a conden-

sation polymer[13]. Suppose that $g(l)dl$ is the frequency of occurrence of tubes having lengths in the range

$$(l - \tfrac{1}{2}\mathrm{d}l) < l < (l + \tfrac{1}{2}\mathrm{d}l).$$

For condensation polymers we expect an exponential dependence of $g(l)$ upon l, the general form being[14]

$$g(l) = \frac{a^{Z+1}}{\Gamma(Z+1)} l^Z \exp(-al) \tag{A-1}$$

The mean and variance of l are given, respectively, by

$$\langle l \rangle = \int_0^\infty l g(l)\,\mathrm{d}l \tag{A-2}$$

$$\langle \Delta l^2 \rangle = \int_0^\infty (l - \langle l \rangle)^2 g(l)\,\mathrm{d}l \tag{A-3}$$

and it is easy to show that

$$Z = \frac{\langle l \rangle^2}{\langle \Delta l^2 \rangle} - 1 \tag{A-4}$$

$$a = \frac{Z+1}{\langle l \rangle} \tag{A-5}$$

From the data of Fig. 4 we find $Z=1.36$, which is not significantly higher than unity, so that we may put

$$g(l) = \left(\frac{2}{\langle l \rangle}\right)^2 l \exp(-2l/\langle l \rangle) \tag{A-6}$$

The expected frequencies in Fig. 4 were calculated from Eqn A-6 as $359g(l)\mathrm{d}l$, where $\mathrm{d}l=0.1$ cm. The agreement with the observed frequencies is fair, considering the small number of tubes in each length interval. It is further to be noted that $\langle l \rangle$ should increase with time as more and more short tubes fuse into longer lengths.

ACKNOWLEDGEMENTS

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