THIN-LAYER CHROMATOGRAPHY OF RED CELL GLYCOSPHINGOLIPIDS FROM VARIOUS MAMMALIAN SPECIES

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SUMMARY

.Two thin-layer chromatography systems for the separation of glycosphingolipids from total lipid extracts of erythrocytes were developed. By system I (Silica Gel HR–magnesium silicate (4:1)/tetrahydrofuran–water (5:1)), ceramide hexosides were separated from all other lipids and fractionated into mono-, di- and triglycosyl ceramides. By system II (Kieselguhr G, impregnated with a 0.60 M boric acid–0.15 M disodium tetraborate buffer (pH 7.9)/chloroform–methanol–water (65:25:4)), complex glycosphingolipids (mucolipids) were separated from all other lipids and fractionated. The red cells of nine different mammals showed no species variation in the qualitative composition of the ceramide hexosides but a marked variation in the mucolipid composition as well as in the concentration of the glycosphingolipids.

INTRODUCTION

According to YAMAKAWA[1], two different types of glycosphingolipids (GSL), ceramide hexosides and mucolipids, are present in the erythrocyte membrane. Unlike ceramide hexosides, mucolipids contain, in addition to hexoses, hexosamine (globosides) or sialic acid (hematosides) or both compounds (gangliosides according to YAMAKAWA). This classification proved to be very useful for the description of the chromatographic behaviour of GSL and the discussion of the GSL patterns in erythrocytes.

In recent years, the predominant GSL of various mammalian erythrocytes have been isolated by many investigators[2-14], and in some cases the chemical structure has been elucidated. The GSL composition in the erythrocytes of rabbit, pig, and man is well known from the work of YAMAKAWA and co-workers[15-18], SWEELEY and co-workers[19,20] and HAKOMORI AND STRYCHARZ[21]. A comparison of the GSL patterns of several mammalian erythrocytes, however, has been made only by YAMAKAWA[1]. This may be due to the fact that only a few methods are available for the separation of GSL from phospholipids because of their very similar chromatographic behaviour in most systems[22]. On the other hand, the content of GSL in the erythrocyte membrane is very low in comparison with that of phospholipids, as shown by NELSON[23]. Previous investigators separated the GSL of erythrocytes by thin-layer chromatography

(TLC) after applying total lipid extracts to different types of columns to concentrate the GSL and to remove neutral lipids and phospholipids. Only SKIPSKI et al.[22] and NESKOVIC et al.[24] separated GSL from total lipid extracts by TLC, but their systems failed to resolve the very complex GSL from phospholipids.

The present paper describes new TLC systems that permit the separation of all red cell GSL, in small amounts of total lipid extracts, without prior removal of neutral lipids and phospholipids. One analytical separation requires at most the lipid extract from 1 ml of packed cells. After visualization of spots, the chromatograms allow an obvious comparison of the GSL patterns of different mammalian species. Our TLC systems may also be used to isolate any GSL preparatively. System I, which separates the ceramide hexosides, is similar to the TLC systems of SKIPSKI et al.[22]. It is based on the facts that mixtures of tetrahydrofuran and water move ceramide hexosides ahead of phospholipids, and that magnesium silicate adsorbs phospholipids more strongly than GSL. System II, in which mucolipids are separated, makes use of the formation of complexes of boric acid with the carbohydrate moiety of the GSL. Kieselguhr, impregnated with a boric acid buffer, allows all lipids without the oligosaccharide portion to move to the top of the chromatograms.

MATERIAL AND METHODS

Extraction of lipids

Blood of pig, sheep, cow and horse was taken from freshly slaughtered animals; heparinized blood of anaesthetised rat, guinea-pig, cat and rabbit was drawn by heart puncture. Freshly donated human acid–citrate–dextrose blood was obtained from Bluttransfusionsdienst der Universitätskliniken, Hamburg, G.F.R. After isolating and washing the erythrocytes as described previously[25], the extraction of lipids was carried out according to a modified procedure of WAYS AND HANAHAN[26]. A given volume (n ml) of packed erythrocytes was added slowly to 5n ml of cold methanol, while stirring on a magnetic mixer; stirring was continued for 30 min. 5n ml of chloroform were added, and after further stirring for 10 min the solvents were filtered, using a fast filter (Selecta 1117 1/2) previously washed with chloroform–methanol (1:1). The residue was washed with 2n ml of chloroform–methanol (1:1). The filtrate was evaporated to near dryness under reduced pressure at 37° and the residue was re-extracted successively with 2.5n ml of chloroform, 7.5n ml of chloroform–methanol (2:1) and 5n ml of chloroform–methanol (1:1). The extracts were filtered through a pre-washed filter into a separatory funnel. 3n ml of 0.1 M KCl were added directly to the separatory funnel to give a final volume of 18n ml of chloroform–methanol–KCl solution (10:5:3). The mixture was shaken and left to stand at 4° overnight. After warming to room temperature, the lower layer was removed and evaporated to dryness. The residue was dissolved in 40n μl of chloroform–methanol (1:1). Only small amounts of the complex GSL were lost in the aqueous upper phases, as indicated by TLC investigation.

TLC methods

For visualization of the GSL patterns, 40 μl of each total lipid extract (only 8 μl of the pig extract) were delivered with a Hamilton microsyringe as a 2-cm long band to TLC plates.

TLC system I. A mixture of Silica Gel HR (Merck, Darmstadt, G.F.R.)–magnesium silicate (Woelm, Eschwege, G.F.R.) (4:1), suspended in water, was applied to 20 × 20 cm glass plates[22]. Plates were activated overnight at 150° in an oven and used immediately after cooling to room temperature. The chromatograms were developed with tetrahydrofuran–water (5:1). The solvent was allowed to run up to a level of about 18.5 cm from the bottom of the plate; this took 75 min.

TLC system II. Kieselguhr G for TLC (Merck, Darmstadt, G.F.R.) was suspended in a 0.60 M boric acid–0.15 M disodium tetraborate buffer (pH 7.9) to yield a thin slurry. Coated plates were air-dried for about 24 h and used without subsequent activation. They were stored no longer than two days. The chromatograms were developed with chloroform–methanol–water (65:25:4). The solvent was allowed to rise 18 cm in 55–60 min.

Preparative TLC. The two systems were also used to isolate individual GSL. Ceramide hexoside bands, separated by system I, were scraped off the plates together, since the very sensitive and sugar-specific orcinol spray (see below) could not be used in preparative TLC. Ceramide hexosides were extracted from the adsorbent with chloroform–methanol–water (3:3:1). Solvents were removed under reduced pressure and the residue was dissolved in a small volume of chloroform–methanol (1:1). The ceramide hexosides were rechromatographed by using a modified preparative TLC system described by SVENNERHOLM AND SVENNERHOLM[27]. Double width (40 × 20 cm) plates, coated with Silica Gel HR, were activated for at least 2 h at 120°. After the development of the chromatograms with chloroform–methanol–water (65:25:4 or 60:35:8), the individual GSL were eluted with chloroform–methanol–water (3:3:1).

Mucolipids, separated by TLC system II, were extracted from the adsorbent with *n*-propanol–water (1:1). The eluate was evaporated to dryness and the residue was transferred quantitatively with a small volume of propanol–water (1:1) to the top of a 1.8 cm I.D. × 25 cm column, packed with coarse Sephadex G-25, which was suspended in propanol–water (1:1). Chromatography on Sephadex was performed to remove boric acid and sodium tetraborate. In a few minutes, mucolipids were recovered completely in the elution front after percolation of 35 ml of propanol–water (1:1) at a flow-rate of 1–3 ml/min.

Detection of spots

The following sprays were used for the detection of spots on the chromatograms.

(a) Orcinol–sulphuric acid spray[22]. After spraying with this sugar-specific detection reagent, the plates were placed into an oven at 120–130° for 7 min. GSL, cholesterol and phospholipids gave red-violet, red and faintly yellow-brown spots, respectively. All GSL detected by this spray were outlined with a needle, since very minor components faded shortly after heating.

(b) Resorcinol–Cu^{2+}–hydrochloric acid spray[28]. Chromatograms were dried very carefully to completely remove developing solvents. They were sprayed, covered with a clean glass plate, and heated at 130–140° for 15 min. Hematosides and gangliosides gave violet-brown spots.

(c) Molybdenum spray[29]. This was used especially in the preparative TLC system I to stain the phospholipids on two narrow guide strips.

(d) Water spray[30]. This was rather sensitive for plates coated with Kieselguhr G and was used mainly in the preparative TLC system II.
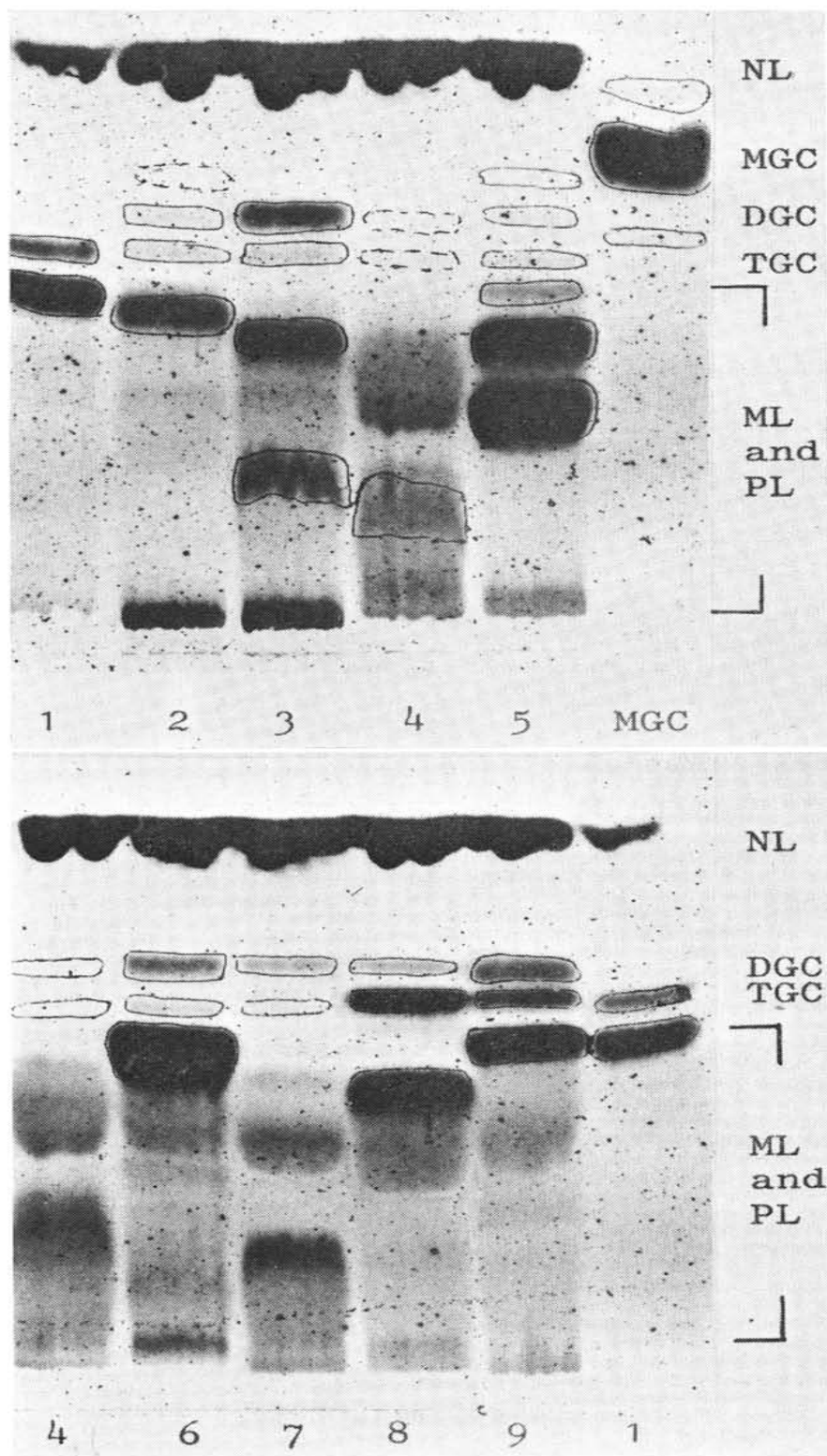
Fig. 1. Separation by TLC system I of erythrocyte ceramide hexosides from pig(1), sheep(2), cow(3), rat(4), horse(5), guinea-pig(6), cat(7), rabbit(8) and man(9). Total lipid extracts from 1 ml of packed red cells were applied from all animals except the pig (0.2 ml). Adsorbent: Silica Gel HR–magnesium silicate (4:1); solvent: tetrahydrofuran–water (5:1); spray: 0.2% orcinol in sulphuric acid, heated at 120–130° for 7 min. All glycolipids visualized by this spray were outlined with a needle. Abbreviations: ML = mucolipids; NL = neutral lipids; PL = phospholipids; MGC = monoglycosyl ceramides (cerebrosides); DGC = diglycosyl ceramides; TGC = triglycosyl ceramides.

Before being sprayed with any detection reagent, analytical chromatograms, developed with tetrahydrofuran–water (5:1), were dried in a vacuum oven at 100° for 1 h to remove the solvents completely.

Identification

GSL were identified mainly by their R_F values in several known TLC systems[1,14,17–19,22,31,32]. Cerebrosides (Koch-Light Laboratories Ltd., Colnbrook, Bucks., Great Britain) and sulphatides from bovine brain extract VI (Sigma Chemical Co., St. Louis, Mo., U.S.A.) were co-chromatographed as comparison standards. A few GSL were isolated by preparative TLC and identified by their galactose/glucose ratios calculated from gas–liquid chromatographic (GLC) data according to VANCE AND SWEELEY[19]. Galactosamine in globoside was detected by GLC after N-acetylation of the methanolyzate of the isolated lipid[33]. Sialic acid in hematosides and gangliosides was demonstrated on the TLC chromatograms with the resorcinol spray.

RESULTS AND DISCUSSION

Separation of ceramide hexosides

Fig. 1 shows the separation of erythrocyte ceramide hexosides by system I. Neutral lipids moved near the solvent front ahead of ceramide hexosides, which were clearly separated from all other lipids and fractionated into monoglycosyl ceramides (MGC), diglycosyl ceramides (DGC) and triglycosyl ceramides (TGC). The more complex GSL were also separated into individual components. Since they ran together with the phospholipids, only major mucolipids could be detected on these chromatograms. A shortcoming of system I is that both brain sulphatides and TGC have the same R_F values. To our knowledge, however, the presence of sulphatides has never been demonstrated in the erythrocyte membrane. System I gave extremely compact spots from the individual ceramide hexosides, and thus permitted the staining of even minor components. The TLC system II of SKIPSKI et al.[22] gave much larger spots so that all minor ceramide hexosides were not detectable, although maximum amounts of total lipid extracts had been applied to the plates. The TLC system B, recently described by NESKOVIC et al.[24], also produces large TGC spots and requires a two-step development.

Ceramide hexoside patterns

A comparison of the ceramide hexoside patterns, as shown in Fig. 1, indicates that DGC and TGC occur in all mammalian erythrocytes. Porcine DGC could be detected only after concentrating it by preparative TLC. Bovine DGC and porcine TGC were identified by GLC analyses, resulting in a molar galactose/glucose ratio of 1:1 (1.05) and 2:1 (1.98), respectively. The integers obtained for the galactose/glucose ratios indicate that in both cases a pure component with a uniform carbohydrate composition had been isolated, *i.e.* galactosylglucosyl ceramide and digalactosylglucosyl ceramide. Nevertheless, it cannot be expected that system I further separates ceramide hexosides with an equal number of hexose units but with different constituent hexoses. On the other hand, MGC, DGC and TGC of all mammalian erythrocytes investigated so far have uniform carbohydrate compositions[18–20]. On our chromatograms, MGC could be found only in the extracts of red cells of sheep and
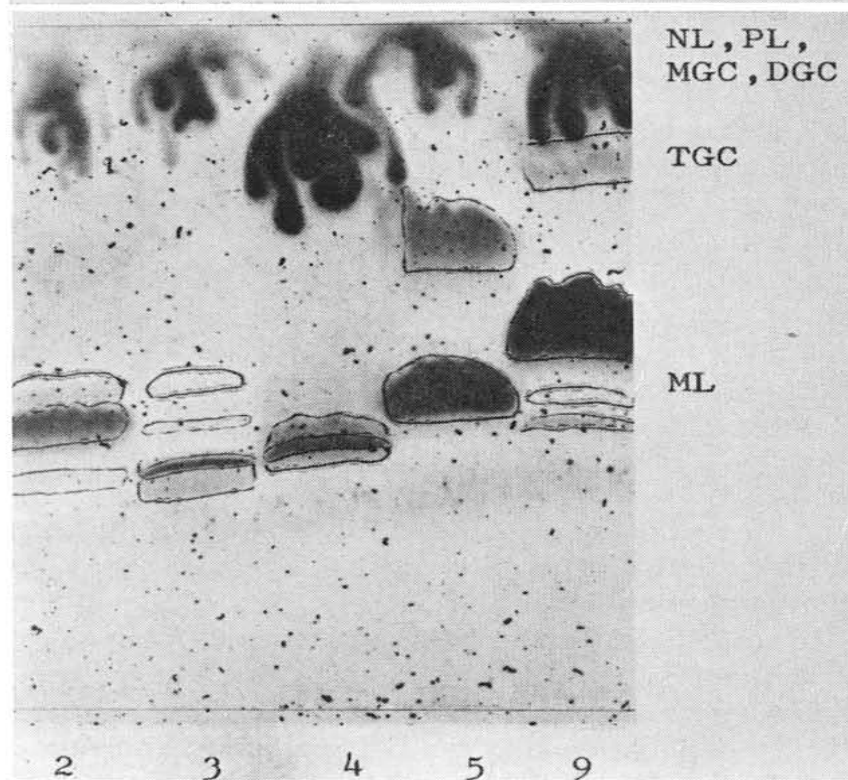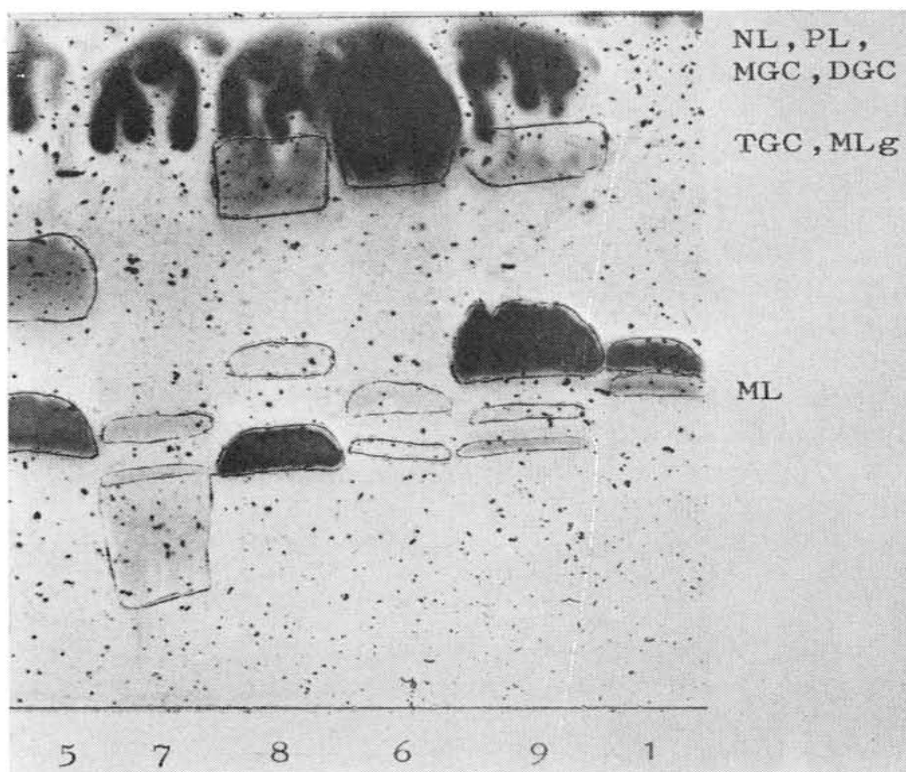
Fig. 2. Separation of erythrocyte mucolipids by TLC system II. Samples as in Fig. 1. Adsorbent: Kieselguhr G impregnated with a 0.60 M boric acid–0.15 M disodium tetraborate buffer (pH 7.9); solvent: chloroform–methanol–water (65:25:4); spray: as in Fig. 1. Abbreviations: ML = mucolipids; ML_g = mucolipid of guinea-pig; NL = neutral lipids; PL = phospholipids; MGC = monoglycosyl ceramides; DGC = diglycosyl ceramides; TGC = triglycosyl ceramides.

horse. The visualization of the minimal amounts of the red cell MGC demonstrated by other authors in rabbit[18], man[19] and pig[20] would require prior concentration by preparative TLC.

Contrary to the similarity in the qualitative composition of the ceramide hexosides, the concentrations of the individual components were highly variable. The red cells of both the rat and the cat had a very low content of ceramide hexosides, whereas in human and porcine cells there was a relatively high concentration (Fig. 1). In the distribution of ceramide hexosides, no obvious conformities were found between related animals such as rat–guinea-pig and sheep–cow. MGC was a minor component in the erythrocytes of all animals. The major ceramide hexoside in the red cells of cow, horse, guinea-pig and cat was DGC. But contrary to the results of YAMAKAWA[1], DGC was not the predominating ceramide hexoside in the erythrocytes of all mammals; TGC was the main component in pig and rabbit.

Separation of mucolipids

As shown in Fig. 2, in TLC system II all lipids except TGC and mucolipids moved with the solvent front, which was therefore overloaded. The TGC migrated close to the front, while all mucolipids except the major component of the guinea-pig (Fig. 2, lane 6: ML_g) were adsorbed more strongly and separated into individual components. Largely responsible for the success of this method is the use of an adsorbent containing kieselguhr, which is so non-polar that only those lipids that form several complexes per molecule with boric acid, do not move with the solvent front.

Mucolipid patterns

2–4 mucolipid fractions per animal were found in the erythrocytes of nine different mammals. Most mucolipid R_F values differed from species to species (Figs. 1 and 2). Even mucolipids of related mammals had different R_F values. Marked species differences thus exist in the qualitative composition of mucolipids in contrast to that of ceramide hexosides.

The main mucolipid of man (Fig. 2, lane 9) was identified by GLC analysis as the globoside I (tetraglycosyl ceramide) of YAMAKAWA et al.[15]. According to the results of VANCE AND SWEELEY[19], galactosamine was detected and a molar galactose/glucose ratio of 2:1 (1.99) was found. The slowest one of the two minor mucolipids of man (Fig. 2, lane 9) gave a positive test for sialic acid. The predominant mucolipid of the rabbit (Fig. 2, lane 8) was judged to be the pentaglycosyl ceramide of the globoside-type elucidated by ETO et al.[18]. The major component of the pig (Fig. 2, lane 1) was identified as the tetraglycosyl ceramide of MIYATAKE et al.[17] with the carbohydrate sequence of human globoside I, since in several TLC systems it had the same R_F value as the latter substance. The second porcine mucolipid may be a globoside with the same oligosaccharide chain but a different acyl residue (monohydroxy fatty acids, see HANAHAN et al.[32]). The main mucolipid of the guinea-pig (Fig. 2, lane 6: ML_g) is probably a triglycosyl ceramide of the globoside-type[1], thus accounting for the high R_F value of the lipid. Sialic acid was detected in both components of horse cells (Fig. 2, lane 5). It can be presumed that the slow mucolipid is the classic hematoside, the chemical structure of which was determined by KLENK AND PADBERG[9] and by HANDA AND YAMAKAWA[4]. The fast-moving mucolipid was identified as the hemato-

side described by HAKOMORI AND SAITO[14], which has an identical carbohydrate struc-
ture to the classic hematoside except that the sialic acid carries both an O-acetyl ester
group and an N-glycolylamide group. We succeeded in converting this hematoside
into a compound with the R_F value of ordinary hematoside by liberating the O-acetyl
group from the sialic acid (according to HAKOMORI AND SAITO[14]). The presence of
sialic acid was also demonstrated in both the fourth (slowest) and the second muco-
lipid of bovine erythrocytes (Fig. 2, lane 3). Two mucolipids containing sialic acid
were detected in cat erythrocytes (Fig. 2, lane 7). The particularly low R_F value of
the major component indicated that this was the disialohematoside of HANDA AND
HANDA[5]. The mucolipids of the sheep (Fig. 2, lane 2) and the rat (Fig. 2, lane 4) are
as yet unidentified.

From this investigation, it is apparent that in nearly all mammalian red cells,
minor mucolipids are present in addition to the known major components. They were
found especially in the erythrocytes of man, cow, sheep, rat and guinea-pig. Probably
some of the fractions, separated by TLC system II, are still heterogeneous and may be
further separated by other systems just as HAKOMORI AND STRYCHARZ[21] separated
the minor mucolipids of man into 6–9 components depending on the ABO blood-
group of the donors.

The mucolipid content was high in porcine erythrocytes, but relatively low in
the red cells of sheep, cow, rat and cat (Fig. 2). In the mucolipid distribution of all
mammals, one mucolipid clearly dominated. This fact supports the hypothesis of
DAWSON AND SWEELEY[20] that in the red cell membrane of all mammals a major
mucolipid is present, which may have a completely different carbohydrate structure,
but is functionally the same in all mammals.

The main value of systems I and II is that they permit direct application of
total lipid extracts, they selectively separate GSL, and even minor components may
be visualized. Both procedures are simple, rapid and suitable for preparative work.
A further application of both TLC systems includes the quantitation of all individual
GSL when combined with the GLC method of VANCE AND SWEELEY[19].

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