(2) Cytosine, $C_4H_5N_3O$, with one molecule in the asymmetric unit of $P2_12_12_1$.[6] 133 reflections (20 %) with $|E|>1.2$ were assigned phases. A comparison with the refined data gave a mean deviation of 14° and a maximum deviation of 64° in α. Most satisfactory E maps with no spurious peaks were also obtained in this case.

Other known structures which have successfully been handled are the ferrocene derivative, $Fe_6C_{70}O_2H_{68}$,[8] and *N*-phenyl-*N'*-benzoylselenourea, $C_6H_5{\cdot}NH{\cdot}CSe{\cdot}NH{\cdot}CO{\cdot}C_6H_5$,[9] with space groups $P\bar{1}$ and $P2_1/c$, respectively. The first unknown structure which was solved with this program set was $K_2CS_3{\cdot}H_2O$.[10]

When coding *GAASA I* and *II*, extensive use was made of *SAP 1* and *2* written by Hall.[11]

The FORTRAN IV listings and a short description of the card input for the program set can be obtained from the authors. A discussion of the methods applied in the programs, and especially in *GAASA IV*, will be published.[7]

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Enrichment of Gangliosides in Plasma Membranes of Hamster Kidney Fibroblasts

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Earlier investigations have suggested that animal plasma membranes may contain gangliosides.[1–6] Plasma membranes of hamster kidney fibroblasts (BHK21 cells) have been isolated in this laboratory by using enzyme and antigen markers to follow the isolation steps.[7] These cells do contain gangliosides,[8] and the present report shows that they are enriched in the plasma membrane to a remarkable extent.

Material and methods. BHK21 cells, clone WI-2, were grown as described elsewhere.[9] Fragments of plasma membrane and endoplasmic reticulum were isolated by the method of Wallach and Kamat [10] as described previously.[7] Protein, marker enzymes, and plasma membrane antigens were assayed as described previously.[7] Lipids were extracted from lyophilized samples (2—30 mg protein) by two treatments at 20° with 3 ml of chloroform-methanol (2:1) for 4 h, followed by two similar extractions with chloroform-methanol (1:2). This procedure is believed to extract kidney gangliosides completely.[11] In some experiments the completeness of extraction was actually determined as described by Weinstein *et al.*;[6] only traces of lipids remained in the extracted protein.

To the combined chloroform-methanol extracts was added 6 ml chloroform and 4.5 ml water. The two liquid phases were equilibrated and separated. The lower phase was washed twice with fresh upper phase. The washed lower layer is called the *phospholipid extract.* The combined upper phases were dialyzed against distilled water and lyophilized. The dry residue was extracted 3 times with 3 ml of chloroform-methanol (2:1) and filtered. This solution is called the *ganglioside extract.*

The gangliosides were assayed by the method of Warren.[12] Semiquantitative analyses were carried out also by thin layer chromatography (TLC) on silica gel G plates with propanol-

water[13] or propanol-concentrated ammonia-water (6:2:1)[14] as solvent; aliquots of the extracts were run together with known amounts of pure compounds, and the plates were stained with the resorcinol reagent.[15]

N-Acetyl and *N*-glycolyl-neuraminyl-lactosyl-ceramide of bovine kidney[16] were gifts from Dr. K. Puro. Lactosyl-ceramide was purchased from Miles Laboratories, Elkhart, Indiana.

Characterization of BHK-cell gangliosides. TLC with propanol-water (7:3) revealed only one resorcinol positive spot in the ganglioside extract prepared from the whole BHK21 cells. It had the same mobility as authentic *N*-acetyl-neuraminyl-lactosyl-ceramide and *N*-glycolyl-neuraminyl-lactosyl-ceramide. TLC with propanol-concentrated ammonia-water (6:2:1) revealed two resorcinol positive spots; the fast one had the mobility of *N*-acetyl-neuraminyl-lactosyl-ceramide, and the slow one had the mobility of *N*-glycolyl-neuraminyl-lactosyl-ceramide. — Sometimes a third, more polar ganglioside was observed in small amounts. — These findings were confirmed by mild acid hydrolysis of the gangliosides. *N*-Acetyl and *N*-glycolyl neuraminic acid were identified by TLC[17] in the hydrophilic fraction of the hydrolysate. The lipophilic fraction of the hydrolysate in turn revealed lactosyl-ceramide on TLC. Gas-liquid chromatography of the neutral sugar components (trimethylsilyl ethers of methyl glycosides) revealed equimolar amounts of glucose and galactose.

These findings confirm those of Hakomori and Murakami,[8] but the *N*-glycolyl-neuraminyl-lactosyl-ceramide is a new component in BHK21 cell gangliosides. The *N*-acetyl and *N*-glycolyl derivatives in the whole cell gangliosides were assayed in one experiment by the method of Suzuki.[18] The *N*-acetyl derivative made up 88 % and the *N*-glycolyl derivative 12 % of the total. The estimated upper limit for any additional ganglioside component is 5 %.

Ganglioside extracts of plasma membrane and endoplasmic reticulum were qualitatively similar to those of whole cells on TLC.

Localization of the gangliosides in the microsomal subfractions. The extent of separation of plasma membranes (PM) and endoplasmic reticulum (ER) can be judged from the enzyme and antigen analyses shown in Table 1. The PM-markers, activity of Na^+-K^+-stimulated ATP phosphohydrolase and concentration of surface antigens, were 5—10 times higher in PM than in ER. The ganglioside concentration was 5 times higher in PM than in ER (Table 1). The distribution of gangliosides in reality is even more skewed; a correction must be made for the fact that the two membrane fractions are contaminated with each other.

In conformity with the findings of Hakomori and Murakami[8] the ganglioside extracts prepared from the intact BHK-cells contained 0.8—1.5 μg neuraminic acid per milligram extracted protein. Thus the ganglioside concentration in PM was about 10 times higher than that in the original cells. The activity of Na^+-K^+-stimulated ATP phosphohydrolase and the surface antigens are also concen-

Table 1. Ganglioside distribution in microsomal subfractions of BHK-cells.

	Ganglioside concentration[a]	Marker enzymes: Na^+-K^+-ATPase[b]	Marker enzymes: NADH-diaphorase[c]	Concentration of plasma membrane antigens[d]
Plasma membranes				
Experiment 1	8.1	3.4	0.40	10
2	13	7.7	0.29	
3	11	6.2	0.54	
Endoplasmic reticulum				
Experiment 1	1.2	0.67	1.4	1
2	2.1	1.7	1.0	
3	2.6	1.2	1.2	

[a] μg lipid-bound neuraminic acid/mg protein.
[b] μmoles P_i liberated/mg protein/h at 37°.
[c] μmoles substrate utilized/mg protein/min at 20°.
[d] units of BHK-cell agglutinin absorbed/μg protein.

trated in PM of these cells to a similar extent.[7] Thus the ganglioside sialic acid is as good a PM-marker in these cells as the enzyme and the surface antigens.

Evaluation of the ganglioside assay. Nonpolar gangliosides are easily lost into the phospholipid extract if salts are present during the solvent partition.[11] Therefore the presence of gangliosides was studied in phospholipid extracts prepared from PM (0.3 mg protein) and ER (1.0 mg protein). TLC did not reveal any resorcinol-positive material in these extracts. The detection limits were such that the phospholipid extracts probably contained less than 1—2 μg neuraminic acid per milligram of extracted protein.

Plasma membranes of animal cells contain sialo-glycoproteins,[19] and one might ask whether the lipid-bound sialic acid of the ganglioside extracts is contaminated by any protein-bound material. This possibility is excluded; TLC in propanol-water (7:3) did not reveal any other sialic acid-containing material besides the gangliosides in the PM- and ER-extracts. Glycoproteins would presumably give resorcinol-positive spots of low mobility in this system.

Discussion. The present work shows that the gangliosides may be added to the array of plasma membrane markers in BHK cells. They possess the great advantage over the marker enzymes that their concentration, instead of activity, can be measured and correlated with other parameters of the membrane.

About 0.5 % of the protein of the original BHK21 cells is recovered in isolated plasma membranes in our procedure.[7] However, the actual amount of plasma membrane protein in these cells is probably higher.* The ganglioside/protein ratio was about 10 times higher in the plasma membranes than in the whole BHK21 cells. Therefore at least 5 % of the total gangliosides, but probably much more, are located in plasma membranes of these cells.

Changes take place in the gangliosides when BHK21 cells [8] and mouse cells[20] are transformed with oncogenic DNA-viruses. Similar changes are observed also in chemically malignized rat liver cells.[21] It now seems possible that these changes reflect alterations on the outer cell surfaces, at least in the BHK21 cells. This is an interesting possibility, since gangliosides are haptens,[22] and may possess other properties of ektobiological [23] importance as well.

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* Weinstein *et al.*[6] isolated 4.7 % of whole cell protein as the plasma membrane fraction of mouse fibroblasts (L-cells).