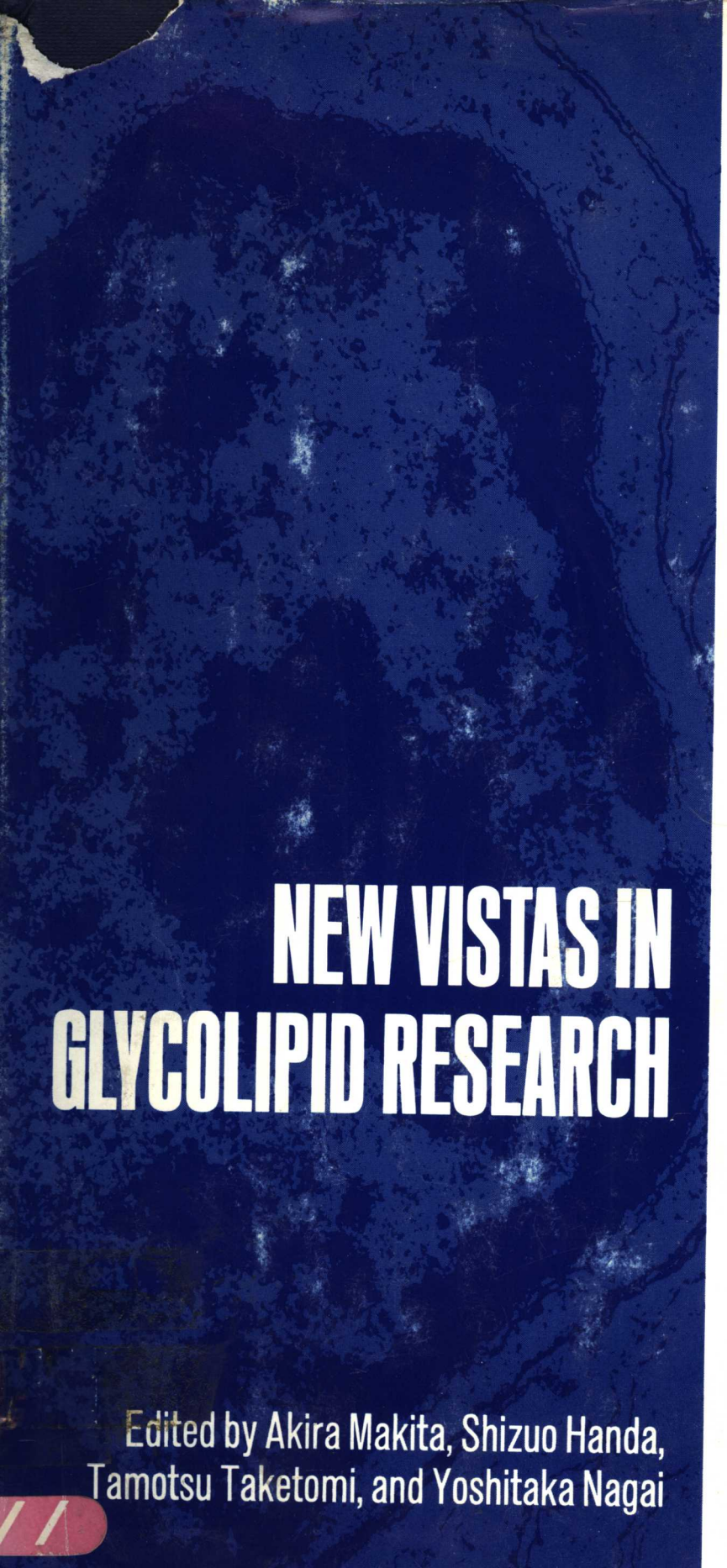

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NEW VISTAS IN GLYCOLIPID RESEARCH

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PREFACE

This book presents the proceedings of the "Biwako Symposium on Glycolipids" which was held in Otsu, Japan, September 17-19, 1981, just before the 6th International Symposium on Glycoconjugates in Tokyo. The meeting was convened by Dr. Taro Hori in commemoration of the discovery of hematoside and globoside by Dr. Tamio Yamakawa in 1951.

Glycolipid research beginning from the age of Thudichum, father of neurochemistry, developed rapidly in the third quarter of this century and is now extensive and interdisciplinary, having progressed to the isolation and chemical characterization of a great number of glycolipids, studies on catabolism and synthesis, biochemical elucidation of inherited sphingolipidoses, immunochemical characterization of various antigens, studies on membrane phenomena and cancer problems, and so on. These developments have been stimulated by both methodological advances (TLC, GLC, MS, NMR and cell cultures) and mutual interactions with many other fields of life science. Although glycolipids are relatively minor components of cells and tissues, and their functions depend essentially on their carbohydrate moiety like other glycoconjugates, they are unique in biochemistry and biophysics by way of their amphipatic properties.

The purpose of the meeting was to bring together scientists involved in a wide variety of aspects of glycolipid biochemistry, and to promote further research. The proceedings also includes the very fruitful discussions at the meeting as well as some papers which were not presented at the meeting but dedicated to Dr. T. Yamakawa on the 60th anniversary of his birth. Regretfully, we cannot communicate the pleasant atmosphere of the "get-together" on the same boat on Lake Biwako, and other informal events during the convention.

We would like to express our thanks to all participants at the meeting and contributors, especially Dr. Sen-itiroh Hakomori for communicating with the publisher.

The Editors

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GLYCOLIPIDS AND I: PAST, PRESENT AND FUTURE

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It was in 1950 that I found a peculiar sphingoglycolipid in a large amount of horse erythrocyte stroma (1). It gave a beautiful purple color when heated with Bial's orcinol reagent due to the presence of neuraminic acid. But I named it hematoside, because of some discrepancy with the reported properties of ganglioside (Fig. 1).

In those days, the situation was very poor both economically and as regards scientific conditions. I was only a beginner at biochemical research and did not have enough experience in the chemistry of glycolipids from natural sources.

I wrote a letter to Prof. Klenk of the University of Colonge and asked his opinion on my finding of hematoside. He was the main authority at the time and had already discovered ganglioside before the 2nd World War. A few months later, Prof. Klenk wrote a letter of reply and mentioned some doubt about the identity between hematoside and ganglioside. In the same letter, he also informed me that he had recently isolated a glycolipid from human erythrocytes which had galactosamine but no neuraminic acid (2).

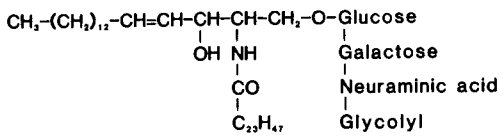


Fig. 1: Structure of hematoside as the major glycolipid of equine erythrocytes

I reexamined his work and separated a glycolipid from human erythrocytes which had the same chemical composition as the one he had given me in his letter (3). I named it Globoside, but the first discoverer of Globoside was Prof. Klenk. And he reconfirmed my work on hematoside immediately (4) (Fig. 2). This was the start of my research work on glycolipids.

In those days, the purification of glycolipids was carried out on the basis of the solubility difference of glycolipids in various organic solvents. After column chromatography using silicic acid as an adsorbent became available, glycolipids were purified more and more by this technique (5). More recently DEAE-Sepharose together with the application of thin-layer chromatography has been a powerful method of purification of individual glycolipids. For example, we now have excellent separation with the ganglioside mapping technique of Nagai and co-workers (6). Thus far, we have applied high performance liquid chromatography to separation of individual glycolipids from erythrocytes by detecting UV-absorption after a p-nitrobenzoyl group is introduced to the acylamide nitrogen of peracetylated glycolipids (7) (Fig. 3).

Recently, Otsuka in my laboratory applied DCC or droplet counter-current chromatography to the separation of glycolipids. The DCC apparatus was devised by Tanimura and himself and they have applied it to the separation of natural products including alkaloids, phenolics, peptides and saponins. The DCC apparatus is equipped with 50 to 500 thin glass columns, connected to each other by Teflon tubings in series and the separation is carried out by passing fine droplets of a mobile phase successively through the columns of the stationary liquid phase in an ascending or descending manner. Thus, in this system, no mechanical shaking is involved and there is no solid support which may reduce the recovery of polar substances by irreversible adsorption or chemical modification.

As much as 1 - 2 grams of crude glycolipid mixture can be applied to DCC. Several examples of separation profiles are presented here (8) (Fig. 4).

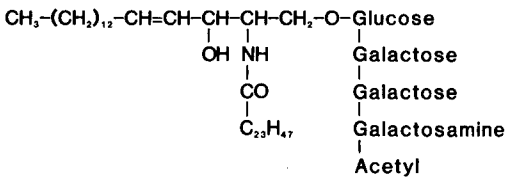


Fig. 2: Structure of Globoside as the major glycolipid of human erythrocytes

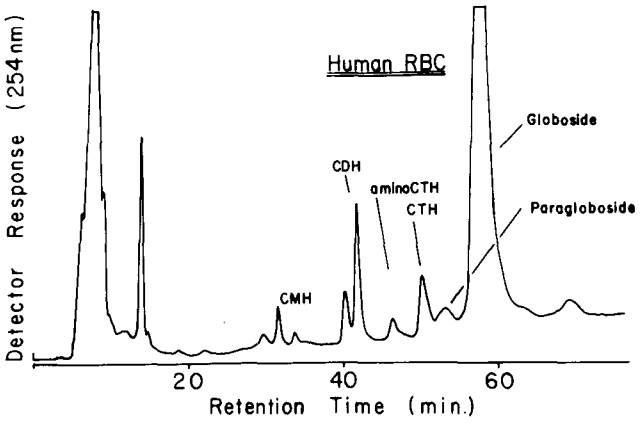


Fig. 3: The analysis of neutral glycolipid composition of human erythrocytes by high performance liquid chromatography using O-acetyl-N-*p*-nitrobenzoyl derivatives

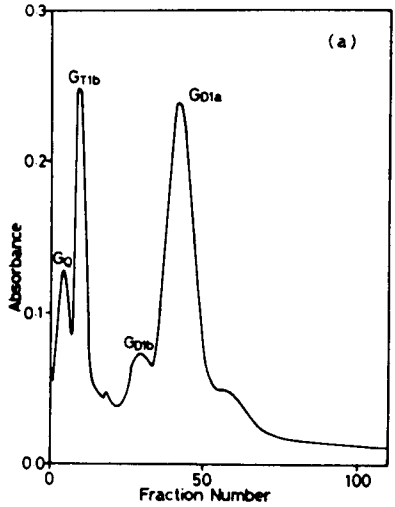
The efficiency of separation by DCC is not so high, but it is a useful technique for the preparation of rather large amounts of glycolipids, and I think it can be applied to a variety of glycolipids by improving the selection of solvents and modification of the mixing proportions.

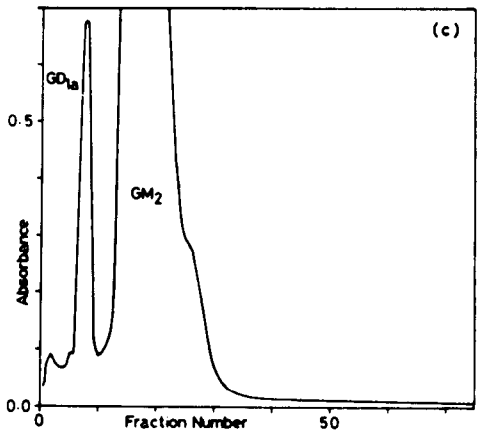
In 1953, we noticed the presence of ABO blood group activity in the crude globoside preparation, it was the first evidence of biological activity of glycolipid (9).

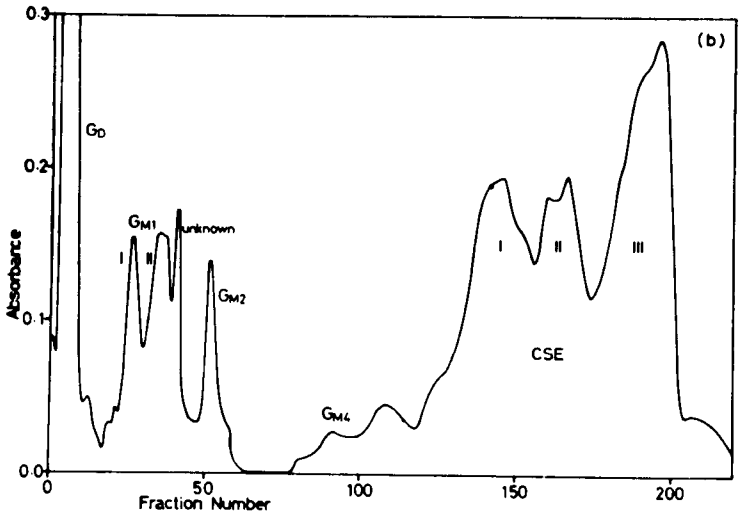
Application of column chromatography made it possible to separate the active portion from the major Globoside I. The activity was further divided into two fractions: Globoside II and Globoside III (10) (Fig. 5). Further purification of these active glycolipids was carried out and their structures were proposed by Hakomori and by Hanfland.

In those days, chemical structure of blood group antigens was investigated by using materials from mucous secretions and the carbohydrate structures of the antigenic determinants were elucidated. You will see that the carbohydrate determinant groups attached to the ceramide moiety are of the same chemical structure as the blood group active glycoproteins isolated from body fluids or secretions which are attached to peptide by O-glycosidic linkages (Fig. 6).

Since then, I have had the idea that the antigenic materials at the surface of erythrocytes are glycolipid in nature and the blood group active material in the secretion is glycoprotein in


(a)
G_{T1b}
G_{D1a}
G_D
G_{D1b}
Absorbance
Fraction Number


(c)
GD_{1a}
GM_2
Absorbance
Fraction Number


(b)
G_D
G_{M1}
unknown
G_{M2}
G_{M4}
I
II
III
CSE
Absorbance
Fraction Number

nature. Our findings were not accepted immediately, but now everybody accepts the existence of ABO active glycolipid.

However, in recent years, ABO blood group activity has still been reported to be present also in glycoprotein from human erythrocyte membrane (11). Several other people isolated a highly polymerized glycolipid called megaloglycolipid or macroglycolipid from human erythrocytes and mentioned that the major activity resides in such high molecular glycolipids (12).

The separation of water-soluble high molecular glycolipids from glycoprotein is difficult and the activity detected in the glycoprotein fraction might be due to contamination by such a high molecular glycolipid.

I think it is not so simple to determine which is the real active group material, because the procedure to separate and purify such a high molecular substance is rather complicated as compared to the methods used for relatively low molecular glycolipids such as ceramide hexa- or octa-saccharide.

A recent examination by Handa, my co-worker, showed the distribution of ABO blood group activity in human erythrocytes (Table 1). He showed that the activity is found mostly in the low molecular glycolipid and only 1% of the activity was found in the megaloglycolipid and no activity was detected in the glycoprotein fraction.

Several workers in Finland stated that the ABO activity was found in the glycoprotein fraction called poly(glycosyl)peptide of erythrocytes (13). However, their assay method for blood group activity was not the hemagglutination inhibition test using isoantibody, they used the binding activity with a lectin, *Bandeiraea simplicifolia*, as a measure of blood group activity. My opinion

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Fig. 4: Separation of glycolipids by droplet counter-current chromatography (DCC). (a) Mouse brain gangliosides (75 mg of crude glycolipids prepared by acetone precipitation and an alkaline treatment) were separated with 500 columns using solvent system of chloroform - methanol - 0.5% NaCl in water - *n*-propanol (50:60:40:6, by volume). (b) Crude glycolipids (150 mg) from whale brain were separated by using chloroform - methanol - water - *n*-propanol (50:60:40:2). G_{M1} was separated into two peaks and CSE into three. (c) Separation of crude glycolipid obtained from the brain of a patient with Tay Sachs' disease. Crude mixture (700 mg) was separated with chloroform - methanol - water - *n*-propanol (50:60:40:5, by vol.) Sugar was determined by phenol-sulfuric acid method.

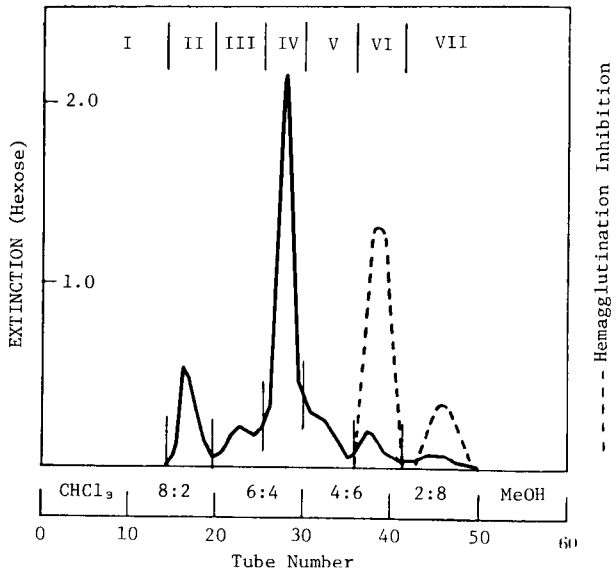

Fig. 5: Silicic acid chromatography of the glycolipids obtained from human erythrocytes with blood group A. Fraction IV is Globoside I and the activity of hemagglutination inhibition was detected with Globoside II in fraction VI and Globoside III in fraction VII. For the inhibition assay anti-A isoagglutinin and blood group A erythrocytes were used.

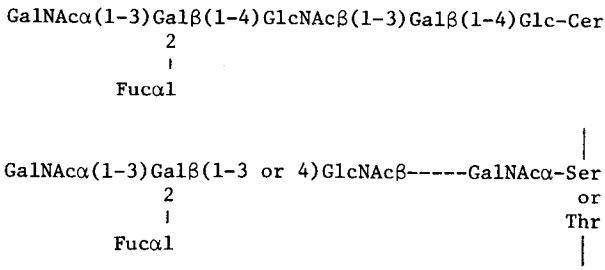

Fig. 6: The carbohydrate structure of a glycolipid and glycoprotein with blood group A activity.

Table 1. Distribution of blood group A activity in the various fractions of human erythrocytes

Fractions	Yield mg/1 liter RBC A	Minimum Amount to Inhibit Agglutination µg B	Total Active Amount A/B
Low-molecular glycolipid			
C-M-W Extract	510	25	26,100
BuOH Extract	8.6	1.5	
Megaloglycolipid	5.3	20	265
Glycoprotein	38.5	>100	-

is that the binding activity with lectin has broader specificity than the immunological antigen-antibody reaction and the 'group activity' revealed by such a poly(glycosyl)peptide does not represent the true antigenic activity of the ABO blood group.

Now, I will mention our recent experience with the molecular difference of erythrocyte glycolipids. As to the hematoside-type of glycolipids, that of horse has 1 mole of N-glycolylneuraminic acid, and that of cat 2 moles of N-glycolylneuraminic acid. Previously, we reported that dog red cell glycolipid consisted of 27% N-glycolylneuraminic acid and 73% N-acetylneuraminic acid (14). On the other hand, when Klenk and Heuer proposed the structure of dog erythrocyte glycolipid in 1960, they had found that the neuraminic acid was exclusively N-acetylated (15). This apparent discrepancy had us puzzled for a long time. Recently, reexamination of this problem revealed that red cell glycolipids from some dogs have N-glycolylneuraminic acid, while others have N-acetylneuraminic acid; no mixed type exists (16).

As our previous study was carried out with pooled blood from several dogs, so the sialic acids found were not structurally uniform.

The dogs examined in these studies were mongrels, we tried to determine which breeds of dog have the N-acetyl and which the N-glycolyl type of hematoside. It was revealed that in the blood of all the European dog breeds examined N-acetylneuraminic acid existed but no hematoside of the N-glycolyl type (Table 2).

On further investigation, we found N-glycolylhematoside or the G-type in some oriental dogs. They are Shiba-dog, Kai-dog,

Table 2. Type of hematoside in dog erythrocytes (European dogs)

Breeds	NeuAc	NeuGc
Afganhound	4	0
Am. cocker spaniel	2	0
Beagle	30	0
Bulldog	1	0
Cairn terrier	2	0
Chihuahua	2	0
Collie	1	0
Dachshund	1	0
Dalmatian	1	0
Fox terrier	1	0
French poodle	1	0
Great dane	1	0
Laika	1	0
Maltese	26	0
Miniature poodle	1	0
Pomeranian	9	0
Poodle	10	0
Setter	3	0
Shephard	6	0
Shetland sheep dog	7	0
Siberian husky	5	0
St. Bernard	2	0
Yorkshire terrier	21	0
Westhighland	1	0
Total	139	0

Kishu-dog, Chin and Pekinese. However, Akita-dogs and Hokkaido-dogs were found to have N-acetylhematoside or the A-type, even though they also live in Japan. Thus, we found that there are two types of dogs in Japan, namely the A-type in northern districts and the G-type in southern districts (Table 3). Shiba-dogs are rather small in size and generally up to 9 kg in weight. The population of Shiba-dogs is decreasing in Japan, so the Society of Preservation of Shiba-dogs has been established to preserve this species as a pure breed.