Investigations on Cellular Blood-Group Substances. I. Isolation and Chemical Composition of Blood-Group ABH and Le^b Isoantigens of Sphingoglycolipid Nature*

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ABSTRACT: Blood-group A and B isoantigens of sphingoglycolipid nature with a single carbohydrate chain have been isolated from human erythrocytes. Three polymorphic types of A-active glycolipids from A_1 cells, two types of A-active glycolipids from A_2 cells, and two types of B-active glycolipids from B cells have been separated. One of the A-active polymorphic types found in A_1 cells was not found in A_2 cells. One glycolipid fraction isolated from O cells was found to be H active as well as Le^b active. None of the Le^a-active glycolipid was found in normal human erythrocytes. All of the glycolipids that carry ABH and Le^b specificities were found to have a high content of fucose. Ceramide pentasaccharide, which consists of 1 mole each of glucose, glucosamine, and fucose and 2 moles of galactose, constitutes the fundamental common skeleton of blood-group ABH and Lewis glycolipids. The fatty acid compositions of these glycolipids are almost identical irrespective of blood type. Thus, the carbohydrate composition of the blood-group glycolipids is very similar to that of a Le^a-active, fucose-containing glycolipid ("tumor glycolipid") accumulating in human adenocarcinoma (Hakomori, S., and Jeanloz, R. W. (1964), *J. Biol. Chem. 239*, pc 3606; Hakomori, S., Koscielak, J., Bloch, K. J., and Jeanloz, R. W. (1967), *J. Immunol. 98*, 31).

Our knowledge about the chemistry of the cellular blood-group ABH isoantigen is very limited, although the chemistry of the ABH isoantigen found in mucous secretions and in glandular tissue has been greatly advanced by the investigations of Morgan and Watkins, and Kabat and his associates (see Watkins, 1966). The work carried out by Yamakawa and Iida (1953), Yamakawa *et al.* (1958, 1960, 1965), Hakomori (1954), Radin (1957), Hakomori and Jeanloz (1961), Handa (1963), and Koscielak (1962, 1963) has established that the cellular blood-group A and B isoantigens of erythrocytes and tissues are glycolipid in nature. Nevertheless, because of the difficulty in obtaining homogeneous material and the extremely low yield of the material obtained from erythrocytes, the chemical composition of the isoantigens has not been settled, and the structure of the antigens is far from being known.

Preparations described by Handa (1963) and also by Koscielak (1963) were characterized by their high content of sialic acid (5–10%) and low content of fucose (1–2%). The blood-group A glycolipid recently described by Yamakawa *et al.* (1965) contained a relatively high content of fucose in addition to glucose, galactose, glucosamine, galactosamine, and sialic acid. The content of fucose is about one-half that of glucose. In either of these studies, attempts to isolate a H-active substance were unsuccessful although the cells were agglutinable by anti-H serum or anti-H lectin. No attempt has been made to elucidate the chemical nature of the Lewis blood-group substances of erythrocytes.

On the other hand, a Le^a-active sphingoglycolipid with the structure: β-galactosyl-(1→3)-[α-fucosyl-(1→4)]-2-acetamido-2-deoxyglucosyl-(1→3)-galactosyl-(1→4)-glucosyl-(1→1)-ceramide was isolated in relatively high yields from different samples of human adenocarcinoma (Hakomori and Jeanloz, 1964, 1965; Hakomori *et al.*, 1967; Hakomori, 1967). It is not known whether or not such a glycolipid is present in even a small amount in normal cells, and represents a "Lewis hapten" of normal cells.

In view of the inconsistent data concerning the chemical composition of the blood-group A and B haptens hitherto isolated from erythrocytes and of an ambiguous chemical nature of the H and Lewis blood-group haptens of erythrocytes, a thorough fractionation and extensive purification of blood-group ABH and Lewis haptens of erythrocytes have been carried out. The results of this study establish that these haptens are fucose-containing sphingoglycolipids whose chemical composition is very similar to that of a Le^a-active sphingoglycolipid previously isolated from human adenocarcinoma.

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Materials and Methods

Preparation of a Crude Blood-Group Glycolipid (Fraction I and Fraction II). A large quantity of sedimented erythrocytes has been obtained regularly through the courtesy of Dr. Robert B. Pennell, Blood Research Institute, and Mr. Lewis H. Larsen of the Massachusetts Department of Public Health, Institute of Laboratories, Boston, Mass. The blood type was confirmed by us and the cells were lysed at about 5° in tap water containing 0.2% acetic acid. After standing overnight, cell ghosts were collected with a Sharples continuous centrifuge. The packed ghosts were homogenized with ten volumes of 95% ethanol, in a Waring Blendor, allowed to stand at room temperature for a day, and filtered with suction. The residue was extracted with five volumes of 90% ethanol for another day and filtered again with suction. Filtrates were combined and the "Protagon" was precipitated at −10° according to Koscielak (1963). After the precipitate had settled the supernatant fluid was removed by aspiration and the precipitate was collected by centrifugation at −10°. The precipitate was washed twice with ten volumes of acetone and centrifuged at room temperature. The bulky precipitate was air dried and then extracted with diethyl ether by stirring at room temperature followed by centrifugation. The precipitate, representing a sphingoglycolipid, was dried *in vacuo*. This fraction (1–2 g) was dissolved in about 30 ml of chloroform–methanol–pyridine (9:1:1, v/v), some insoluble material (proteolipid) was removed by filtration, and the filtrate put onto a column of silicic acid (3 × 40 cm, Biosil A, Bio-Rad Laboratories, Richmond, Calif.) which was prepared from a suspension in chloroform–methanol (9:1). Elution was carried out with 400 ml each of the following mixtures of chloroform–methanol: 9:1, 8:2, 7:3, 6:4, and 4:6, respectively. Aliquots of 20 ml were taken by fraction collector and a few drops of each fraction were analyzed by thin-layer chromatography on silica gel H (Brinkmann Instruments, Inc., Westbury, L. I., N. Y.) plates (0.3 mm thickness; activated at 110° for 1 hr) developed with chloroform–methanol–water (65:30:8, lower layer). Under these conditions, ceramide lactoside, ceramide trihexoside (galactosylgalactosylglucosylceramide), and globoside (2-acetamido-2-deoxygalactosyl-(1→3)-galactosyl-(1→3)-galactosyl-(1→4)-glucosylceramide) were eluted by chloroform–methanol ratios of 9:1, 8:2, and 7:3, respectively. None of the blood-group-active lipid was eluted by chloroform–methanol over this concentration range.

After the globoside had been eluted, two minor fractions were subsequently eluted by chloroform–methanol 6:4 and 4:6, respectively (designated fraction I and fraction II). These may correspond to "globoside II" and "globoside III" of Yamakawa *et al.* (1965). Both of these fractions were active in the inhibition of a specific hemagglutination, but the components present in the two fractions were completely different on thin-layer chromatography. Fraction I contained at least four components which were distinguishable on thin-layer chromatography, one of which was blood-group active. This active fraction was further separated into polymorphic forms after acetylation.

Fraction II consisted of slowly migrating components; it was extremely difficult to attain further purification. The characterization of the active components in this fraction is, therefore, not described in this article.

Separation of an Apparently Homogeneous Blood-Group Glycolipid (Fraction I-1). Fraction I was separated into four bands by thin-layer chromatography on silica gel H plates (0.3 mm thickness; activated at 110° for 1 hr), developed twice with chloroform–methanol–water (65:30:8, lower phase, solvent 1). The bands were revealed by exposure of the plates to a 3-mm channel of iodine vapor contained in a plastic container. The bands were extracted with chloroform–methanol–water (1:1:0.1). The blood-group-active material, irrespective of the blood type of the starting material, was always found in the second slowest band (about 2 cm from the origin). This fraction was further separated into two fractions by thin-layer chromatography on silica gel H impregnated with sodium tetraborate. The plates were prepared by a modification of the method of Kean (1966) and developed three times with solvent 1. Silica gel H (30 g) was mixed with 75 ml of 1% sodium tetraborate to yield a slurry of pH 7.2, and six 0.3-mm thin-layer plates (20 × 20 cm) were prepared. The plates were dried at room temperature and used without subsequent activation. The first (slowest) band (fraction I-1) was found to be blood group active irrespective of whether the blood group of starting cells was A or B. The second band (fraction I-2) was characterized as *N*-acetylneuraminyllactosylceramide (hematoside of Yamakawa and Suzuki, 1951; Makita *et al.*, 1966; Svennerholm, 1963). Fraction I-1 was completely devoid of sialic acid, greatly enriched in fucose, and gave a single spot by thin-layer chromatography on silica gel H and silica gel G, each developed with solvent 1 and propanol–water (7:3). However, this fraction still possessed a galactosamine-containing contaminant. The heterogeneity of this fraction was demonstrated by means of paper chromatography of the oligosaccharides liberated from the glycolipid by osmium-catalyzed periodate oxidation followed by alkaline degradation (Hakomori, 1966). The same glycolipid fraction (corresponding to fraction I-1) obtained from blood type O cells was found to be only very weakly H active, and the fucose content was very low (1%). The major glycolipid of this fraction of O cells is a blood-group-inactive glycolipid containing glucose, galactose, glucosamine, and galactosamine.

Isolation of Active Glycolipids with a Single Carbohydrate Chain and the Procedure for Distinguishing Polymorphic Components. Blood-group-active glycolipids were isolated in the form of their acetylated derivatives by means of thin-layer chromatography, using less polar solvents than those used for separating free glycolipids. Fraction I-1 was acetylated overnight in pyridine–acetic anhydride (3:2) and the reaction mixture was evaporated in the presence of toluene. The residue was separated into three to four bands by

means of thin-layer chromatography on silica gel H plates and developed with chloroform–methanol–water (90:10:1) or with 1,2-dichloroethane–methanol (95:5). Four fractions were separated by either of these solvents from fraction I-1 of A_1 cells; *i.e.*, the fast-moving band (component I, R_F value 0.75–0.8), the second band (component II, R_F value 0.60–0.65), the third (R_F 0.35), and the fourth band (R_F 0.2). Each fraction was eluted with the chloroform–methanol mixture and evaporated. The dried residues were saponified with 5% methanolic ammonia at 4° for 18 hr. Component I and component II were highly A active, while the third and the fourth components were completely inactive. Thus, two A-active fractions were separated from fraction I-1 of A_1 cells. From A_1 cells, therefore, three active fractions were separated which are chemically and chromatographically distinguishable; two of them were isolated from fraction I-1 in the pure state and one was found in fraction II. The last component (component III) has not been purified as yet. The same fractionation procedure was applied for separating B- and H-active glycolipids. The pattern of polymorphism is different in B and O cells (see under Results). Component I and component II, isolated either from A or B cells, were homogeneous on thin-layer chromatography and gave homogeneous oligosaccharides by splitting the carbohydrate–lipid bond.

Method of Analysis. The homogeneity of the glycolipid was examined by thin-layer chromatography on silica gel H and silica gel H impregnated with sodium tetraborate (Kean, 1966). The following solvents were used: (1) chloroform–methanol–water (65:30:8, lower phase; solvent 1), (2) chloroform–methanol–water (60:35:8), and (3) propanol–water (7:3). Development of the chromatograms three times with intermediate drying, using solvent 1 and borate-impregnated silica gel H without activation, was found to be most effective for separation of blood-group A and B substances. Spots were detected by the iodine vapor and orcinol–sulfuric acid methods. Oligosaccharides were released from the acetylated sphingoglycolopid by osmium-catalyzed periodate oxidation followed by alkaline treatment (Hakomori, 1966). Sugar components were determined qualitatively by paper chromatography and by thin-layer chromatography. Hexoses and methylpentoses were analyzed by means of the cysteine–sulfuric acid method (Dische, 1955), and hexosamines were determined by the modified Elson–Morgan reaction (Rondle and Morgan, 1955). Sialic acid was determined by the Warren method (Warren, 1959) and through the use of Bial's reagent (Werner and Odin, 1952). The molar ratio of fucose:galactose:glucose:hexosamine was determined by gas chromatography according to the method of Sweeley and Walker (1964) ("procedure B") using an F & M Model 402. Separation was carried out in a 6-ft SE-52 glass column. The ratio of hexoses to hexosamine was calibrated according to the results of analysis of a pure globoside, in which the ratio of glucose:galactose:galactosamine is known as 1:2:1. The ratio of glucosamine to galactosamine was determined by paper chromatography according to the method of Fischer and Nebel (1955). Following methanolysis, the composition of the fatty acids was determined by gas chromatography employing a SE-52 column. Presence or absence of α-hydroxy fatty acid was determined by thin-layer chromatography on silica gel G, developed with methylene chloride (Mårtensson, 1966). Sphingosine bases were qualitatively determined by thin-layer chromatography after methanolysis (Sambasivarao and McCluer, 1963). Blood-group activities of glycolipids were measured by inhibition of specific hemagglutination with or without the addition of "carrier" or "auxiliary" lipid (Koscielak, 1963; Rapport *et al.*, 1959). The substance to be tested was dissolved in chloroform–methanol (1:1) and was added with five times its weight of a mixture of lecithin–ceramide dihexoside (1:1). The solvents were evaporated under nitrogen, a suitable amount of saline was added, and the tubes were vigorously agitated with a Vortex mixer and heated at 60° for 10 min. Specific anti-A and anti-B antisera, anti-H lectin (*Ulex europeus* and *Lotus tetragonolobus*), and anti-Le^a and anti-Le^b sera were purchased from the Blood Grouping Laboratory, Boston. The agglutination by anti-H lectin was determined by centrifugation 10 min after the addition of cells; the agglutination by anti-A or anti-B sera was determined by the conventional test-tube method and also by means of a micromethod with a Microtiter apparatus. Agglutination by anti-Le^a or anti-Le^b antiserum and its inhibition were carried out using ficin-treated erythrocytes. The potency of the anti-A and anti-B sera used was 4 hemagglutination units/0.2-ml sample. The potency of anti-H lectin as well as anti-Le^a and anti-Le^b antisera was 3 hemagglutination units/0.2-ml sample. The immunological specificity of the highly purified A, B, and H glycolipids was further tested by use of the respective precipitating antisera. Anti-A and anti-B precipitating human sera were kindly donated by Dr. G. F. Springer, Evanston Hospital, Northwestern University; anti-Le^a precipitating goat antiserum was donated by Dr. Donald Marcus, Albert Einstein College of Medicine. A part of the anti-A human sera was kindly donated by Dr. Lloyd of Professor Kabat's laboratory, Columbia University. γ-Globulin was precipitated from the later serum by ammonium sulfate and the precipitate was dissolved in saline and dialyzed against saline.

Results

Separation of Blood-Group A Glycolipids and Their Polymorphism. Three fractions with blood-group A specificity have been separated. Two of them (components I and II) were separated from fraction I-1 as acetylated compounds by thin-layer chromatography; one of them (component III) was in fraction II. Both components I and II showed equally high activities and 0.4 μg of these preparations could inhibit the A hemagglutination, evoked by 0.2 ml of anti-A serum which contain 4 hemagglutination units (Table I). This is the same degree of activity as is usually demonstrated by A-active glycoproteins in mucous secretion.

TABLE I: Chromatographic Distinction of Polymorphic Types of Blood-Group-Active Glycolipids and Their Blood-Group Activities.

	Component	Migration Rates on Thin-Layer Chromatography		Blood-Group Activities[a]				
		R_F Value[b]	$R_{globoside}$ Value[c]	A	B	H	Le[a]	Le[b]
A-active glycolipid of A_1 cells	I	0.75–0.8	0.6	0.4+	>100−	>100	>100−	>100
	II	0.60–0.65	0.6	0.2+	>100−			
	III	0.4–0.5	0.2	12+	>100−			
(Component III is lacking in A_2 cells)								
B-active glycolipid	I	0.75–0.8	0.4	>100−	0.4+	>100	>100−	>100
	III	0.4–0.5	0.15	>100−	25+	>100		
(Component which corresponds to component II of A_1 cells is lacking in B cells)								
H- and Le[b]-active glycolipids of O cells	I	0.8–0.85	0.7	>100−	>100−	12	>100−	6+
Le[a]-active glycolipid of human cancer		0.8–0.85	0.75	100−	100−	12	6+	25

[a] Numbers are the minimum amount (in micrograms) of glycolipid which inhibit specific hemagglutination, evoked by 4 hemagglutinating units of anti-A and anti-B antisera. Three hemagglutinating units of anti-Le[a] or anti-Le[b] antisera and of anti-H *U. europeus* lectin per 0.2 ml. Sign + or − after the numbers indicates the positive or negative precipitin reaction tested with respective precipitating antisera; no sign indicates no experience of test. [b] On silica gel H as acetylated compound. On activated plate, developed with chloroform–methanol–water (9:1:0.1). [c] On borate-impregnated silica gel H as free glycolipid. No activated plate, developed three times with chloroform–methanol–water (60:35:8); $R_{globoside}$ value means migration rate under this condition as compared with that of globoside of human erythrocytes (migration distance of substance/migration distance of globoside).

The ratios of the carbohydrate components as determined by gas–liquid chromatography and paper chromatography are shown in Table II. The results suggest that component I contained 2 moles of galactose, while component II contained 3 moles of galactose. In addition, each component contained 1 mole of fucose, glucose, glucosamine, and galactosamine. Component I was therefore deemed to be a ceramide hexasaccharide and component II could be a ceramide heptasaccharide. Both components were clearly separated as acetylated compounds on thin-layer chromatography, while as free glycolipids both migrated together and were not separable by thin-layer chromatography. Component III, which was found in fraction II, showed much weaker activity (12 μg/0.2 ml) and contained sialic acid. This fraction was heterogeneous, consisting of components with close R_F values, and acetylation did not improve separation. Further purification to isolate an active component has not been attained at the present time. Although this fraction has not been purified, it gave at least three oligosaccharides by osmium-catalyzed periodate oxidation and alkaline degradation. None of these corresponded with the oligosaccharides of component I and component II.

A-Active Glycolipids from A_2 Cells. Applying the same procedure as used for separating A-active glycolipids from A_1 cells, two active components were isolated from fraction I-1 of A_2 cells. One major component (component I) had the same R_F value as component I isolated from A_1 cells and the other component (component I) was present only in trace amounts but had the same R_F value as component II from A_1 cells. A_2 cells contain, therefore, only one major active glycolipid (component I), a trace of component II, and none of component III. Chemistry of the active component of A_2 cells was not carried out because of the extremely limited quantity of the material.

Separation, of B-Active Glycolipids, Their Carbohydrate Composition, and Polymorphism. Two fractions with blood-group B activity have been isolated. One component (component I) was isolated from fraction I-1 which had the same R_F value as component I of A_1 or A_2 cells. Another component (component II) was present in fraction II of B cells. Component I is highly active as compared to the B-active glycoproteins of mucous secretion (limit of inhibition, 0.4 μg/0.2 ml), while fraction II was very weakly active (limit of inhibition, 25 μg/0.2 ml). Component I, as isolated after acetylation, did not contain galactosamine. The ratios of the component sugars found for galactose:glucose:fucose:glucosamine was about 3:1:1:1, indicating a ceramide hexasaccharide. The absence of galactosamine in this highly B-active glycolipid is of significance. Fraction I-1 of B cells did contain,

TABLE II: Chemical Composition of Blood-Group Glycolipids.

	Chemical Composition in Wt %				Molar Ratio of Carbohydrates[d]					
	Total Fatty Moiety Sphingosine and Fatty Acids[a]	Hexose[b]	Fucose[b]	Hexosamine[c]	Glu	Gal	Fuc	$GlNH_2$	Gal-NH_2	Sial
A_1 glycolipid										
Component I	38.5	30.2	9.5	15.2	1	2.1	1.1	1.0	1.2	0
Component II	40.5	35.2	9.2	15.5	1	3.3	0.9	1.0	1.1	0
Component III					1	3.5	0.5	1.5	1.1	1.5
B glycolipid										
Component I	38.0	38.5	10.1	9.5	1	3.2	1	1	0	0
Component III					1	4.2	0.5	1.5	0.5	1.3
H and Le^b glycolipids	40.5	32.5	12.1	10.5	1	2.2	1.5	1	0	0
Le^a-active glycolipid of human cancer	42.5	35.3	10.1	9.5	1	2.1	1	1.1	0	0

[a] Weight of the fatty material liberated on acid hydrolysis. [b] Cysteine–sulfuric acid method of Dische (1955). [c] The method of Rondle and Morgan (1955). [d] Gas chromatography (Sweeley and Walker, 1964) for the ratios of neutral sugars and the ratios of neutral sugars, total amino sugars, and sialic acid. Paper chromatography (Fischer and Nebel, 1955) for the ratio of glucosamine ($GlNH_2$) and galactosamine ($GalNH_2$). Sialic acid by the Warren (1959) method.

however, varying amounts of galactosamine which was due to the presence of an inactive glycolipid-containing galactosamine.

Separation of H- and Le^b-Active Glycolipids from O Cells. Fraction I-1 of O cells was prepared and was further separated, after being acetylated, into three bands (R_F 0.85, 0.6, and 0.4) by thin-layer chromatography. Only the fraction with R_F 0.85 showed H as well as Le^b activity. The substance showed a weak, but definitive, inhibition of H hemagglutination evoked by *U. europeus* agglutinin and showed a positive precipitin reaction with anti-H human goat serum (the reaction was performed by Dr. Donald Marcus of Albert Einstein College of Medicine). The same fraction also showed a high inhibition potency of Le^b hemagglutination evoked by anti-human Le^b agglutinin as well as precipitation with Marcus' anti-Le^b goat serum (Marcus and Grollman, 1966). No activity was demonstrated on fraction II of O erythrocytes. None of these fractions showed inhibition of Le^a hemagglutination evoked by anti-Le^a human serum nor precipitate formation with anti-Le^a goat serum of Marcus and Grollman. The active fraction had glucose, galactose, fucose, and glucosamine in the molar ratios of 1:2:1.5:1. Neither galactosamine nor sialic acid was demonstrated in this substance.

Composition of Fatty Moiety. Sphingosine and dihydrosphine were detected in all of these glycolipids; scarcity of the material did not permit analysis of the presence of C_{20}-sphingosine.

The fatty acid compositions of different blood-group glycolipids are shown in Table III. Nearly identical patterns in the composition of fatty acids were observed irrespective of blood type. Scarcity of the material did not permit analysis of the α-hydroxy fatty acids; however, qualitative analysis by means of thin-layer chromatography of the fatty acid fractions indicated that no detectable amount of α-hydroxy fatty acids was present in blood-group glycolipids (Mårtensson, 1966).

Discussion

The results of these studies indicate that all of the glycolipids of erythrocytes carrying blood-group ABH and Lewis specificities are characterized by a high content of fucose. Evaluation of molar ratios of carbohydrates in glycolipids by the conventional test-tube analysis is sometimes difficult. Lower values of hexosamine (Elson–Morgan) and higher values of hexose and methylpentose (cysteine–sulfuric acid) as compared with the results of gas chromatographic analysis were observed (Table II). The molar ratio of the component sugars in some of the purified glycolipids yielded integral numbers basing on the results of gas chromatography and paper chromatography, and the oligosaccha-

TABLE III: Fatty Acid Compositions of Blood-Group Glycolipids (component I of A- and B-active glycolipid).[a]

	A Glycolipid	B Glycolipid	H- and Le^b-Active Glycolipid
C_{14}	2.8	2.8	2.5
C_{14}=	7.5	4.5	5.2
C_{16}	3.8	5.1	4.6
C_{16}=	4.4	3.9	4.2
C_{18}	1.2	1.2	1.5
C_{18}=	2.2	1.4	2.0
C_{20}?[b]	11.4	11.6	9.8
C_{21}	4.2	2.2	2.0
C_{22}	9.2	12.8	10.5
C_{22}=[c]	16.2	16.7	14.5
C_{24}	19.3	19.5	20.5
C_{24}=[c]	17.9	18.1	16.1

[a] Numbers indicate percentage of individual fatty acids to the total fatty acids. [b] Do not correspond exactly to C_{20}, unidentified. [c] No control run with standard substance. The = marking indicates an unsaturated acid.

rides liberated from them showed a single band on paper chromatography. The identities of the essential carbohydrate components of the blood-group ABH glycolipid have not been elucidated at the present time. It has become apparent, however, from this investigation that fucose, galactose, glucose, and glucosamine are common components, constituting an essential common structure of the ABH and Lewis glycolipids. Sialic acid and galactosamine are not essential components of blood-group B or H glycolipids, since these two components are completely absent in the highly active component of B or H glycolipids. Because of the extreme scarcity of the pure materials, the exact structure of ABH glycolipids is far from known at the present time. For this purpose, a much larger scale approach, in particular, a larger supply of erythrocytes, is required. Such an approach is currently being pursued.

From the results of the carbohydrate composition of purified haptens, it is clear that the chemical composition of the H- and Le^b-active glycolipid is similar to that of the Le^a-active glycolipid of human adenocarcinoma. The carbohydrate composition of the B-active glycolipid is also similar to that of the tumor glycolipid except that the former contains one additional mole of galactose. Similarly, the carbohydrate composition of one of the A-active glycolipid (component I) is identical with that of the tumor glycolipid except that component I contains 1 additional mole of galactosamine.

Separation of glycolipids with different blood-group activities was hardly possible by thin-layer chromatography on regular silica gel plate; separation between A and B glycolipid was possible on borate-impregnated silica gel H plate by three times development with solvent 1. $R_{globoside}$ values (see footnote *c* of Table I) of A and B glycolipids were 0.6 and 0.4, respectively; thus the presence of both A and B glycolipids in the blood-group AB erythrocytes was demonstrated when fraction I-1 of AB erythrocytes was analyzed. H- and Le^b-active glycolipid of O erythrocytes and tumor glycolipid had almost the same migration rate ($R_{globoside}$ 0.7 and 0.75), while tumor glycolipid and A and B glycolipid were easily distinguishable (see Table I).

Analogous to the structure of the active carbohydrate chain, established in ABH blood-group substances of glycoprotein nature, the structural feature which determines the ABH specificities of glycolipids is the terminal α-galactosyl residue in the B-active chain and the α-*N*-acetylgalactosaminyl residue in the A-active chain; the fucosyl residue (probably linked at the subterminal galactose) is also essential for the maintenance of high blood-group activity in blood-group glycolipids. It is possible that the immunospecific conformation of the determinant structure is stabilized by the presence of the fucosyl residue as suggested by Lloyd *et al.* (1966) for the glycoprotein blood-group substances. Removal of the fucosyl residue from blood-group A and B glycolipids by heating in 1 M acetic acid at 100° for 2 hr caused a marked decrease in the activities, but did not abolish them.

From a genetical point of view, it is interesting to note that three polymorphic types of glycolipid carrying A specificity are present in the glycolipid fraction of A_1 cells. One of them (component III) is lacking in A_2 cells, whereas two types corresponding to components I and III are present in B glycolipids. Since these results were obtained from pooled blood samples, it would be desirable to study the glycolipids in the blood of individuals subjects, although this may be very difficult because of the extremely low yield of the active glycolipids. Yamakawa *et al.* (1958) have noticed that two blood-group A active peaks were invariably observed on silica gel chromatography of glycolipids of A erythrocytes. They differ both in carbohydrate composition and rates of migration on thin-layer chromatography (Yamakawa *et al.*, 1965).

As pointed out by Koscielak (1963) as well as by Handa (1963), it is an enigma that no H-active glycolipid or H-active glycoprotein has been detected in O erythrocytes, although they are agglutinable by anti-H lectin or serum. This may simply be owing to the extremely low quantity of the H-active glycolipid present in erythrocytes as compared to the quantities of A or B glycolipid.

A remarkably high Le^b activity was detected for the H-active glycolipid fraction on the basis of inhibition of hemagglutination as well as by immune precipitation. This glycolipid fraction inhibits H hemagglutination evoked by *U. europeus* phytoagglutinin and gave a precipitating reaction with anti-human H serum, but it did not precipitate with the anti-hog H serum, recently produced in goat (Marcus and Cass, 1967). The results of these precipitin experiments with the new precipitating anti-H antisera, all of which were

kindly carried out by Dr. Donald M. Marcus of Albert Einstein College of Medicine, New York, indicate that the glycolipid may have a structure: α-L-fucosyl-(1→2)-β-galactosyl-(1→4)-*N*-acetylglucosaminyl, since Marcus' anti-H serum has an unusually narrow specificity and is only reactive to the structure α-L-fucosyl-(1→2)-β-galactosyl-(1→3)-*N*-acetylglucosaminyl. This fraction also gave a precipitin reaction with anti-Le^b-precipitating antiserum produced in goat (Marcus and Grollman, 1966). Plausibly this glycolipid is still a mixture of H-active and Le^b-active glycolipid, since the ratios of the component sugars are not integral numbers although it is homogeneous on thin-layer chromatography. Further extensive study is required for the complete understanding of H and Lewis blood-group substances in erythrocytes. The demonstration of a Le^b glycolipid in erythrocytes is contrary to a general theory that Lewis blood-group substances in erythrocytes are mucopolysaccharides which are absorbed by cells from serum (see Race and Sanger, 1962).

The amount of fucose-containing sphingoglycolipids and its distribution in normal mammalian tissue are extremely limited. Thus far we have studied in erythrocytes only those glycolipids containing fucose, *i.e.*, those carrying blood-group ABH and Lewis specificities. However, Suzuki *et al.* (1967) and McKibbin (1967) have reported the presence of fucose-containing glycolipid in the intestinal tract. These are, in general, very minor components of erythrocytes and tissues; only a few milligrams of A- or B-active glycolipids and less than 1 mg of the H-active glycolipid were obtained from 500 g of the packed erythrocyte ghosts, which, themselves, were obtained from more than 30 l. of blood (see Table IV). This yield is surprisingly low as compared to that of other glycolipids; *e.g.*, approximately 150 mg of globoside, 50 mg of ceramide lactoside, and 20 mg of ceramide trihexoside were obtained in the pure state from 500 g of erythrocyte ghosts. The low yield of a fucose-containing glycolipid from normal erythrocytes and normal tissue contrasts greatly with the much larger yield of a similar fucose-containing glycolipid (tumor glycolipid) from human adenocarcinoma tissue.

TABLE IV: Yield of the Blood-Group Glycolipid in Milligrams from 500 g of Wet Stroma.

A-active glycolipid of A_1 cells	Component I	1.8
	Component II	1.2
	Fr-I-1	3.5
B-active glycolipid	Component I	1.6
H- and Le^b-active glycolipids	Component I	0.5

It is also noteworthy that no detectable Le^a-active glycolipid was found in erythrocytes, although Le^b-active glycolipid is present in the erythrocytes. This is in striking contrast to human tumor tissue which contains a Le^a-active glycolipid as the major glycolipid component (Hakomori *et al.*, 1967). The distribution of the glycolipid with H, Le^a-, and Le^b activities in various tissues of different individuals, as well as those in various neoplastic tissues, is of great interest, since the fucose-containing glycolipid accumulating in human adenocarcinoma tissue is Le^a active and has a chemical composition similar to that of the blood-group ABH and Le^b glycolipids.

The accumulation of a Le^a-active, fucose-containing glycolipid in human adenocarcinoma occurs, most probably, as a result of a biosynthetic failure involving blood-group A and B glycolipids.

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Self-Association of Cholesterol and Its Interaction with Triglycerides. An Infrared Study*

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ABSTRACT: The self-association of cholesterol due to hydrogen bonding has been studied by infrared measurements of its OH-stretching band. At concentrations below 0.014 M in CCl_4, cholesterol exists only as a monomer.

As the concentration is increased it associates to form a dimer and at a concentration of ~0.06 M a higher aggregate begins to form which becomes the predominant species at a concentration of ~0.2 M. The dimerization constant has been determined ($K_{d,23°}$ = 4.5 l. mole^{-1}) at different temperatures from which the enthalpy of dimerization has been evaluated (ΔH = −1.8 kcal mole^{-1}). The ν_{max} of the OH-stretching bands of the dimer and trimer have also been reported. Infrared spectra of mixed solutions of cholesterol and the triglycerides gave evidence of formation of a 1:1 hydrogen-bonded complex. The equilibrium constants and enthalpies of formation of the complexes of cholesterol with triacetin, tributyrin, and trilaurin have been reported ($K_{23°}$ = 2.4–3.7 l. mole^{-1}; ΔH = −3.5 to −5.4 kcal mole^{-1}). The hydrogen-bonding properties of cholesterol are suggested as factors in the mechanism of plaque formation in atherosclerosis.

The mechanism of the deposition of cholesterol in atherosclerotic plaque is not well understood, and attention to some physicochemical factors may be of value in helping to understand this process. Recent studies attest to the need for further investigation of the physical chemistry of cholesterol, triglycerides, and other substances associated in the plasma with them (Sodhi and Gould, 1967; Chapman and Penkett, 1966; Paton, 1964). The proton-donating OH group of cholesterol immediately suggests hydrogen bonding as a possible mechanism of its interaction with triglycerides, which have proton-accepting carbonyl groups, and other acceptor molecules. Surprisingly enough there have been no reports of any such investigations on the hydrogen-bond interactions of cholesterol in the literature. There is only a passing reference to hydrogen-bonded aggregates of cholesterol as a probable cause for the line broadening of its nuclear magnetic resonance spectrum (Varian, 1957). We have carried out detailed studies of the hydrogen-bond interactions of cholesterol in terms of both its self-association and its association with triglycerides.

Experimental Section

Chromatographically pure (99+ %) cholesterol (General Biochemicals), reagent grade triacetin and tributyrin (Fisher), and trilaurin (Mann Research Labs) were used in the present studies. Spectrograde carbon tetrachloride (Merck) was dried over P_2O_5 in a desiccator

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