ABO-Blood-Group Lipoids in Human Liver.

First Report.*

By

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From human livers dehydrated with cold alcohol, lipids extractible with boiling alcohol and ether were separated and therefrom by the aid of organic solvents a group-active fraction was obtained. Though devoid of an affirmative evidence, I assumed the products pretty pure and examined their properties and composition. The present account deals with the preparation procedure and analytical results. Shortage of material did not allow sugar determination in the Group B lipid but it appeared that A, B and O group lipoids from human liver contain generally galactose, hexosamine, P and N in approximate equivalent proportions of 1:1:10:10 and possess minimum molecular weight of about 6500, (computed from the galactose content). N.E.=600~700. Nitrogen proved to be partially present in the form of primary amine.

The group lipoids here showed similar solubility and qualitative reactions as those from a liver metastasis of gastric cancer (Aikawa¹⁾) and erythrocytes (Hamamoto²⁾) despite of a different composition.³⁾

EXPERIMENTAL.

Preparation of the Group Lipoids.

The group lipoids were separated, taking the group activity as an indication, by a procedure resembling those of the predecessors in our Institute. A typical example will be given below.

Livers from two persons⁴⁾ belonging to Group A, weighing 2000 g. in total, were cut into small pieces, washed with physiological saline to remove blood and bile and kept in 5 volumes of alcohol (95%) for about a month. The tissue was then squeezed and ground in a masticator. The obtained pulp was boiled with 3 volumes of alcohol (95%) for 4 hours under reflux.

* 4th report of Masamune and co-workers' "Chemistry and Biology of Lipids."

While hot the extract was separated by decantation and by means of a press. The residue was subjected again to extraction with 2 volumes of alcohol (95%) in a like manner and then, after drying up in a Faust apparatus, pulverized in a mortar and treated with ether (200 cc.) in a Soxhlet apparatus exhaustively. The alcoholic extracts were filtered from yellowish flocculent precipitates, that had come out on standing, and distilled in vacuo as far as possible. The syrupy residue changed mucilaginous after long time (about 2 weeks) in a vacuum desiccator (H_2SO_4), whereupon it was also treated with ether. The ether extracts were combined and distilled in vacuo and dried as above. The remaining dark brown gelatinous substance amounted to 28 g.—Fr. Es.

Fr. Es was washed with six 40 cc. portions of cold acetone. And the part undissolved and that dissolved and distilled were both placed in a desiccator. The latter—Fr. Acs—remained as a viscous brown fluid. The former—Fr. Acn—was hygroscopic and brown, gelatine-like and weighed 22 g. This was extracted with benzene (Five 20 cc. portions of benzene employed). The insoluble substance here—Fr. Bzn—was colored grayish brown and not more than 0.1 g. (96 mg.). The benzene solutions were evaporated together in vacuo with following drying (20 g. of a brown solid were left) and removed from a petroleum-ether insoluble substance (a trace—Fr. Pen). Glistening dark brown gelatine-like substance obtained was washed with acetone thoroughly and dried—Fr. Pes-acn. The yellow colored washings left a residue on distillation—Fr. Pes-acs. Fr. Pes-acn and Fr. Pes-acs were dark brown and yellowish respectively. Fr. Pes-acn (15 g.) was further subdivided into the absolute alcohol-soluble and -insoluble portions. The solvent was changed many times until no coloring matter came out. The insoluble (6 g. when dry)—Fr. Pes-an—was yellowish non-sticky powder. The soluble (Fr. Pes-as) weighed 8 g. after expelling off the solvent.

In the next Fr. Pes-an was kneaded with 30 cc. of acetone. Since, here, acetone did not get colored, the mixture was boiled under reflux for 2 hours and centrifuged while hot. The yellowish supernatant liquid gave 150 mg. of brown resinous substance on vacuum distillation and drying. The centrifugate was dried and after taking up in 30 cc. benzene (The whole substance went into solution), 5 volumes of acetone added. Yellowish brown precipitates came out. Centrifuged. The residual after distillation of the supernatant fluid (yellowish) was again dissolved in 10 cc. of benzene and precipitated with acetone (50 cc.). Centrifugation, distillation of the supernatant liquid, dissolution of the remainder and precipitation was again repeated. This time precipitates were few (The mother liquid was almost non-colored). The three successive lumps of precipitates were united and dried (Cr. S.S.A, 2.3 g.).

Final purification was carried out by transfer of the substance from water to organic solvents:—

TABLE I.

Occurrence of Agglutination of Erythrocytes by Normal Antisera Treated with Fractions of a Lipid-mixture (obtained with boiling alcohol and ether) from Livers of Group A Individuals.

(— no agglutination, $\pm$ faint aggl., + slight aggl., ++ marked aggl., ## strong aggl.)

Fraction	Group of serum	Dilution of fractions							
		1% Original	$\times 2$	$\times 5$	$\times 10$	$\times 1.6 \times 10$	$\times 3.2 \times 10$	$\times 6.4 \times 10$	$\times 1.28 \times 10^2$
Es	A	++	++	++	++	##	##		
	B	—	—	+	++	##	##		
Acs	A	##	##	##	##				
	B	##	##	##	##				
Bzn	A	##	##	##					
	B	##	##	##					
Pen	A	##	##	##					
	B	##	##	##					
Pes-acn	A	##	##	##					
	B	##	##	##					
Pes-as	A	##	##	##	##				
	B	$\pm$	++	##	##				
Pes-an	A	##	##	##	##	##			
	B	—	—	—	+	++			
Pes-achs	A	##	##	##					
	B	##	##	##					
Cr. S.S.A	A	##	##	##	##	##	##	##	
	B	—	—	—	$\pm$	++	##	##	
S.S.A	A	##	##	##	##	##	##	##	##
	B	—	—	—	—	—	—	$\pm$	##
(Pes-as)'	A	++	##	##	##				
	B	$\pm$	++	##	##				

200 mg. of Cr. S.S.A were dissolved in 20 cc. of water.⁵⁾ At first 1.0 n. NaOH was added till pH of 9.0 and the brown solution shaken with an equal volume of benzene. The watery layer was separated, acidified to pH 1.0 with 1 n. H₂SO₄⁶⁾ and extracted with benzene (two 20 cc. portions). The acid benzene extracts were washed together with changes of water until a renewed washing remained at a pH above 6.0 and distilled. The residue when dried up approached to 130 mg. This was further taken up in water, acidified and shaken with chloroform similar to above. The chloroform solution, too, was washed with water with following distillation to dryness, 82 mg. of substance were yielded—S.S.A. The product inhibited isoagglutination of A erythrocytes at a dilution of 1:3200 (s. below).

Fr. Pes-as, whose 1% solution was faintly group-active, was also treated with boiling acetone, and from the undissolved fraction the part soluble in benzene and precipitable with acetone was separated—Fr. (Pes-as)' (yellowish non-sticky powder). The attempt, however, failed to raise the potency.

Anti-isohemoagglutinative Potency of the Lipid Fractions.

Table I shows the serological potencies of the lipid fractions in the run taken as an example of preparation. The assay was effected as by Hamasato²⁾, but Cr. S.S. and S.S. were dissolved in physiological saline without the use of alkali. Regarding the corresponding fractions from the liver of a Group B person, of which the active ones were B specific, the results were resembling, too. The dilution of A- and B-S.S.'s, whereat the inhibition of the isoagglutination manifested itself, varied from 1:1600 to 1:3200. The lipid mixture from the liver of an O person gave no fraction reactive to A or B serum.

Physical and Chemical Properties of S.S.'s.

1) Solubility.

S.S.'s had similar solubility irrespective of their blood group. (Table II)

TABLE II.

Solubility of Group Lipoids (S.S.'s) from Human Liver.

(— insoluble, + partly sol., ++ mostly sol., ### completely sol. "hot" signifies "heated just to boiling," except when glacial acetic acid was employed.)

Temp.	Solvent									
	Water	Methanol	Ethanol	Ether	Petr.-ether	Acetone	Chloroform	Benzene	Tetralin	Glacial acetic acid
Cold	##	—~+	—	—	—	—	##	##	##	##
Hot	##	+~++	+	—	—	—	##	##	##	## (100°C)

2) Qualitative tests.

All the S.S. preparations belonging to different blood groups resembled

one another in qualitative chemical property. Biuret negative. Tests for glycerol (acrolein reaction), pentose (with aniline acetate), methylpentose (Rosenthaler), uronic acid (Neuberg-Saneyoshi), sulfur (Lassaigne) and cholesterol (Liebermann-Burchard) also resulted in negative. Detected were nitrogen (Lassaigne), phosphorus (ammonium molybdate test with oxidation), ordinary sugar (Molisch) and amino sugar (Hamasato-Akakura). Saccharose-conc. H_2SO_4 test (Pettenkofer) gave a purple colour.

3) Quantitative analysis.

In addition to analytical figures of nitrogen (micro Kjeldahl), phosphorus (Haneda, Sinokawa and Yasuoka's modification of Plimmer method) and ash (Pregl), are given in Table III the colorimetric results, expressed as galactose, of immediate reactions of the substances with indole (Dische), orcinol (Ozaki's modification of Tillmans and Philippi method) and α -naphthol (Reaction I of Dische). Galactose can be accurately determined by the aid of these reagents only after acid hydrolysis of the substances with following removal of water-insoluble components and phosphotungstic acid-precipitable ones. Thus the values in the table are remote from the actual, being much larger, but they will serve the purpose of comparison with the corresponding values in those investigations¹⁾²⁾ when we were

TABLE III.
Composition of Group Lipoids (S.S.'s) from Human Liver.
(Figures express percentages.)

Preparation	Analysis					
	Direct colour development as galactose			Phosphorus	Nitrogen	Ash
	Indole r.	Orcinol r.	α -Naphthol r.			
S.S.A Prep. I	9.4	7.3	8.0	4.4	1.86	5.9
S.S.A Prep. II	9.2	6.8		4.1	1.84	6.5
S.S.A Prep. III	9.0	6.1		4.0	1.88	6.0
S.S.B Prep. I	9.4	6.9		5.4	2.24	5.2
S.S.O Prep. I	8.3	5.0		5.2	2.10	4.8
Average	9.1	6.4		4.6	1.98	5.7

unaware of the mentioned fact. The table indicates that, at least so far N, P and the components reacting to indole and orcinol concern, the S.S. preparations all coincided mutually and the equivalent ratio of the average values of N and P was 1:1.

As will be seen in a forthcoming report,⁷⁾ I have isolated galactose and glucosamine from the present A group lipid after hydrolysis. Thereby 2.7% galactose and 2.0% glucosamine were obtained. On the other

hand, Masamune-Tanabe procedure⁸⁾ for fractionated analysis of so-called glycoproteins was adapted⁹⁾ to a preparation of the Group O lipid (S.S.O Prep. I) except which no enough material was available, and the hydrolyzate-fractions exhibited reduction and coloration corresponding to 2.5% galactose and 2.0% hexosamine. Not only from these data but also from previous findings in group carbohydrates it is regarded as justified to conclude that A, B and O group lipid from the same organ or tissue are not divergent in galactose and hexosamine content. Furthermore, the analysis and isolation of hexosamine above are rather accepted to have given lower values than the virtual and it is assumed that in fact the materials involved an amount of hexosamine equimolecular to galactose, because acid hydrolysis gave in both cases an voluminous insoluble mass involving palmitic acid and phosphate rest to which adsorption of a part of the hexosamine probably occurred.

In short, in group lipoids of human liver hexosamine, galactose, P and N are considered to be present in approximate equivalent ratios of 1:1:10:10 (basis of calculation: 2.6% galactose (average value), 2.6% hexosamine, 4.6% P, 1.98% N, 5.7% ash (s. the table)). Calculated from the galactose content the minimum molecular weight approximates to 6500 $[(100/2.6 \times \frac{100}{94.3}) \times 180 = 6528]$.

Amino nitrogen (micro Van Slyke) amounted to 1.26% in S.S.A Prep. I and 0.79% in S.S.B Prep. I.

S.S.A, Prep. I.

100 mg. substance, 0.21 cc. N₂ (11°C, 770 mm. Hg). Found, 1.26%.

S.S.B, Prep. I.

11.32 mg. substance, 0.15 cc. N₂ (11°C, 770 mm. Hg). Found, 0.79%.

Whether free amino-nitrogen varies in amount according to the blood group or the discrepancy is due to contaminants will be settled by a future study.

Neutral equivalent. It was measured by formol titration. 3 cc. of a solution were titrated with 0.01 n. NaOH, and the total alkali used before and after addition of dilute neutral formalin (three times diluted formalin was neutralized to phenolphthalein and 3 cc. of it were added) was taken for the calculation. The blank experiment was made in parallel.

S.S.A, Prep. I.

$$\text{N.E.} = \frac{0.0207 \times 1000}{3.13 \times 0.01} = 661 \text{ (703 on ash-free basis).}$$

S.S.B, Prep. I.

$$\text{N.E.} = \frac{0.0236 \times 1000}{3.75 \times 0.01} = 629 \text{ (664 on ash-free basis).}$$

SUMMARY.

1. From human liver group active lipoids were separated. Of them A and B group substance inhibited isoagglutination of erythrocytes at a dilution from 1:1600~3200 specifically.

2. These group lipoids dissolved in water, benzene, chloroform, tetralin and glacial acetic acid, but not in ethanol, ether, petroleum ether and acetone. They dissolved in methanol scarcely in the cold but to a certain extent when boiled.

3. The group lipoids contained galactose, hexosamine, P and N in equivalent proportions of roughly 1:1(most probably):10:10. Their minimum molecular weight (The substances were presumed as nearly pure) of 6500 was computed out from galactose content. N.E., 600~700.

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H. Masamune.

References and Notes.

- 1) Aikawa, Tohoku J. Exp. Med., 1949, **51**, 367.
 - 2) Hamasato, *ibid.*, 1950, **52**, 17.
 - 3) Aikawa and Hamasato estimated galactose in group lipoids by direct application of colour reactions to the substances. But group lipoids involve components which influence the results enormously, so that the galactose values of these authors are not reliable. The equivalent ratio of P to N was 1:3 in the group lipid from the liver metastasis of gastric cancer and 2:3 in the ones from erythrocytes. Regarding the content of the components see the original papers.
 - 4) Livers were afforded from the Institute of Forensic Medicine (Director: Prof. T. Murakami) where the autopsy had been made.
 - 5) A perfect solution was given.
 - 6) The solution was completely precipitated at pH 1.0.
 - 7) Matuda, Tohoku J. Exp. Med., in press.
 - 8) Masamune and Tanabe, J. Bioch., 1938, **28**, 19.
 - 9) 130 mg. of the substance were hydrolyzed with 10 cc. of 1 n. H_2SO_4 and a 1 and a 5 cc. portion of the hydrolysate were used respectively for the total-sugar (galactose and glucosamine) and galactose determination.
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