magnitude of the formation constants of the magnesium ion complexes of the nucleotides. It should be noted that the value of Vladimirov, *et al.*,[12] ($\Delta Fh_{7.25}{}^{0\prime} = -5.6$ kcal./mole), measured from the same reactions used by Robbins and Boyer, differs considerably from the above values. Part but not all of the difference in this last instance can be traced to the use of the value of Ginodman (*vide supra*) for the free energy change in the hydrolysis of glucose 6-phosphate.

Acetyl Phosphate.—The values quoted in Table II for the hydrolysis of acetyl phosphate were derived from the data of Burton[19] on the hydrolysis of acetyl coenzyme A and the measurements made by Stadtman,[37] apparently performed at *p*H 6.8 and 28°, on the equilibrium constant (K_{Total} = 74) for the transfer of acetyl from phosphate to coenzyme A.

(37) E. R. Stadtman in McElroy and Glass, "Mechanism of Enzyme Action," Johns Hopkins Press, Baltimore, Md., 1953, p. 581; *J. Cell. Comp. Physiol.*, Supplement 1, **41**, 89 (1953).

Creatine Phosphate.—The measurements of Noda, Kuby and Lardy[11a] on the equilibrium constant ($K_{\text{Total}} = 0.25$) in the transfer of phosphate from ATP to creatine at *p*H 8.9, 30° and 0.02 *M* $MgSO_4$ were combined with the values of Robbins and Boyer for ATP hydrolysis in the presence of excess Mg^{++} and the stability constants of Smith and Alberty[11b] for the magnesium complexes of the various components of the system.

Acknowledgments.—The author would like to thank Professor Henry Borsook for use of some of the unpublished solubility data. I also wish to thank the students of Biochemistry 290-E for checking the numerous calculations involved in preparing this article.

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[CONTRIBUTION FROM THE DEPARTMENTS OF MICROBIOLOGY AND NEUROLOGY, COLLEGE OF PHYSICIANS AND SURGEONS, COLUMBIA UNIVERSITY AND THE NEUROLOGICAL INSTITUTE, PRESBYTERIAN HOSPITAL, AND THE MASSACHUSETTS GENERAL HOSPITAL]

Immunochemical Studies on Blood Groups.[1] XXIV. Some Oligosaccharides Isolated from Dialysates After Mild Acid Hydrolysis of Human Blood Group B Substances from Ovarian Cyst Fluid

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Blood group B substance derived from human pseudomucinous ovarian cyst fluid was hydrolyzed at *p*H 1.6 for 2 hr. at 100° and dialyzed. The dialysate was fractionated by charcoal column chromatography. Five fucose-containing oligosaccharides were isolated. The structures of these compounds are inferred from the composition, behavior on oxidation with sodium periodate before and after sodium borohydride reduction, reaction with triphenyltetrazolium chloride and optical activity. They are: (1) fucosyl (1 → 6) galactose, (2) fucosylgalactose, (3) fucosyl (1 → 3) fucose, (4) (fucosyl, galactosyl) (1 → 3) N-acetylglucosamine, (5) fucosylfucosyl (1 → 3) N-acetylglucosamine. None of the compounds isolated was as active in inhibiting B-anti-B precipitation as galactinol or melibiose, two of the most potent inhibitors available. Only 2.6% of the total fucose in the dialysate was free.

Studies on blood group substances[2-5] have clearly shown that biological activity is associated with oligosaccharide side chains which are split off by mild acid hydrolysis[6] at *p*H 1.5 to 2.0 for 2 hr. at 100° leaving a high molecular weight non-dialysable fraction,[7] PI, possessing little or none of the original blood group activity. This procedure also splits from these substances mono and other oligosaccharides unrelated to the blood group activity. Coté and Morgan[4,8] have identified six disaccharides from a partial acid hydrolysate in 0.1 *N* HCl for 3 hr. or in 1.0 *N* HCl for 30 minutes both at 100°, of blood group A substances. One of these, O-α-N-acetylgalactosaminoyl 1 → 3-D-galactose, was more active in inhibiting the hemagglutination of A cells by anti-A than any other compound thus far reported, in agreement with earlier work showing the importance of a terminal non-reducing N-acetylgalactosamine residue in the specificity of blood group A substance in its reaction with a plant hemagglutinin[9] and with human anti-A.[10]

This report describes the chromatographic separation and isolation of five hitherto undescribed oligosaccharides from the dialysate of a human ovarian cyst B substance which had been hydrolyzed in mild acid. The composition, partial identification and biological activity of these compounds is given.

Experimental

Materials and Methods.—Blood group B substance, Beach phenol insol 12.1 g., isolated from human ovarian cyst fluid, was hydrolyzed at *p*H 1.6 for 2 hr. at 100° at a concentration of 35 mg. per ml., dialyzed and the dialysate, 2.8 g., lyophilized. Two hundred seventeen mg. of the dialysate was chromatographed on a 100 g. Darco–100 g. Celite column[11] with an ethanol gradient as previously described.[12] The chromatogram thus obtained showed a

(1) Aided by grants from the National Science Foundation (G-5208) and the William J. Matheson Commission.

(2) E. A. Kabat, "Blood Group Substances," Academic Press, Inc., New York, N. Y., 1956.

(3) E. A. Kabat, *Behringwerk-Mitteilungen*, **34**, 39 (1957).

(4) W. T. J. Morgan, *Naturwiss.*, **46**, 181 (1959).

(5) W. T. J. Morgan and W. M. Watkins, *Brit. Med. Bull.*, **15**, 109 (1959).

(6) E. A. Kabat, H. Baer, A. E. Bezer and V. Knaub, *J. Exper. Med.*, **88**, 43 (1948).

(7) P. Z. Allen and E. A. Kabat, *J. Immunol.*, **82**, 340 (1959).

(8) R. H. Coté and W. T. J. Morgan, *Nature*, **178**, 1171 (1956).

(9) W. T. J. Morgan and W. M. Watkins, *Brit. J. Exper. Pathol.*, **34**, 94 (1953).

(10) E. A. Kabat and S. Leskowitz, THIS JOURNAL, **77**, 5159 (1955).

(11) R. L. Whistler and D. F. Durso, *ibid.*, **72**, 677 (1950).

(12) G. Schiffman, C. Howe and E. A. Kabat, *ibid.*, **80**, 6662 (1958).

large number of inadequately resolved oligosaccharides. Only the monosaccharides and the two most rapidly eluted disaccharides were sufficiently purified to attempt identification. Paper chromatography using propanol:ethyl acetate:water (7:1:2)[10] and developed with trichloroacetic acid–aniline also showed no resolution of the many oligosaccharides. It was, therefore, decided to fractionate the dialysate initially by stepwise elution from a Darco–Celite column followed by rechromatography of the fractions with gradient elution. The main fraction of dialysate, 1.749 g., was placed on a 100 g. Darco–100 g. Celite column which was developed batchwise with 2-liter portions: fractions 1 to 7 with water, 8 to 22 with 4% ethanol, 23 to 35 with 15% ethanol, 36 to 51 with 20% ethanol, 52 to 65 with 30% ethanol, 66 to 71 with 50% ethanol and 71 to 78 with a solution of 50% ethanol, 20% propanol and 30% water. All fractions were analyzed for reducing sugar before and after concentrating *in vacuo* to 8.0 ml.

Reducing sugar, methylpentose, galactose, hexosamine, N-acetylhexosamine and periodate were measured by methods previously reported.[12] Formaldehyde was determined by the method described by Smith and Montgomery[13] using smaller volumes. A sample containing 0.1 to 1.0 μ g. of formaldehyde, usually 10 to 20 μ l., was removed from a reaction mixture, the periodate was destroyed by addition of excess arsenite and the volume adjusted to 0.10 ml. with water. To this was added 1.0 ml. of chromotropic acid reagent, and the mixed solution was heated in a boiling water-bath for 20 to 30 minutes, cooled and read at 5700 Å.

Borohydride Reduction.—In the investigation of sequence and linkage of unknown oligosaccharides, analyses were performed before and after borohydride reduction. A sample of unknown sugar solution at neutral reaction containing 100 to 200 μg. was added to an equal volume of ice-cold aqueous sodium borohydride, 50 mg./ml. The solution was allowed to stand at room temperature for 4 hr. or overnight at 4°. After this time the completeness of reaction was established by the Park–Johnson assay on a 20-μl. aliquot acidified with an equal volume of 0.4 *N* HCl. This aliquot should contain less than 0.5 μg. of reducing sugar if the reduction is sufficiently complete. If the reduction is not complete, additional standing at room temperature is allowed. Since all methods used to analyze the reduced compounds are spectrophotometric micro determinations, several controls are included. When a disaccharide consists of two different sugars, each monosaccharide is reduced separately; two water blanks are treated simultaneously. After reduction is complete and the excess borohydride is destroyed with 0.1 volume of 4.0 *N* HCl, the sugar to be assayed is added to one of the water blanks and nothing to the other. A quantitative recovery of the sugar added to the control tube indicates that there is no interference by the borate ion, and total loss of reactivity of the reduced sugar demonstrates that the sugar alcohol does not react in the assay for that sugar. Oligosaccharides composed of methylpentose, galactose and hexosamine can all be studied in this manner. The formed borate ion interferes with the N-acetylhexosamine reaction, and therefore the results after borohydride reduction by this method are only qualitative.

Periodate Oxidation.—Periodate oxidation studies were performed at room temperature in the dark in an unbuffered solution on the original oligosaccharides and on these substances after reduction with sodium borohydride and acidification as above. One tenth to two tenths of a micromole of oligosaccharide in a volume of 0.20 ml. is oxidized with 2 μM sodium periodate, the final volume of the reaction mixture is 0.25 ml. The uptake of periodate was measured spectrophotometrically at 2225 Å. in quartz cells. A blank and several known disaccharides were included in each study.

Results

Fractions 1 to 8 contained only monosaccharides as follows: fraction 1, 9 mg. of glucosamine, fractions 2 and 3, 3 mg. of galactose, fractions 3 to 8, 45 mg. of fucose and fraction 8, 14 mg. of N-acetylhexosamine. Fractions 9 to 14 were pooled and rechromatographed on a 10 g. Darco–10 g. Celite column using a two stage 0 to 25% ethanol gradient as described.[12] The peaks eluted may be seen in Fig. 1. Peaks labelled A and B were mixtures of compounds which could not be identified. Peak B, 600 μg. of reducing sugar, was rechromatographed on a 20 g. Darco–20 g. Celite column; the main peak representing 60% of the material put on the column was still a mixture of oligosaccharides. Peaks I and II (Fig. 1) were rechromatographed separately and each yielded essentially one component. Analytical data given in Table I show that compounds I and II are isomeric fucosylgalactoses. On oxidation with periodate after borohydride reduction compounds I and II liberated 0.9 and 1.0 moles of formaldehyde, respectively, indicating linkage of carbon 1 of fucose to the hydroxyl on carbon 2 or 6 of galactose. To distinguish between these two possibilities, the triphenyltetrazolium reaction of Wallenfels[14] was used with modification. To 0.1 ml. of aqueous sample containing about 50 μg. of the compound to be tested is added 50 μl. of a reagent made by mixing equal volumes of 4% triphenyltetrazolium chloride and 2 *N* NaOH just before use. After mixing, the solution is heated at 60 to 70° for 10 minutes. A blank, 200 μg. of kojibiose[15a] and 300 μg. of 2-O-methylglucose[15b] were completely colorless; 50 μg. of lactose, cellobiose, maltose, laminaribiose[15c] and melibiose gave dark red formazan precipitates. Compound I, 50 μg., gave about the same reaction as did lactose, *i.e.*, a dark red formazan precipitate. Compound II, 50 μg., did not give the red precipitate but turned the solution pink. In a series of determinations comparing 16 and 32 μg. of compound I and 18 and 36 μg. of compound II with 5, 10, 15 and 20 μg. of cellobiose, lactose and isomaltose, it was seen that 16 μg. of compound I gave a similar reaction to 15 μg. of the known disaccharides while 18 μg. of compound II gave a reaction intermediate between 5 and 10 μg. of the known disaccharides and 36 μg. of compound II gave less reaction than 15 μg. of cellobiose and lactose and less than 10 μg. of isomaltose but more than 10 μg. of the former two sugars and more than 5 μg. of the latter. From these reactions com-

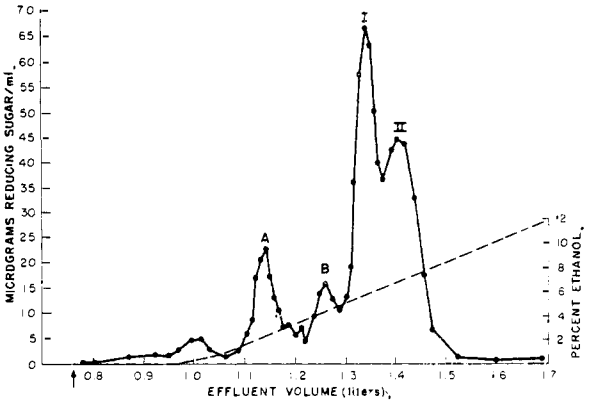

Fig. 1.—Rechromatography of fractions 9 through 14 on a 10 g. Darco–10 g. Celite column. The arrow indicates where the two stage ethanol gradient was begun. The dashed line represents the ethanol concentration in the effluent.

(13) F. Smith and R. Montgomery, "Methods of Biochemical Analysis," Vol. III, Interscience Publishing Co., New York, N. Y., 1956, p. 197.

(14) K. Wallenfels, *Naturwiss.*, **37**, 491 (1950).

(15) Kindly supplied by (a) Dr. K. Aso, (b) Dr. N. K. Richtmyer, (c) Dr. P. A. Rebers.

TABLE I

ANALYTICAL DATA ON ISOLATED OLIGOSACCHARIDES[a]

Compound	I		II		III		IV		V	
Condition	Unred.	Reduc.[b]	Unred.	Reduc.[b]	Unred.	Reduc.[b]	Unred.	Reduc.[b]	Unred.	Reduc.[b]
Reducing sugar, mg.	2.5	0.1	1.4	0.1	5.2	0.1	0.9	0.2	0.9	0.2
Methylpentose, mg.	2.7	2.4	1.3	.9	7.0	3.7	.8	.6	1.2	.9
Galactose, mg.	2.2	0.6	1.4	.3	(1.6)[d]	(1.4)[d]	.6	.5	0.1	.1
N-Acetylhexosamine, mg.	0.0	.0	0.0	.0	0.3	0.0	.5	.0	.6	.0
Hexosamine, mg.	0.3	.1	0.1	.1	0.9	0.2	.5	.0	.6	.0
$[\alpha]_d$[c]	−25°		−9°		−44°		0°		−18°	

[a] All values in table give quantities actually isolated from final chromatogram. [b] Values for reduced oligosaccharides were obtained after sodium borohydride treatment. [c] Optical rotations were taken with the Keston polarimeter attachment to the Beckman spectrophotometer in a 0.5 decimeter cell of 2.5-ml. capacity. With the small quantities of available material, values are considered as only approximate. [d] Values represent galactose equivalent but probably not pure galactose; see text for further discussion.

pound I would appear to be fucosyl 1 → 6 galactose. Compound II reacted more weakly in the triphenyltetrazolium test; if this is truly a positive reaction, it would make compound II the anomeric fucosyl 1 → 6 galactose.

A pool of fractions 15 through 21 of the original chromatogram gave only one peak, compound III, on rechromatography. From the data in Table I it can be seen that of the 7 mg. of fucose only half, 3.7 mg., remains after borohydride reduction. Thus compound III is fucosylfucose. On periodate oxidation after borohydride reduction 1 mole of formaldehyde is released (1.0, 1.3 see Table II) as is 1 mole of acetaldehyde (0.7, 0.9, 0.9, 1.1) as measured by the *p*-hydroxybiphenyl method.[16] From these data it is inferred that compound III is fucosyl 1 → 3 fucose. Small amounts of other materials are present.

Figure 2 shows the results of rechromatography of fractions 23 to 30 on a 25 g. Darco–25 g. Celite column. The two peaks preceding the four main

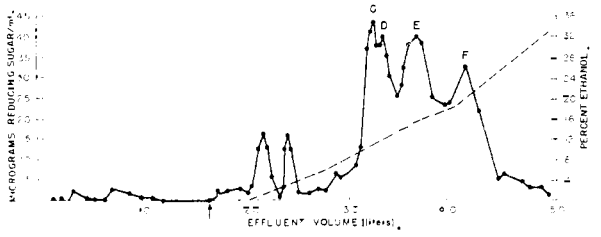

Fig. 2.—Rechromatography of fractions 23 through 30 on a 25 g. Darco–25 g. Celite column. Arrow and dashed line as in Fig. 1.

peaks, C to F, were fucose and N-acetylhexosamine probably due to some hydrolysis in concentrating the fractions. Each major peak was pooled separately and refractionated on a larger column. Thus far only peak C has yielded compounds sufficiently pure for characterization. When peak C was rechromatographed on a 50 g. Darco–50 g. Celite column, an asymmetrical peak was obtained. Galactose was found only in the first half of the peak but methylpentose and N-acetylhexosamine were found throughout. The peak was subdivided so that the galactose containing portion, IV, was separated from that devoid of galactose, V. Each was rechromatographed and gave a single peak, compounds IV and V. Compound IV, eluted at the lower alcohol concentration, contained 1 residue each of galactose, hexosamine and fucose (1:1:1.3). The other, V, contained 2 residues of fucose to 1 of hexosamine with a trace of galactose. The actual quantities obtained may be seen in Table I. Both compounds gave the N-acetylhexosamine reaction and both lost hexosamine on borohydride reduction indicating a reducing N-acetylhexosamine with carbon 4 unsubstituted.[17] Formaldehyde was split off on oxidation with periodate indicating that carbon 6 of the hexosamine was also unsubstituted. Assuming a pyranose structure, only the hydroxyl group on carbon 3 is available for linkage by the fucose or galactose to the amino sugar. Thus both trisaccharides are linear and possess a terminal reducing end of N-acetylhexosamine substituted in position 3.

Figure 3 gives the results obtained when the various purified fractions were tested for their ability to inhibit[10] precipitation of 0.5 ml. of an anti-B se-

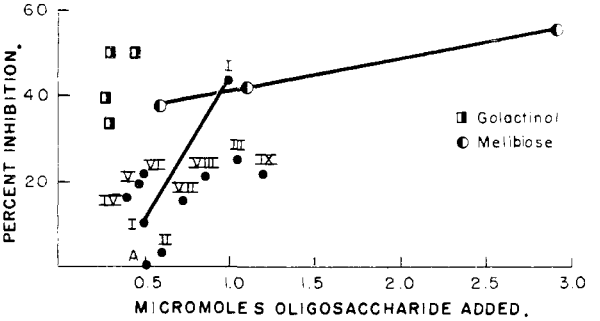

Fig. 3.—Oligosaccharide inhibition of precipitation of an anti-B serum, 0.5 ml. Als 2 → 3, by various dialysable fractions isolated by chromatography from a partial hydrolysis of human ovarian cyst B substance. The antigen was 25 μg. of PM phenol insol.

rum,[18] Als diluted 2 to 3, by 25 μg. of blood group B substance, PM phenol insol.[19] The volume of each determination was adjusted to 2.0 ml. by addition of 1.0 ml. inhibitor or saline. In the absence of inhibitor 24 ± 2 μg. of antibody N was precipitated. Included for comparison are melibiose and galactinol, among the best inhibitors thus far encountered

(16) R. P. Hullin and R. L. Noble, *Biochem. J.*, **55**, 289 (1953).

(17) R. Kuhn, A. Gauhe and H. H. Baer, *Chem. Ber.*, **87**, 1138 (1954).

(18) S. Leskowitz and E. A. Kabat, *J. Immunol.*, **75**, 171 (1955).

(19) H. Baer, E. A. Kabat and V. Knaub, *J. Exper. Med.*, **91**, 105 (1950).

TABLE II

PERIODATE OXIDATION STUDIES ON ISOLATED OLIGOSACCHARIDES AND VARIOUS KNOWN COMPOUNDS[d]

Compound	Condition	Moles of periodate consumed at various times (hr.) 2–6[c]	19–24	44–48	Theory	Moles of formaldehyde liberated at various times (hr.) 1.5–6	20–24	44–48	Theory
I	Reduced	4.4	5.1	5.8	6	1.0	0.8	0.9	1
	Unreduced	4.4	5.8	6.2	5	0.4	0.4	0.3	0
II	Reduced	3.8	4.4	5.5	6	1.1	1.1	1.0	1
	Unreduced	3.7	4.6	5.6	5	0.3	0.4	0.4	0
III	Reduced	3.7	4.7	5.2	4	1.1	1.1	1.2	1
	Unreduced	2.6	3.7	4.1	3	0.3	0.3	0.1	0
IV	Reduced	3.6	4.7			0.8			1
	Unreduced	4.2	5.9	6.7		0.6	0.9	0.8	1
V	Reduced	3.7	4.6			0.5			1
	Unreduced	4.5	9.5	10.9		0.7	0.9	0.9	1
Melibiose	Reduced		4.3	5.0	6	0.8		0.8	1
	Unreduced		5.3	5.3	5	0.1	0.2	0.1	0
Laminaribiose	Reduced	1.9	3.6	5.8	5	1.4	1.7	1.9	2
	Unreduced	2.0	5.8	6.7	5[a]		1.0	1.2	1[a]
Kojibiose	Reduced	2.7	4.4	4.3	5	1.2	1.2		1
	Unreduced	0.7	4.3	5.2	5	0.6	0.9		1
Isomaltose	Reduced	4.1	5.8	5.8	6	1.6	1.6		1
	Unreduced	3.9	4.8	4.9	5		0.0	0.0	0
Maltose	Reduced	3.3	5.5	5.4	5	1.7	1.8	1.8	2
	Unreduced	2.5	4.0	4.6	4	0.3	0.3		0
Lactose	Reduced	5.7	5.8	5.8	5	1.9	2.0	2.0	2
	Unreduced	3.5	4.0	4.3	4	0.0	0.1	0.0	0
Cellobiose	Reduced	2.9	4.7	5.4	5	1.4	1.9	1.8	2
	Unreduced	2.2	3.4	3.9	4	0.0	0.2	0.2	0
α-Methylglucoside	Reduced	1.3	1.5	1.6	2		0.0	0.1	0
	Unreduced	0.9	1.5		2	0.0	0.0		0
2-O-Methylglucose	Reduced	2.2	2.4	2.4	3		0.6	0.6	1
6-O-Methylglucose	Reduced	3.0	3.0	3.1	4		0.7	0.6	1
Galactosyl 1 → 3β N-acetylglucosamine	Reduced	2.0	3.3	3.8	4		0.7	1.1	1
	Unreduced	2.0	2.9	2.8	4	0.6	0.7	0.5	1
Galactosyl 1 → 4β N-acetylglucosamine	Reduced	1.5	2.8	2.8	3		0.7	1.0	1
Glycerol	Reduced[b]	1.8	2.0	1.8	2	2.0	1.9	1.9	2
	Unreduced	2.1	2.0	2.0	2	2.1	2.1	1.9	2

[a] Calculated for oxidation in acyclic form. [b] Carried through reducing procedure. [c] Mainly 2–3 hour determinations. [d] Data in the table represent means of two or more independent determinations in which measurements were made at fairly comparable time intervals. This was done at the suggestion of a referee to reduce the size of the table. An adequate but less complete indication of the variability may be obtained by comparison with theoretical values for the various known compounds.

in this system. It can be seen that none of the purified fractions is as active as either galactinol or melibiose.

Discussion

A dialysate from a blood group B substance which had been partially hydrolyzed in mild acid has been fractionated to yield five fucose-containing oligosaccharides. Compound I appears to be fucosyl 1 → 6 galactose. From 1749 mg. of dialysable material 4.9 mg. (sum of methylpentose and galactose) of compound I was obtained (0.3%). The molar ratio of methylpentose to galactose is 1.3 and the fraction contains 0.3 mg. of hexosamine indicating that some impurities are present. On periodate oxidation fucosyl 1 → 6 galactose should behave like melibiose and isomaltose, *i.e.*, consume 5 moles of periodate and liberate no formaldehyde. It can be seen from Table II that compound I actually consumed about 6 moles of periodate (5.7 to 6.2) in 1 to 2 days, melibiose 5.1 to 5.6 moles and isomaltose 5.8 moles. No formaldehyde was liberated from isomaltose, 0.1 mole from melibiose and 0.3 to 0.4 mole from compound I. After reduction with borohydride compound I gave almost theoretical results. The small excess periodate consumption and formaldehyde liberation observed in the determinations on the unreduced compound might well be within the limits of uncertainty of the method or may be caused by the small amounts of impurities known to be present, or both. In Table I it can be seen that the fucose content was reduced by 11% (0.3 mg.) and 0.6 mg. of galactose remained after reduction with borohydride. These values further indicate that small amounts of impurities are present.

Compound II showed a decrease in fucose after reduction and the galactose was only 79% destroyed. On oxidation with periodate compound II consumed 5.5 and 5.6 moles in 2 days in the reduced and unreduced forms, respectively, showing a behavior similar to compound I. If compound II were fucosyl 1 → 2 galactose theoretical considerations[20] should allow the liberation of formaldehyde from C_6 of galactose as oxidation proceeds between C5 and C6 of the acyclic form. Unfortunately this

(20) J. M. Bobbitt, *Advances in Carbohydrate Chem.*, **11**, 1 (1956).

is an extremely slow reaction[12] and is incomplete in the 2 day period used in these studies. Thus, 2-O-methylglucose liberated 0.6 mole of formaldehyde and galactosyl 1 → 3 β N-acetylglucosamine 0.5 mole in 2 days. Compound II liberated 0.4 mole of formaldehyde as did compound I. After reduction 1.0 mole of formaldehyde was found. Compound II, therefore, behaves similarly to compound I in the periodate studies, indicating that both are probably anomers of fucosyl 1 → 6 galactose. However, the periodate data are not sufficiently precise to preclude the possibility that similar results could not be obtained with fucosyl 1 → 2 galactose. In the triphenyltetrazolium test compound I was shown definitely not to be 2 linked; compound II gave equivocal results. In copper reducing sugar assays, 2-O-substituted glucoses show an anomalous behavior.[21-23] A similarly low result was obtained with kojibiose using the Park–Johnson ferricyanide method; kojibiose giving only 17.5% reducing sugar equivalent to glucose. Compounds I and II give 51 and 52%, respectively. Therefore, although the periodate data and the triphenyltetrazolium reaction were indicative of 2 or 6 linked fucosylgalactose for compound II, the reducing sugar evidence strongly suggests the latter. Thus both compounds I and II appear to be fucosyl 1 → 6 galactoses. In the L series the α isomer is the higher levorotating compound. Thus compound I might be fucosyl 1 → 6α galactose and compound II would be fucosyl 1 → 6β galactose. Compound II represents 0.2% of the original dialysable material fractionated.

Compound III consisted of 7.0 mg. of fucosylfucose equivalent to 0.4% of the starting material. In the assay for galactose the color equivalent of 1.6 mg. was obtained. This color was dependent on cysteine but was not the usual blue obtained with pure galactose. Fucose does not interfere in the test. The color obtained might in part be due to galactose but may also be augmented by small amounts of amino acids that are present. After hydrolysis in 6 *N* HCl for 4 hr. at 100°, 4.2 μ*M* amino nitrogen were observed; 1.3 μ*M* were found before hydrolysis. Also present is 0.9 mg. of hexosamine. It is estimated that the fraction containing compound III is about 70% fucosylfucose with about 30% impurities. On periodate oxidation 5.3 and 3.5 moles were consumed by the reduced and unreduced compound while liberating 0.9 to 1.2 and 0.4 mole formaldehyde, respectively. In two series of periodate oxidation studies 7.4 and 8.6 moles were unaccountably consumed.

The analytical data on compounds IV and V clearly indicate their composition and the existence of the hexosamine on the reducing end. Both liberate formaldehyde on periodate oxidation. However, the data on periodate consumption are not sufficiently precise to indicate a unique structure. Compound V tends to consume periodate far in excess of any expected amount but does so fairly reproducibly.

(21) S. A. Barker, E. J. Bourne, P. M. Grant and M. Stacey, *Nature*, **178**, 1221 (1956).

(22) S. Haq and W. J. Whelan, *ibid.*, **178**, 1221 (1956).

(23) R. W. Bailey, S. A. Barker, E. J. Bourne and M. Stacey, *ibid.*, **176**, 1164 (1955).

Until quite recently the chemistry of fucose-containing oligosaccharides had been only slightly developed. In 1954 O'Neill[24] isolated fucosyl 1 → 2α fucitol from fucoidin. Morgan[4] mentions that Coté has also isolated from fucoidin the 1 → 2, 1 → 3 and 1 → 4α fucosylfucoses.[25] In addition Kuhn and co-workers in an extensive series of investigations[26-29] on the identification of oligosaccharides from human milk have isolated and identified at least 6 fucose-containing oligosaccharides; lacto-N-fucopentaose I, lacto-N-fucopentaose II, lactodifucohexaose, fucosido-lactose, fucosyl 1 → 2α galactose and lactodifucotetraose. Lacto-N-fucopentaose II is capable of inhibiting the agglutination of Le^a cells by an anti-Le^a serum, only 1.0 μg. is required, whereas 60 μg. of lacto-N-difucohexaose are required.[4,30] The other compounds are not active. All the fucosides tested were inactive in inhibiting the agglutination of O cells by eel serum, with the exception of fucosido-lactose, which has only slight activity, although all have an α-fucosyl structure.[4]

In addition to the six disaccharides[4,8] isolated by Coté and Morgan from blood group A substance, note must be taken of the first disaccharide isolated from hog gastric mucin; galactosyl 1 → 4 β N-acetylglucosamine.[31,32] No oligosaccharides have hitherto been reported from blood group B substance. The oligosaccharides isolated from blood group B substance as described in this paper were found to have less activity in inhibiting precipitation of anti-B by a blood group B substance than either galactinol or melibiose, the two most potent inhibitors of this system thus far available. Neither galactinol nor melibiose represents structures which could occur in the B substance since it does not contain either inositol or glucose.[2,3] The lower activity of the compounds tested suggest, therefore, that they do not represent the sequences determining blood group B specificity. This is not surprising since all compounds isolated probably have fucose as the non-reducing end group, a structure not compatible with current information as to the grouping determining blood group B activity.

Watkins[33] reported the synthesis by *T. foetus* enzymes of galactosyl 1 → 6α N-acetylglucosamine and subsequently stated[34] that this compound is not active in hemagglutination inhibition of B cells with anti-B. It was predicted that this compound should be a good inhibitor.[10] However, the evidence presented for the specific structure is depend-

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(30) W. T. J. Morgan, "Ciba Foundation Symposium on the Chemistry and Biology of Mucopolysaccharides," J. and A. Churchill Ltd., London, 1958, p. 200.

(31) Z. Yosizawa, *Tohoku J. Exptl. Med.*, **51**, 51 (1949); **52**, 111 (1950); **52**, 145 (1950).

(32) R. M. Tomarelli, J. B. Hassinen, E. R. Eckhardt, R. H. Clark and F. W. Bernhart, *Arch. Biochem. and Biophys.*, **48**, 225 (1954).

(33) W. M. Watkins, *Nature*, **181**, 117 (1958).

(34) W. M. Watkins, discussion following E. A. Kabat, "Ciba Foundation Symposium on the Chemistry and Biology of Mucopolysaccharides," J. and A. Churchill Ltd., London, 1958, p. 60.

ent on qualitative color tests and on R_f. The periodate oxidation information given is difficult to interpret in terms of a unique structure. Quantitative data on the amounts of formaldehyde liberated during periodate oxidation before and after reduction with borohydride would be useful in determining whether the compound tested and found to be inactive in hemagglutination inhibition tests in the blood group B system was galactose linked α to the 6 carbon of N-acetylglucosamine; inhibition of precipitation, which is a more sensitive test, was not carried out.

The fucose in blood group B substance from ovarian cyst appears to be linked very differently from the fucose in blood group A and O(H) substances from hog gastric mucin. Only 45 mg. of free fucose (2.6%) of 1.7 g. dialysable constituents were found when ovarian cyst B substance was hydrolyzed at *p*H 1.6 for 2 hr. at 100°, whereas 47 to 95% of the fucose in the dialysate was free when hog gastric mucin was treated similarly.[10] A blood group A substance derived from human ovarian cyst fluid and partially hydrolyzed at *p*H 1.9 also liberated most of the dialysable fucose as free fucose.[35] These observations would suggest that part of the fucose in blood group A substance is linked quite differently from that in blood group B substance. Thus if the hypothesis of Watkins and Morgan[36] were correct that A and B substances are derived from H substance under the influence of the A and B genes, respectively, these genes would also have to modify the fucose portion of the H substance in addition to introducing the specific A and B oligosaccharides side chains to account for the differences in lability of the fucose. Moreover, the findings that the P1 fractions[7] liberated by mild acid hydrolysis of B and A substance give rise to antibodies which were different in specificity from each other and from the original A and B substances also indicate that the genes determining A or B specificity control other steps in the synthesis of their respective substances in addition to the synthesis of the specific oligosaccharide side chains which are recognized using anti-A and anti-B serum.

(35) Unpublished observations.

(36) W. M. Watkins and W. T. J. Morgan, *Vox Sanguinis*, **4**, 97 (1959).

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[CONTRIBUTION FROM JOHN L. SMITH MEMORIAL FOR CANCER RESEARCH]

E-73: An Antitumor Substance. Part I. Isolation and Characterization[1]

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The isolation of E-73 with antitumor properties together with two diastereoisomeric forms of cycloheximide, fungicidin and two inert crystalline compounds from the culture filtrates of *Streptomyces albulus* is described.

In our cancer-screening program, a new species of actinomycete was isolated from the soil by our microbiology division. Broths from this culture, when tested at the Sloan–Kettering Institute as well as at our laboratories showed significant activity against mouse Sarcoma 180 and against human tumor transplants grown in rats. This organism was designated as *Streptomyces albulus*,[2] and the isolation of the active principle is reported here.

The organism is grown in the usual natural media in submerged culture for 3–6 days. The broths are active against rodent tumors such as S-180 and HS#1 and against various fungi, especially yeast-like organisms *in vitro*. It appeared early in the work that the antitumor activity might parallel the activity against yeasts. The conventional disk-plate assay with *Saccharomyces cerevisiae* grown in glucose–yeast extract-agar was used during the isolation work. The samples were plated at serial dilutions and the last dilution which gave a distinct zone was expressed as the number of dilution units per ml. The antitumor activity of the various fractions was checked from time to time against Sarcoma 180 in mice as well as by the heterologous screens of the type HS#1 and HEp#3 in rats.[3]

Extraction of the broths with 1-butanol and concentration gave a product designated as A-73 and which agreed in its properties with fungicidin.[4] Ethyl acetate extracts of the broth on processing gave products which were free from fungicidin and showed antitumor activity at levels at 0.5 to 2.0 mg./kg. in S-180 tumors. Paper chromatography showed possible presence of cycloheximide.[5] However, the latter is known to be active only at levels of 30–80 mg./kg. against Sarcoma 180.[6] Thus, it appeared that the concentrates from *Streptomyces albulus* contained a highly active compound in addition to the possible cycloheximide.

Partition chromatography on a silica gel column was employed for the separation of the active components. Aqueous methanol and diisopropyl ether were selected as stationary and mobile phases, respectively. When the progress of the column was followed by gravimetric and microbiological

(1) Presented at the 134th Meeting of the American Chemical Society, Chicago, Ill., September, 1958.

(2) The organism was characterized by Dr. J. B. Routien, Mycology Department, Chas. Pfizer & Co., Inc.

(3) The antitumor assays were carried out by our Assay Department. For methods see H. W. Toolan, *Cancer Research*, **14**, 660 (1954), and ref. 5.

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(6) H. C. Reilly, C. C. Stock, S. M. Buckley and D. A. Clark, *Cancer Research*, **13**, 684 (1953).