

## Carbohydrate Composition of Hepatitis B Surface Antigen

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### SUMMARY

The content and composition of carbohydrate in hepatitis B surface antigen (HB<sub>s</sub>Ag) were clarified by gas chromatography. A value of 75.8 µg carbohydrate per mg of protein was obtained. The main components were *N*-acetylglucosamine, mannose, galactose and sialic acid and the minor one was fucose. No *N*-acetylgalactosamine was detected. The fact that no sugar was detected in lipid fractions suggests that the sugar in HB<sub>s</sub>Ag exists almost exclusively in the form of glycoprotein and there is no glycolipid.

Burrell *et al.* (1973) reported that carbohydrate was necessary for the serological activity of HB<sub>s</sub>Ag and in the same year, Chairez *et al.* (1973) found that the carbohydrate content in 20 nm spherical particles, estimated by the phenol-sulphuric acid method, was in the range of 3.6 to 6.5%. The presence of glycosphingolipid (Steiner, Huebner & Dressman, 1974), sialyl residues (Neurath, Hashimoto & Prince, 1975) and glycoprotein (Chairez *et al.* 1973) in HB<sub>s</sub>Ag have since been reported. Because of the reported involvement of carbohydrate and lipid (Burrell *et al.* 1973) in the antigenicity of HB<sub>s</sub>Ag, the exact determination of carbohydrate composition was attempted in this study.

Almost 20 mg highly purified HB<sub>s</sub>Ag, with a reversed passive haemagglutination (RPHA; Schuur & Kačaki, 1974) of 1:64000, was obtained from 2 l of plasma from blood donors (mixture of subtypes adr and adw) in a reproducible manner by the method shown below. The pellet obtained by centrifugation at 30000 rev/min for 16 h was suspended in 0.01 M-phosphate buffer containing 0.15 M-NaCl, pH 7.2 (PBS), and the HB<sub>s</sub>Ag was then precipitated by addition of ammonium sulphate to 50% saturation, followed by re-suspension and dialysis against PBS. After addition of solid potassium bromide to give a concentration of 30% (w/v), the preparation was subjected to two cycles of equilibrium centrifugation at 40000 rev/min for 40 h, followed by rate zonal sedimentation in glycerol (10 to 50%, v/v) at 40000 rev/min for 4 h, and KBr (10 to 50%, w/v) at 30000 rev/min for 16 h. The fractions containing HB<sub>s</sub>Ag were monitored by RPHA, combined and dialysed against PBS. Finally, the preparation was again subjected to equilibrium centrifugation in KBr followed by dialysis. In the preparations obtained, the human serum components were not detected by a complement fixation (CF) test with rabbit anti-human serum antibody (Behring-Werke AG, Marburg-Lahn, Germany).

Delipidation of HB<sub>s</sub>Ag was performed by the method of Hakomori & Murakami (1968). The purified HB<sub>s</sub>Ag (about 2 mg) in PBS was subjected to freeze-drying, followed by the addition of chloroform-methanol (2:1). The mixture was shaken vigorously for 5 min and centrifuged at 3000 rev/min for 10 min. The solvent phase (Ex-1) was removed and the pellet obtained was again extracted by chloroform-methanol (2:1; fraction Ex-2), followed by hot chloroform-methanol (1:1; fraction Ex-3) and hot chloroform-methanol (1:2; fraction Ex-4). The residual pellet (Fr-1, apoprotein) was dried over P<sub>2</sub>O<sub>5</sub> *in vacuo* for gas chromatography. The four fractions (Ex-1, Ex-2, Ex-3 and Ex-4) containing lipid were combined and evaporated to dryness under N<sub>2</sub> below 25 °C. A mixture of chloroform-

methanol (2:1) and water at a ratio of 6:1 was added to the evaporated lipid fraction which was then shaken vigorously and subsequently centrifuged. The upper layer (Fr-2) was separated from the lower layer (Fr-3) and each was freeze-dried.

Carbohydrate analysis by gas chromatography was performed essentially by the method of Clamp, Bhatti & Chambers (1972). Each freeze-dried sample containing 0.5 to 1.5 mg of intact HB<sub>s</sub>Ag, Fr-1, Fr-2 and Fr-3 was methanolysed with 5% HCl in anhydrous methanol by heating at 100 °C for 5 h under N<sub>2</sub> gas in a sealed tube. After neutralization with silver carbonate, an appropriate amount of acetic anhydride was added and the mixture was subjected to re-*N*-acetylation for 12 h. D-Mannitol was added as an internal standard to each fraction. After centrifugation at 3000 rev/min for 5 min, the solvent phase of each fraction was separated and the pellet was triturated with methanol and centrifuged again. The residue was evaporated to dryness, and then kept for 24 h over P<sub>2</sub>O<sub>5</sub>.

Table 1. Carbohydrate compositions of intact and treated HB<sub>s</sub>Ag

	Intact HB <sub>s</sub> Ag	Treated HB <sub>s</sub> Ag*		
		Fr-1	Fr-2	Fr-3
Fucose	1.1† [6.7]‡	Trace	ND§	ND
Glucose	Trace	ND	ND	ND
Mannose	12.1 [67.2]	12.4 [68.8]	ND	ND
Galactose	8.3 [46.1]	8.8 [48.8]	ND	ND
<i>N</i> -acetylglucosamine	45.8 [207.0]	42.1 [190.3]	ND	ND
Sialic acid	8.5 [27.5]	7.8 [25.2]	ND	ND

\* Refers to method, Fr-1: delipidated HB<sub>s</sub>Ag, apoprotein; Fr-2: upper layer of re-extraction of chloroform-methanol-water from lipid fraction (Ex-1, Ex-2, Ex-3 and Ex-4); Fr-3: lower layer of re-extraction of chloroform-methanol-water from lipid fraction (Ex-1, Ex-2, Ex-3 and Ex-4).

† Value represents µg/mg of protein and the average of two independent experimental preparations.

‡ Value expressed in brackets represents nmol/mg protein.

§ ND: not detected.

|| Sialic acid was determined by the method of Aminoff (1961) using 2-thiobarbituric acid.

Each sample was dissolved in dry pyridine and subjected to trimethylsilylation with hexamethyldisilazane and trimethylchlorosilane by heating at 100 °C for 10 min. The mono-saccharide methylether thus trimethylsilylated was examined by gas chromatography using a Model GC-6A Shimadzu gas chromatograph (Shimadzu Co. Ltd., Kyoto, Japan), equipped with a flame ionization detector. Separation was carried out on a 2 m glass column packed with 3% OV-1 (Nihon Chromato Co. Ltd., Tokyo, Japan). The column temperature was programmed from 140 to 200 °C at 0.5 °C/min from the time of injection and the injector and detector temperatures were 220 °C. Carrier gas (N<sub>2</sub>) flow rate was 60 ml/min, H<sub>2</sub> gas 50 ml/min and air 850 ml/min. Peak areas were measured with a digital integrator Model ITG-4A (Shimadzu Co. Ltd.).

Total hexose was determined by the phenol-sulphuric acid method (Hodge & Horfreiter, 1962), sialic acid by the method of Aminoff (1961) using 2-thiobarbituric acid, and protein by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

The results obtained are given in Table 1. The main components were *N*-acetylglucosamine, mannose, galactose, and sialic acid, and the minor one was fucose. No *N*-acetylgalactosamine was detected. The hexose contents in intact HB<sub>s</sub>Ag and Fr-1 by the phenol-sulphuric acid method were 25.0 and 23.1 µg/mg of protein respectively. These values were very close to those (20.4 µg, mannose and galactose) obtained by gas chromatography. Results previously reported with native HB<sub>s</sub>Ag were obtained only by the phenol-sulphuric acid method

and no reference was made to the presence of any hexosamine and sialic acid (Burrell *et al.* 1973; Chairez *et al.* 1973). The high contents of *N*-acetylglucosamine and mannose detected here are in accordance with the data that HB<sub>s</sub>Ag binds concanavalin A (Neurath, Prince & Lippin, 1973), since concanavalin A reacts with mannose, glucose and *N*-acetylglucosamine. Furthermore, the fact that no sugar was detected either in Fr-2 or Fr-3 suggests that sugar in HB<sub>s</sub>Ag exists almost exclusively in the form of glycoprotein. Although glycolipids have been reported by Steiner *et al.* (1974), no evidence was found here for their presence in HB<sub>s</sub>Ag.

Recently it was shown that oligosaccharide chains are joined to the glycopeptide backbone by asparagine-*N*-acetylglucosamine linkage in vesicular stomatitis virus (Moyer *et al.* 1976), and serine- or threonine-*O*-acetylgalactosamine linkage in reovirus (Krystal, Perrault & Graham, 1976). That a large amount of *N*-acetylglucosamine was present and *N*-acetylgalactosamine was absent may suggest that the linkage between protein and sugar in HB<sub>s</sub>Ag glycoprotein should occur via *N*-acetylglucosamine to asparagine and not via *N*-acetylgalactosamine to serine or threonine. It is also known that at least two types of structural distinctive oligosaccharide commonly occur in glycoprotein molecules, one of which is acidic and complex in sugar composition, and the other which consists only of mannose and *N*-acetylglucosamine (Johnson & Clamp, 1971). From the sugar composition of HB<sub>s</sub>Ag (Table 1), it is possible that both types of sugar chains occur in HB<sub>s</sub>Ag. Finally, because of the small size of the putative genome of this virus (Robinson, Clayton & Greenman, 1974), it is unlikely to carry the structural genes for enzymes necessary for oligosaccharide synthesis, as has been pointed out for other small enveloped viruses (Sefton, 1976).

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