Studies in Immunochemistry

17. THE DECOMPOSITION OF SEROLOGICALLY SPECIFIC H SUBSTANCE ISOLATED FROM PIG GASTRIC MUCIN AND HUMAN OVARIAN CYST FLUIDS BY AN ENZYME PREPARATION OBTAINED FROM *CLOSTRIDIUM WELCHII* (TYPE B)*

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Schiff (1935, 1939) reported that bacteria-free filtrates from cultures of certain strains of *Clostridium welchii* destroyed the serological character of the blood-group A substance in commercial pepsin and human saliva. This finding has been confirmed by many workers. Meyer (1945) observed that an enzyme obtained from *Cl. welchii* liberated free reducing sugars from a neutral mucopolysaccharide which had been isolated from pig gastric mucin (Meyer, Smyth & Palmer, 1937) and which possessed high blood-group A activity. This carbohydrate was later shown to contain two neutral mucopolysaccharides, each of which had similar physical and chemical properties but showed a different serological specificity (Bendich, Kabat & Bezer, 1946; Aminoff, Morgan & Watkins, 1946; Chadwick, Smith, Annison & Morgan, 1949). The digestion of those pig-stomach linings which were free from blood-group A activity gave a neutral mucopolysaccharide which was closely similar in its physical, chemical and serological properties to a mucopolysaccharide found in the water-soluble secretions of the majority of persons belonging to blood group O (Landsteiner & Harte, 1941; Witebsky & Klendshoj, 1941; Morgan & Waddell, 1945). The serological specificity of this material has been designated by the symbol H (Morgan & Watkins, 1948, 1955). The H substance obtained from pig gastric mucosa is of special interest owing to its close similarity to the human H substance and because it can be more easily obtained in a high state of purity and in amounts sufficient for detailed structural studies.

An enzyme which brings about the rapid inactivation of H substances of human and animal origin was obtained from *Cl. welchii* (type B) culture filtrates by Stack & Morgan (1949), who devised a procedure for its purification, described a simple semi-quantitative method for the measurement of enzymic activity and recorded some properties of the enzyme, such as its heat stability, pH of optimum activity, etc. This enzyme preparation was used by Crumpton & Morgan (1953) to inactivate and degrade purified H substance obtained from pig-stomach linings, and it was found that fucose, *N*-acetylglucosamine, galactose and a small amount of a disaccharide composed of *N*-acetylgalactosamine and galactose were liberated from the macromolecule.

Iseki & Tsunoda (1952) obtained from soil an organism which produced an enzyme that specifically inactivated H substance, and Iseki & Masaki (1955) reported that when this enzyme acted on H substance a large part of the fucose contained in it was liberated in a dialysable form but that no other component was set free and no change was induced in the moiety containing amino acid.

Watkins (1955*b*) showed that an enzyme could be extracted from the protozoan flagellate *Trichomonas foetus* which rapidly inactivated H substance and liberated reducing sugars. Part of the material became diffusible through a cellophan membrane and chromatographic examination showed that L-fucose was the main sugar liberated together with a small amount of *N*-acetylhexosamine; galactose appeared to be absent.

The investigations recorded in the present paper are an extension of earlier work (Crumpton & Morgan, 1953) and include some preliminary observations on the enzymic degradation of the H-specific substance of human origin.

METHODS

The determinations of reducing sugars and hexosamines were carried out on materials after hydrolysis with 0·5 N-HCl for 16 hr. at 100°. All other estimations were made on intact materials.

Nitrogen. Nitrogen was determined by the Kjeldahl method with the apparatus of Markham (1942).

Methylpentose. The method of Dische & Shettles (1948), with procedure CyR 10, and later the method as modified by Gibbons (1955), was employed. Fucose standards were included in each determination. Fucose was also determined as acetaldehyde after its liberation from mucopolysaccharide by mild acid and oxidation with periodate in

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Conway units, according to the method of Winnick (1942) as used by Aminoff, Morgan & Watkins (1950).

Hexosamine. A modification (Rondle & Morgan, 1955*a*) of the method of Elson & Morgan (1933) was used. Glucosamine hydrochloride standards in the range 10–50 μg. were included in each determination. Extinctions were read at 530 mμ. with a spectrophotometer. Results are given as hexosamine base.

Reducing sugars. Determined by the method of Somogyi (1937) or by the colorimetric technique of Nelson (1944). Reducing values are expressed throughout as percentage reduction of an equal weight of glucose or as mg. of glucose.

Equivalent N-acetylglucosamine value. This was determined under the conditions recommended by Aminoff, Morgan & Watkins (1952). The results are expressed as a percentage of the colour given by an equal weight of *N*-acetylglucosamine.

Physical examinations. Ultracentrifugal and electrophoretic examinations were carried out as described by Kekwick (1950).

Enzyme activity. Measured as described by Stack & Morgan (1949). The unit of enzymic activity is that amount of activity which will bring about an extent of inactivation of 1 mg. of H substance in 2 hr. at 37° and pH 7 which results in the destruction of 94% of its serological activity. In practice this consists of bringing about a lowering of the inhibiting titre by four tubes when the usual twofold geometrical-dilution scale is employed.

Separation of glucosamine and galactosamine. According to the method of Gardell (1953) as described by Rondle & Morgan (1955*b*), with a column filling of Zeo-Karb 225 and 0·34 N-HCl as eluent.

Chromatography. Chromatograms were run on Whatman no. 1 or 4 paper with (*a*) *n*-butanol–acetic acid–water (4:1:5, by vol.), or (*b*) *n*-butanol–pyridine–water (6:4:3, by vol.), or (*c*) water-saturated *S*-collidine, both the descending and ascending methods being used. In certain instances, in order to obtain a better separation of the components, the papers were dried and run a second time in the same solvent system. Aniline hydrogen phthalate (Partridge, 1949), *p*-anisidine–HCl (Hough, Jones & Wadman, 1950) and benzidine–acetic acid (Horrocks, 1949) sprays were used to detect reducing sugars; ninhydrin was used for amino acids and amino sugars, and alkaline acetylacetone followed by the *p*-dimethylaminobenzaldehyde–HCl reagent as a more specific test for hexosamines (Partridge, 1948). The method of Gardell, Heijkenskjöld & Roch-Norlund (1950), as modified for paper chromatography by Stoffyn & Jeanloz (1954), was used to distinguish glucosamine from galactosamine.

Serological activity. The H activity of the original materials and of the enzymic- and acid-hydrolysis products was determined by the method of inhibition of haemagglutination with (*a*) an immune rabbit anti-H serum (Morgan & Waddell, 1945), (*b*) the human anti-H serum 'Warboys' (Watkins, 1952), and (*c*) the human serum 'Tomlinson' (Watkins & Morgan, 1954). These sera reacted preferentially with group O cells and were completely inhibited by extremely small amounts of H substance. The pneumococcus type XIV activity of the different materials was measured by precipitation-inhibition tests as described by Kabat, Baer, Bezer & Knaub (1948).

MATERIALS

Pig H substance. The preparations of H substance were obtained from pig gastric mucosa according to the method of Bendich *et al.* (1946). The crude mucin obtained from several stomachs, each of which possessed H activity only, was purified by extraction with 90% (w/v) phenol (Morgan & King, 1943) and precipitated from solution in phenol by addition of ethanol to a concentration not exceeding 10% (v/v). The mucopolysaccharide was fractionated further either from solution in 90% (w/v) phenol with ethanol or from aqueous solution with ethanol, or from both. The material precipitating from aqueous solution between 50 and 53% (v/v) ethanol concentrations was most active serologically; it was dissolved in water, dialysed and dried from the frozen state.

Three preparations made from batches of stomach linings were used in the main experiments. The analytical figures for the H materials are given in Table 1. The values given are uncorrected for ash, which was less than 2% of the weight of H substance. Preparations nos. 2 and 3 gave single, symmetrical boundaries in U-tube electrophoresis at pH 4·0 (acetate buffer *I* 0·1) and at pH 8·0 (phosphate buffer *I* 0·2). Examination at pH 8·0 in a Svedberg ultracentrifuge revealed in both preparations a single major component and a small amount of a second substance which sedimented slightly faster. The ultraviolet-absorption spectrum showed only a weak general absorption between 260 and 310 mμ. A fractional-solubility test carried out as described by Aminoff *et al.* (1950), with water as solvent, failed to reveal any gross inhomogeneity.

Human H substance. Isolated from a human ovarian-cyst fluid (no. 247), as described by Morgan & Waddell (1945) and Annison & Morgan (1952). The analytical figures are given in Table 1.

Enzyme preparations. The enzyme was isolated from *Cl. welchii* (type B) culture filtrates (strains CN 1253 and CN 1990, National Type Culture Collection) grown in peptone medium (3% of Evans peptone and 2·5% of sodium β-glycerophosphate) freshly supplemented with glucose and thioglycollic acid as described by Stack & Morgan (1949). The enzyme was recovered by precipitation of the culture filtrate with an equal volume of acetone and purified by reprecipitation from aqueous solution between 25 and 31%

Table 1. *Analytical figures of H substances*

Preparation	N (%)	Fucose (%)	Hexosamine* (%)	Reduction* (%)	Equivalent *N*-acetylglucosamine (%)
H substance no. 1	5·5	9·8	27	49	10·5
H substance no. 2	6·0	10·6	32	51	12·8
H substance no. 3	5·9	10·0	29	50	12·6
Human H substance	5·5	9·8	27	49	4·5

* After hydrolysis with 0·5 N-HCl for 16 hr. at 100°.

saturation of $(NH_4)_2SO_4$, or up to 40% (v/v) of ethanol. Fractionation with $(NH_4)_2SO_4$ was carried out at about 2°, and with ethanol and acetone at about −10°. The selected fractions were finally dialysed against water and dried from the frozen state. All enzyme experiments were made in the presence of McIlvaine buffer (McIlvaine, 1921) at pH 6·8–6·9.

Morgan (1946), Stack & Morgan (1949) and Crumpton (1953) showed that the enzyme which inactivated the H substance was stable on heating in buffer at pH 6·8 for 10 min. at 55°, whereas many of the accompanying enzymes were destroyed. To reduce the number of enzymes present which were not directly involved in the destruction of the serological activity, all the enzyme preparations used in the experiments to be described were heated at 55° for 10 min. The analytical figures for enzyme preparation no. 1 were: N, 8·5%; reducing power, 1·1 and 16% before and after acid hydrolysis respectively; hexosamine about 2% and fucose 0·5%. These values were typical of the other enzyme preparations examined, the N values of which were between 8 and 12%; the reducing values after acid hydrolysis were usually low and the fucose and hexosamine content was less than 2%.

EXPERIMENTS AND RESULTS

Decomposition of H substance by Clostridium welchii *enzyme*

Expt. 1. H substance (prep. 1, 896 mg.) was dissolved in 80 ml. of water saturated with toluene and incubated for 7 hr. at 37° with 10 ml. (about 4000 units) of 1% (w/v) solution of enzyme (prep. 1) in buffer. After incubation less than 5% of the original H activity, measured by immune-rabbit anti-H serum, remained. The mixture of inactive H substance and enzyme was quantitatively transferred to a thoroughly washed cellophan bag (Cellophane sheet no. 300 supplied by British Cellophane Ltd.) and dialysed for a week at 2° against daily changes of water to separate the hydrolysis products into diffusible and indiffusible materials. The diffusates were stored with added toluene at 2° until dialysis was complete, concentrated under reduced pressure at about 15° and made up to a known volume. The indiffusible material was recovered by drying the contents of the cellophan bag from the frozen state.

The total nitrogen, fucose, hexosamine and reducing sugar in the diffusible and indiffusible hydrolysis products were determined.

In order to interpret quantitatively the results it is necessary to know how much of the added enzyme preparation was diffusible and the composition of the diffusible and indiffusible material. As a control in Expt. 1 the enzyme [10 ml. of 1% (w/v) solution in buffer] was therefore dialysed after it had been incubated for 7 hr. in the same final volume (90 ml.) as that used in the experiment containing H substance. This treatment was considered to simulate as closely as possible the conditions to which the enzyme preparation was exposed during incubation with the substrate. The analytical figures for the diffusible and indiffusible part of the enzyme were determined as in the main experiment.

Expt. 2. The procedure used was the same as given in Expt. 1 above. H substance (prep. no. 2, 444 mg.) dissolved in 39 ml. of water was incubated for 7 hr. at 37° with 5 ml. (about 590 units, 4·4 mg. of nitrogen) of 1% (w/v) solution of enzyme (prep. no. 2) in buffer. After incubation not more than 3% of the original serological activity remained. The products were separated into diffusible and indiffusible materials and analysed as described in Expt. 1. The enzyme control (50 mg. of enzyme in 44 ml. of dilute buffer, incubated for 7 hr. at 37°) was similarly treated. In Expt. 2, however, an additional or 'substrate' control was included. H substance (90 mg. in 8 ml. of water) was incubated for 7 hr. with 4 mg. of enzyme which had previously been heated in 1 ml. of buffer at 100° for 10 min. The substrate was recovered after dialysis and the analytical composition found to be close to that of the original material. The slight differences found between the recovered material (nitrogen, 6·1; fucose, 9·7; hexosamine, 30%; reduction, 50%), which was serologically fully active, and the original H substance (prep. no. 2) were most probably due to the presence of some enzyme material.

Expt. 3. H substance (prep. no. 3, 862 mg.) dissolved in 83 ml. of water and incubated for 7 hr. with 78 mg. (2200 H units, 8·7 mg. of nitrogen) of enzyme (prep. no. 3) in 10 ml. of buffer. The specific serological activity of the substrate measured against the rabbit anti-H serum was completely destroyed. The products of hydrolysis were recovered and analysed as were the corresponding fractions obtained from the enzyme control.

Expt. 4. Human H substance (779 mg.) in 80 ml. of water was incubated with 4 ml. of 1% (w/v) enzyme solution (about 1600 units). After incubation for 4 hr. a further 4 ml. of enzyme solution was added and incubation continued for an additional 3 hr. The products of hydrolysis were separated into diffusible and indiffusible fractions and analysed. An enzyme control experiment was included. The results are given in Table 4.

Examination of the products of enzymic hydrolysis

Reliable weights for the diffusible substances, which were recovered as hygroscopic sugar syrups, were not obtained; an approximate estimate of their amount was given by deducting the weight of the indiffusible substances, corrected for enzyme content, from the weight of the original substrate. The results showed that one-fifth to one-third of the substrate became diffusible in different experiments, a variation which is presumably due largely to the

enzyme preparation employed. Further decomposition then ceased, although the enzyme, after dialysis and removal of certain of the hydrolysis products, was found to be active when tested on fresh substrate. In each of the four main experiments described not less than 95% of the original serological activity was destroyed.

Physical properties. The immediate action of the enzyme preparation on solutions of the H substance was to bring about a rapid fall in their viscosity. The H substance in Expt. 2, for example, showed a relative viscosity of 26 when measured in an Ostwald-type viscometer at 1% (w/v) concentration in 0·85% saline at 37°, whereas the indiffusible material remaining after the action of the enzyme and accounting for about three-quarters of the original substrate gave a value close to 5 (saline 1). In Expts. 2 and 3 the indiffusible residues (1%, w/v) were examined in the ultracentrifuge at pH 8 (sodium phosphate buffer, *I* 0·2, 0·15M-NaCl). Apart from a small quantity of rapidly sedimenting substance, which was visible in the early schlieren diagrams only and was most probably enzyme protein, the materials gave sharp boundaries, sedimented at a slightly slower rate than did the original H substances and behaved as might be expected for a single substance. The indiffusible material obtained from Expt. 3 was examined electrophoretically at pH 8 and was found to contain one major (about 90%) and at least one minor component. The ultraviolet-absorption spectrum of the product was similar to that of the original H substance.

Chemical properties. The analytical values obtained for the original substrates and for the diffusible and indiffusible products in Expts. 1–3 are given in Table 2. The values shown are corrected for the contribution made by the enzyme to the particular material estimated. The corrections necessary in each instance are relatively small (Table 3).

The figures given (Table 2) show that fucose was preferentially liberated, for whereas in Expts. 1–3 about 31, 25 and 29% of the substrate became diffusible, 37, 57 and 71% of the total fucose respectively was liberated in each instance.

Table 2. *Amounts and distribution of nitrogen, fucose, hexosamine and reducing sugars in the enzymic hydrolysis products of H substance*

	N			Fucose			Hexosamine*			Reducing sugar*		
Expt. no.	1	2	3	1	2	3	1	2	3	1	2	3
Original H substance (mg.)	49	27	51	88	47	86	245	140	247	435	227	433
Indiffusible material (mg.)	39	25	47	61	23	25	179	96	144	339	157	256
Diffusible material (mg.)	9	3	7	32	27	61	53	38	80	98	78	158
Components accounted for (%)	96	103	106	106	106	100	95	96	91	101	103	96
Amount diffusible (%)	18	11	14	37	57	71	22	27	32	23	34	36

* After hydrolysis with 0·5N-HCl for 16 hr. at 100°.

Table 3. *Values of the corrections applied to the enzymic hydrolysis products for the enzyme material present in (a) the diffusible material and (b) the indiffusible residue*

	Nitrogen (mg.)		Fucose (mg.)		Hexosamine* (mg.)		Reduction* (mg.)	
Expt. no.	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
1	2·0	5·3	<1	<1	<1	1·2	3	13
2	0·4	2·4	<1	<1	<1	<1	<1	<1
3	2·2	4·9	<1	<1	<1	1·1	4	9
4	2	5·5	<1	<1	<1	1·5	3·3	16

* After hydrolysis with 0·5N-HCl for 16 hr. at 100°.

Table 4. *Distribution of components after treatment of human H substance with* Clostridium welchii *enzyme*

Component	H substance (mg.)	Indiffusible material (mg.)	Diffusible material (mg.)	Amount of component accounted for (%)	Component diffusible (% of total)
Nitrogen	59	39	5·8	76	10
Reduction	9	27	60	—	—
Reduction*	372	232	81	84	22
Fucose	114	65	29	83	26
Hexosamine*	160	115	38	96	24

* After hydrolysis with 0·5N-HCl for 16 hr. at 100°.

Concomitant with the release of diffusible material during enzymic hydrolysis of H substance there was a rapid formation of reducing groups in both the diffusible and indiffusible products. The H substance used in Expt. 1 (896 mg.) gave a reducing value equivalent to 9 mg. of glucose or about 1% reduction, whereas the indiffusible residue (616 mg.) showed a reducing power equal to 33 mg. of glucose or nearly 5·4% reduction. The diffusible material contained a much larger proportion of reducing groups, equivalent in reduction to 59 mg. of glucose, or about 21% on a weight basis. If the whole of the fucose (32 mg.) in the diffusible material had been present as free sugar, the reduction expected would correspond to about 15 mg. of glucose, as fucose has a reducing power equivalent to about 45% of its weight of glucose. It is evident, therefore, that a part of the reducing power of the diffusate arises from sugars other than fucose. Similar results were obtained in Expts. 2 and 3, where the diffusible materials gave a reducing power equivalent to 63 and 113 mg. of glucose respectively, or 57 and 45% reduction of the weights (111 and 251 mg.) obtained by subtracting the weights of the indiffusible materials, corrected for enzyme content, from the weights of H substances used.

In Expt. 1 (Table 2), of the 245 mg. of total hexosamine in the original substrate 179 mg. remained in the indiffusible residue, and the diffusible material contained 53 mg.; thus 95% of the total hexosamine was accounted for. The ratio of glucosamine to galactosamine in the H substance used was 3·2:1, whereas in the diffusate the ratio was found to be 5·5:1; a preferential release of glucosamine had occurred during the early stages of hydrolysis.

Amino acids. The amino acids contained in the H substance were not liberated to any appreciable extent in a diffusible form during the early stages of enzymic hydrolysis. The analytical figures on which this statement is made, however, are based on the difference between the total nitrogen and the total hexosamine nitrogen, and not on a direct determination of amino acid nitrogen. A part of the small amount of amino acids found in the diffusates undoubtedly comes from autolysis of protein in the enzyme preparation.

Action of mild acid. The rate of liberation of fucose from H substance and from the indiffusible residue obtained in Expt. 2 during hydrolysis with N acetic acid at 100° is shown in Fig. 1. The liberation of fucose from H substance ceased after about 36 hr. and the indiffusible material gave no further diffusible fucose after hydrolysis for about 18 hr. The maximum amounts of fucose set free were 8·6 and 4·2% respectively of the materials used. The total fucose content of the H substance and of the indiffusible material was 10·6 and 6·8% respectively and therefore these two materials contained 2·0 and 2·6% of 'acid-stable' fucose. The original H substance contained 8·9 mg. (2·0% of 444 mg.) of fucose resistant to hydrolysis with mild acid, whereas the indiffusible residue (333 mg.) obtained from 444 mg. of H substance (Expt. 2) contained 8·7 mg. (2·6% of 333 mg.) of 'acid-stable' fucose. This result suggests that in the H substance there are fucose molecules not readily liberated by the enzyme or by acid hydrolysis.

Action of alkali. It has been long known that treatment of the H substance with dilute alkali under carefully controlled conditions gives a chromogenic structure which forms a coloured complex with *p*-dimethylaminobenzaldehyde–HCl reagent (Morgan & King, 1943; Morgan, 1947; Morgan & Waddell, 1945; Annison & Morgan, 1952), and that the formation of the chromogen depends on the presence of *N*-acetylhexosamine containing a reducing end-group (Aminoff *et al.* 1952) and on the presence of an unsubstituted OH group attached to C-4 (Kuhn, Gauhe & Baer, 1954*b*). A preformed chromogenic structure was present neither in the H substance nor in the diffusible or indiffusible materials derived from it by enzymic hydrolysis. However, the products of enzymic hydrolysis gave a chromogenic structure on treatment with dilute alkali which showed an absorption spectrum closely similar to that given by H substance or *N*-acetylglucosamine after similar treatment.

The maximum colour intensity obtained with known weights of these materials can be expressed

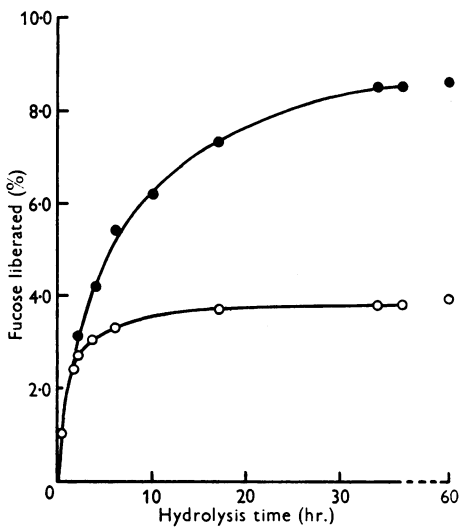

Fig. 1. Rate of liberation and amount of fucose liberated by mild acid hydrolysis from H substance (prep. no. 2; ●) and from the indiffusible material (○) formed by treatment with *Cl. welchii* enzyme preparation.

in terms of the maximum colour intensity developed under closely defined conditions by *N*-acetylglucosamine (see Aminoff *et al.* 1952). It is necessary, however, to determine for each product the optimum time of heating at 100° with alkali (0·05 N-Na_2CO_3) which subsequently gives the maximum colour intensities at 550 mμ. and 590 mμ. on the addition of the *p*-aminodimethylbenzaldehyde–HCl reagent. For *N*-acetylglucosamine and *N*-acetylgalactosamine the optimum heating period under the conditions employed was 4 min. The undegraded H substance required 8–12 min. heating with alkali, the indiffusible materials 6–8 min., and the diffusible products 3–4 min., the times varying somewhat with the products obtained in different experiments.

The maximum colour intensities determined under these conditions for the materials obtained in Expts. 1–3, and expressed as mg. of *N*-acetylglucosamine and as per cent of the weight of the fractions, are given in Table 5. It is known that there is some destruction of the chromogen under the conditions selected for its formation, that free *N*-acetylgalactosamine gives only about one-fifth of the colour given by an equal weight of *N*-acetylglucosamine (Aminoff *et al.* 1952), that *N*-acetylglucosamine substituted in the fourth hydroxyl group gives no colour (Kuhn *et al.* 1954*b*) and that certain oligosaccharide structures give more colour than their content of hexosamine would lead one to expect (Kuhn, Gauhe & Baer, 1954*a*). For these reasons, the full significance of the final equivalent *N*-acetylglucosamine values obtained for each material cannot be fully appreciated; nevertheless, the figures are of considerable value for comparative purposes and show that the indiffusible products give a similar amount of '*N*-acetylhexosamine' colour to that found for the original H substance, whereas the diffusible materials give a colour equivalent to up to one-half their weight of *N*-acetylglucosamine.

The diffusible materials, on treatment with 0·05 N-Na_2CO_3 at room temperature, formed a certain amount of chromogen, whereas after similar treatment *N*-acetylglucosamine and *N*-acetylgalactosamine failed to undergo this change. A substance reactive in alkali under these conditions was isolated by paper chromatography (see below), but was not completely identified. In view of the fact that *O*-β-D-galactopyranosyl-(1 → 3)-*N*-acetyl-D-glucosamine, an alkali-sensitive disaccharide, has been isolated from group A substance (Côté & Morgan, unpublished work) it seems probable that a similar structure is present in H substance and is responsible for the sensitivity to alkali and the ease of chromogen formation. The substance gave a chromogenic structure on treatment in aqueous $BaCO_3$ suspension (pH 8·4) for 15 min. at 100° (cf. Knox, 1954; Knox & Morgan, 1954), as did the *O*-β-D-galactopyranosyl-(1 → 3)-*N*-acetyl-D-glucosamine. Under these extremely mild conditions *N*-acetylglucosamine and *N*-acetylgalactosamine require about 4 hr. and H substance 48 hr. to give maximum chromogen formation.

The results obtained in Expt. 4, in which the products of enzyme hydrolysis of the human H substance were treated with alkali to determine the ease and extent of chromogen formation (Aminoff *et al.* 1952), are given in Table 5; the materials behaved as did those obtained from the pig H substance.

Serological properties. The H substances and their indiffusible and diffusible enzymic hydrolysis products were examined serologically with different anti-H sera. Tested for activity against rabbit-immune H serum and the human anti-H serum 'Tomlinson' the indiffusible materials from Expts. 1, 2 and 4 showed only a few per cent of the activity of the original H substance, whereas the products obtained in Expt. 3 possessed no demonstrable activity. However, the capacity of these materials to react with the anti-H sera 'Warboys' was unimpaired.

The indiffusible material obtained from Expt. 2, when tested at 0·1 % (w/v) concentration, failed to neutralize the following agglutinins: human anti-A with A_1 or A_2 test-cells; natural human and immune-rabbit anti-B, anti-Le^a, anti-Le^b, anti-M, anti-N, anti-P and anti-*Rhesus* sera (including anti-D, anti-C, anti-c and anti-E test reagents). The diffusible material was inactive with all the test antisera.

Table 5. *Equivalent* N-*acetylglucosamine value of the H substance and of the enzymic hydrolysis products after treatment with alkali and the addition of the* p-*dimethylaminobenzaldehyde*–HCl *reagent*

Maximum colour intensity obtained expressed as (*a*) mg. of *N*-acetylglucosamine and (*b*) as % of weight of fraction.

Expt. no.	H substance		Indiffusible material		Diffusible material	
	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)
1	93	10	78	11	72	25
2	57	13	44	13	50	45
3	109	13	74	12	104	41
4	34	4	44	7	45	19

The capacity of the H substance and of the indiffusible enzymic hydrolysis products to react with the anti-H serum 'Warboys' was readily destroyed on treatment with N acetic acid at 100°.

The enzymic hydrolysis products obtained from H substance failed to precipitate with horse antipneumococcus type XIV serum. The products obtained from the H substance after hydrolysis with acetic acid for 2 hr., however, reacted strongly with this serum, but the products lost this property on treatment with the *Cl. welchii* enzyme. The type XIV serum failed to precipitate with its homologous specific polysaccharide after this material had been treated with the enzyme.

Chromatographic analysis. The hydrolysate obtained by treating the diffusible materials with 0·5N-HCl for 16 hr. at 100° was subjected to paper chromatography in some or all of the solvent systems given under Methods. It gave spots whose R_F values and colours were identical with those of fucose, galactose and the two hexosamines run at the same time; no other sugars were detected.

The unhydrolysed diffusible materials gave two strong spots and one weak spot with aniline hydrogen phthalate which agreed in position with those found for fucose, galactose and *N*-acetylglucosamine run in *s*-collidine at the same time. Two reddish purple spots appeared after spraying with the hexosamine reagents. One of these occupied a position identical with that found for *N*-acetylglucosamine (R_F 0·50); *N*-acetylgalactosamine moves at about the same speed in *s*-collidine. The other spot, R_F about 0·25 in *s*-collidine, could not be identified with any of the simple sugars included for comparison. The material responsible for this unidentified spot was characterized as follows: unhydrolysed diffusate was taken to dryness, reconstituted in 1 ml. of water and examined by the usual method of band chromatography with marginal vertical strips to detect the position of the different components. The chromatograms were developed in *s*-collidine for 40 hr. The eluate containing the material (R_F 0·25) was taken to dryness, the residue dissolved in 1·5 ml. of water and 1 ml. of this solution was hydrolysed at 100° in 0·5N-HCl for 16 hr. The hydrolysed and unhydrolysed eluates were then re-examined chromatographically with *s*-collidine as solvent. The unhydrolysed eluate gave a weak or negative reaction with aniline hydrogen phthalate, but after acid hydrolysis gave a strongly positive reaction; the position of the spot with this reagent now corresponded with that of galactose run at the same time. The unhydrolysed eluate gave with the hexosamine reagents a reddish purple colour and possessed an R_F value identical with that found for the unidentified sugar in the original diffusate. The eluate after hydrolysis with 0·5N-HCl at 100° for 16 hr. and running in *s*-collidine gave two spots with the hexosamine reagents which corresponded in position and colour (cherry-red) with those expected for glucosamine and galactosamine (see Aminoff & Morgan, 1948). The galactosamine spot was much less intense than that due to glucosamine. These results suggested that the unidentified material could be a mixture of two (or more) disaccharides composed of *N*-acetylglucosamine or *N*-acetylgalactosamine joined to galactose. The presence of a trisaccharide was not excluded but it seemed improbable that higher oligosaccharides were involved.

The results obtained by spraying the chromatograms of the diffusible material with ninhydrin, aniline hydrogen phthalate and hexosamine reagents indicated that probably a relatively small amount only of the total diffusate remained on or close to the base line in the paper chromatograms.

Chromatograms of the indiffusible materials, which had been hydrolysed at 100° with 0·5N-HCl for 16 hr., or with 6N-HCl for 2 or 4 hr., revealed the presence of fucose, galactose, glucosamine and galactosamine and the same amino acid mixture as was present in the original H substance.

Repeated treatment with enzyme

Pig H substance. Treatment of the indiffusible residue obtained in Expt. 1 with a further amount of enzyme showed that some additional hydrolysis occurred. The analytical figures obtained for the diffusible and indiffusible materials are given in Table 6. About one-third of the substrate became diffusible and this material after treatment with dilute alkali and *p*-dimethylaminobenzaldehyde reagent gave a colour equivalent to 27 mg. of *N*-acetylglucosamine. Chromatographic examination of the diffusate showed that oligosaccharide units larger than disaccharides were probably not present to any appreciable extent. The reducing power of the diffusible material, equivalent to 33 mg. of glucose, indicated that a considerable part of the carbohydrate consisted of small reducing sugar units.

The indiffusible residue was recovered and treated again with enzyme. About 15% of the substrate became diffusible and this material contained 3·7 mg. of nitrogen or about 14% of the original total nitrogen (26 mg.). A much smaller amount (3 mg.) of the total fucose (29 mg.) was released in a diffusible form this time and was accompanied by 10 mg. or about 10% of the total hexosamine. The reducing power of the diffusible material, before and after acid hydrolysis, was equivalent to 11 and 21 mg. of glucose respectively, which indicated again that the major part of the diffusible carbohydrate was made up of small reducing sugar units.

Table 6. *Distribution of components after treatment of the indiffusible material from Expt. 1 with additional* Clostridium welchii *enzyme*

Component	Substrate indiffusible material from Expt. 1 (mg.)	Indiffusible material (mg.)	Diffusible material (mg.)	Amount of component accounted for (%)	Component diffusible (% of total)
Nitrogen	41	28	6·1	85	15
Reduction	31	19	33	—	—
Reduction*	327	221	58	85	18
Fucose	57	34	18	91	32
Hexosamine*	167	111	26	83	16

* After hydrolysis with 0·5N-HCl for 16 hr. at 100°.

Human H substance. The indiffusible material (397 mg.) obtained in Expt. 4 was treated with a further amount of enzyme (40 mg.) for 7 hr. at 37°. An indiffusible residue (261 mg.) was obtained which contained 15 mg. of nitrogen, 105 mg. of reducing sugar after acid hydrolysis, 48 mg. of hexosamine and 28 mg. of fucose. The diffusible material contained 5·6 mg. of nitrogen, 23 and 47 mg. of reducing substances (as glucose) before and after acid hydrolysis, 12 mg. of fucose and 18 mg. of hexosamine. About one-quarter of the starting material diffused through the cellophan membrane after treatment with enzyme and it is again evident that there was a considerable increase in the number of reducing groups present, especially in the diffusible material.

Action of other enzyme preparations on H substance

The following materials, shown to contain an α-glucosaminidase, failed to destroy the serological activity of H substance in 24 hr. at pH 6·9: crude ox-testis extract; Rondase (hyaluronidase, Evans) and an extract of snail (*Helix aspersa*) digestive tract. Similarly, preparations of β-galactosidase derived from emulsin, snail juice and *Bacterium coli* (strain M.L. 308) did not cause any measurable loss of serological activity of the H substance.

DISCUSSION

An attempt has been made to determine the changes induced in H substances of animal and human origin by an enzyme preparation obtained from culture filtrates of *Cl. welchii* (type B). The animal H preparations were obtained from pig-stomach linings and the human specific substance from an ovarian-cyst fluid. The materials possessed similar physical, chemical and serological properties and showed no gross inhomogeneity when examined by fractional-solubility tests, by electrophoresis or by ultracentrifuging. Each material contained fucose, galactose, glucosamine, galactosamine and eleven amino acids, and inhibited, in extremely small amounts, the agglutination of O cells by certain human and animal anti-H agglutinins. The serological specificity of the materials was almost completely destroyed by 10% (w/v) of the enzyme preparation acting for 7 hr. at pH 6·8–7·0 at 37°.

The action of the enzyme on the H substances caused an immediate and rapid fall in the viscosity and serological activity, and liberated in a diffusible form a part of the fucose, galactose and *N*-acetylhexosamine present. After hydrolysis sufficient to bring about almost complete serological inactivation of the substrate, measured against a rabbit-immune anti-H serum, the products were in each instance separated into materials which were diffusible or indiffusible through a cellophan membrane. The extent to which the products were contaminated by the enzyme preparation was determined from control experiments and a correction made to the analytical figures obtained.

The distribution of the various components of the macromolecule in the diffusible material was found to be very different from that which might be expected if each sugar and amino acid residue had been liberated to a similar extent in a diffusible form and it was immediately apparent that there was a preferential release of fucose.

Chromatographic examination of the diffusates indicated that a considerable part of these materials consisted of single sugar residues together with small amounts of di- and possibly higher saccharides. This conclusion is supported by the high reducing power found for the diffusates which contain much fucose, a sugar which gives a reduction equivalent to rather less than one-half of its weight of glucose. Although it is possible that oligosaccharide units greater than disaccharides exist in the diffusates to a small extent, it is now known from the results of partial hydrolysis of the group substances with mild acid that oligosaccharides which contain fucose and *N*-acetylhexosamine residues move relatively fast on paper chromatograms in the solvents used, and might in consequence appear in a position normally occupied by smaller saccharide units. Until a full identification of the sugar units liberated has been made it is not possible to state with any certainty to

what extent oligosaccharides greater than disaccharides occur in the diffusible materials.

Some of the analytical figures (expressed in mg.) which were obtained for the diffusible and indiffusible hydrolysis products are expressed as ratios in Table 7. The values for the ratio of amounts of reducing sugar to nitrogen, reducing sugar to non-hexosamine nitrogen and glucosamine to galactosamine for the diffusible material show a considerable increase over similar ratios obtained for the unchanged substrate. The ratio of reducing sugar to fucose, however, is much reduced. These findings indicate that there is a preferential liberation of fucose and glucosamine in the early stages of the hydrolysis.

The original H substance gave a reduction equivalent to less than 2% of its weight of glucose, whereas the corresponding reduction given by the indiffusible material was 5%. If it is assumed that the indiffusible residue is essentially a single molecular species, as appears possible from the limited physical measurements made, then 5% reduction would suggest that the residue contains straight chains each made up of about 20 sugar units. Such a unit-chain structure would possess a minimum molecular weight of about 3000, or rather more if the reducing end-unit in the chain was fucose or *N*-acetylhexosamine.

A part of the *N*-acetylhexosamine liberated from the H substance was in a form which was readily diffusible and reactive in dilute alkali to give a chromogenic structure. It was possible by paper chromatography to isolate from the diffusates a small amount of material—most probably of disaccharide nature—which formed chromogen under alkaline conditions unable to bring about chromogen formation with *N*-acetylglucosamine or *N*-acetylgalactosamine. Kuhn *et al.* (1954*b*) obtained from a tetrasaccharide present in human milk a disaccharide which reacted more readily with alkali than did *N*-acetylglucosamine, and Kuhn, Baer & Gauhe (1954) subsequently identified the alkali-labile material as 3-β-galactosyl-*N*-acetylglucosamine. This alkali-sensitive disaccharide has also been isolated from the partial acid hydrolysis products of group A substance (unpublished results).

An attempt has been made to determine the changes which occur when the indiffusible material resulting from the first treatment of H substance with enzyme is recovered and treated again with the enzyme preparation. An indiffusible material remained which seemed to be increasingly resistant to further breakdown by the enzyme preparation. The qualitative composition of the indiffusible residue was similar to the original H substance; but the extent of the quantitative changes were more difficult to determine as the analytical figures are not entirely reliable, owing to the steadily increasing contamination of the indiffusible residues with the unspecific material which accompanies the enzyme. For this reason the results are considered provisional. However, the ratio of the amount of hexosamine to fucose, two substances which occur in small amounts only in the enzyme preparations, changed steadily from 2·8 in one preparation of H substance to 4·4 in the indiffusible material obtained after three treatments with the enzyme preparation. The corresponding ratio found for the indiffusible residues obtained from the human H substance likewise showed the same upward trend.

The enzyme preparation liberated up to one-third of the *N*-acetylhexosamine units from the H substances (Expt. 3), yet the resulting indiffusible residue showed about the same percentage of '*N*-acetylhexosamine' colour (see Table 5) as did the original H substance. It seems probable, therefore, that the structure in the H substance which gives this colour cannot be solely responsible for its serological reactivity with anti-H serum.

The serological properties of the H substance, measured by rabbit anti-H serum or the human serum 'Tomlinson', were rapidly destroyed by the enzyme preparation but the hydrolysis products still reacted strongly with the serum 'Warboys'. The capacity to react with this reagent was confined to the indiffusible material.

Treatment of the H substance with the enzyme caused a considerable part of the fucose, together

Table 7. *Ratios of the analytical values (mg.) obtained for the H substance and the indiffusible and diffusible materials after treatment with enzyme in Expt.* 1

Ratio	Original H substance	Materials obtained after enzyme treatment	
		Indiffusible	Diffusible
Reducing sugar:N	8·9	8·7	10·9
Reducing sugar:non-hexosamine N	14·6	12·6	22·0
Reducing sugar:hexosamine	1·8	1·9	1·9
Reducing sugar:fucose	4·9	5·6	3·0
Glucosamine:galactosamine	3·2	2·9	5·5
Hexosamine:fucose	2·8	2·9	1·6
Non-hexosamine N:hexosamine N	1·6	2·3	1·1

with some *N*-acetylhexosamine and galactose, to be released, and it is known that treatment of the H substance with N acetic acid at 100° likewise sets free, in a diffusible form, the major part of the total fucose and destroys the capacity of the material to react with all the anti-H reagents tested, including the serum 'Warboys'. The serologically reactive structure detected by the serum 'Warboys' is therefore different from that reactive with the other anti-H reagents and presumably does not depend on the structural contribution made by the sugars liberated by the enzyme. It appears that some acid-labile structure other than that associated with the sugars liberated by enzymic hydrolysis is essential and must remain intact for reactivity with the serum 'Warboys'. Presumably the 'Warboys' reactive structure is a part only of that responsible for full H specificity. A mucopolysaccharide which reacts with 'Warboys' serum but not with rabbit or human anti-H sera has been encountered in cyst fluids (cf. substance 126/1, Table 1, Gibbons, Morgan & Gibbons, 1955). This material, however, has a fucose content of about 25%.

At the same time as the indiffusible material lost its reactivity with the serum 'Warboys' during hydrolysis with acetic acid, there developed the property of forming a precipitate with horse anti-pneumococcus type XIV serum. This new serological activity was, however, always less pronounced than was the same activity developed by heating the original H substance with mild acid. Kabat *et al.* (1948) suggested that the reactivity of the blood-group substances with type XIV serum after acid hydrolysis was due to the exposure of an *N*-acetylglucosamine–galactose structure similar to that present in the pneumococcus type XIV polysaccharide. If this suggestion is correct then it seems that such an *N*-acetylglucosamine–galactose type of unit is not exposed in a serologically reactive form during enzymic hydrolysis of H substance, or if exposed is rapidly destroyed, as the products fail to react with the anti-pneumococcus serum.

Watkins & Morgan (1955) showed that L-fucose inhibits the enzyme in *Cl. welchii* culture filtrates, which brings about the serological inactivation of H substance, and concluded that L-fucose is an important part of the chemical structure which is responsible for H specificity. The methyl fucopyranosides and furanosides (Watkins, 1955*a*) fail to inhibit the inactivation of H substance by *Cl. welchii* enzyme, but it is known that the similar H-inactivating enzyme in extracts of *Trichomonas foetus* has no action on these fucose derivatives. The presence of a fucosidase in the *Cl. welchii* enzyme preparation, however, must be assumed because of the rapid liberation of free fucose when the enzyme acts on H substance. However, nothing definite is known as to the influence of the nature of the aglycon on the activity of fucosidases, but it seems probable that if the aglycon had been a carbohydrate residue the glycoside bonds would have been readily split. In the light of the earlier observation that the H agglutinins in eel serum (Watkins & Morgan, 1952) and those in an aqueous extract of the seeds of *Lotus tetragonolobus* (Morgan & Watkins, 1953) are inhibited in their action on group O cells by L-fucose, and even more strongly by the glycoside α-methyl-L-fucopyranoside, but by no other simple sugar component of the H substance, it seems probable that an L-fucose end-group plays an important part in the structure responsible for H activity.

The presence of many enzymes in the partially purified *Cl. welchii* enzyme preparation used throughout the present work complicates the interpretation of the results, and for this reason it would be unwise to draw any firm conclusions from the results obtained concerning the structure of the H substance. Since most of the work described in this paper was completed much progress has been made in the separation of mixed enzyme preparations by electrophoresis on starch and cellulose columns, and it is already evident that the application of this and other techniques to the purification of the *Cl. welchii* enzyme preparation is an indispensable step in studies of this kind. Further purification of *Cl. welchii* enzyme, and the discovery (Watkins, 1953, 1955*b*) of a potent source of H enzyme in the protozoan flagellate *T. foetus*, will allow the enzymic approach to the problem of mucopolysaccharide structure to be extended.

SUMMARY

1. Some changes brought about by the action of an enzyme preparation obtained from *Clostridium welchii* (type B) culture filtrates on the serologically specific H substances of animal and human origin are described.

2. When H character, determined by the inhibition of the agglutination of group O cells by immune-rabbit anti-H sera, is almost completely destroyed about one-quarter of the substrate is diffusible through a cellophan membrane.

3. The diffusible material contains the major part of the total fucose, some *N*-acetylglucosamine and galactose and small amounts of a material, possibly a disaccharide, and amino acid-containing residues. The diffusates contain a carbohydrate material which, in dilute alkali at room temperature, forms a chromogen which reacts with *p*-dimethylaminobenzaldehyde.

4. The indiffusible material probably consists largely of a single molecular species and, although containing considerably less fucose and a smaller amount of *N*-acetylhexosamine and galactose, is qualitatively similar to the original H substance.

5. Repeated treatment of the H substance with the enzyme fails to render it completely diffusible through cellophan.

6. The indiffusible residue, which has no capacity to inhibit the agglutination of O cells by immune-rabbit anti-H serum or by the human serum 'Tomlinson', is nevertheless fully active when tested with the anti-H serum 'Warboys'.

7. The capacity of the H substance to react with the anti-H serum 'Warboys' does not depend on the presence of acid-labile fucose within the mucopolysaccharide but probably does depend on some other, equally acid-labile structure.

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