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DETERMINATION OF N-ACETYLGLUCOSAMINE IN HUMAN IMMUNOGLOBULIN M AND ITS FRAGMENTS

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The determination of N-acetylglucosamine (AGA) is an essential component of the investigation of glycoproteins. For this purpose a method is frequently used which includes the hydrolysis of the glycoprotein with hydrochloric acid followed by the determination of the glucosamine hydrochloride formed on an automatic amino acid analyzer. The concentration of hydrochloric acid and the temperature and time of hydrolysis vary in the work of different authors, but the majority of workers adhere to averaged conditions of hydrolysis: 3-4 N HCl, 100°C (more rarely 110°C), 4 h [1]. Strictly speaking, the use of such averaged conditions for the accurate determination of AGA in different glycoproteins is illegitimate and the conditions and temperature of hydrolysis should be selected for each concrete case.

We have carried out such work in the course of the isolation and determination of the characteristics of human immunoglobulin M (IgM) (Waldenström's disease) [2] and have found that the maximum splitting out of glucosamine is achieved in the hydrolysis of IgM with 3 N HCl at 110°C for 4 h. By using these conditions for the determination of AGA in the $(Fc)_5$ and Fab fragments of the same IgM we found that it is impossible to draw up a balance: the total amount of AGA found in the $(Fc)_5$ and Fab fragments was substantially lower than in the initial IgM. This induced us to consider in more detail in the present work the dependence of the amount of AGA found in these three preparations on the temperature and time in the hydrolysis of the samples with 3 N HCl.

As can be seen from Table 1 and Fig. 1, the amount of AGA found in IgM and its $(Fc)_5$ and Fab fragments during hydrolysis at 110°C reach a maximum with time for all three preparations, after which they fall again. However, the positions of these maxima are different, being 4, 6, and 8 h, respectively, for IgM and the $(Fc)_5$ and Fab fragments, respectively. During the hydrolysis of the samples at 100°C, a similar situation is observed, but the process takes place considerably more slowly and at the same time it does not reach those maximum values that were obtained at 110°C (see Table 1). Apparently, the length of the hydrolysis process at 110°C leads to the predominance of reactions involving the secondary destruction of AGA in the process of its liberation.

The results obtained suggest the possibility of achieving still higher yields of hydrolyzed glucosamine with a rise in the temperature of hydrolysis (above 110°C). However,

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TABLE 1. Amounts of N-Acetylglucosamine Found in IgM and Its Fragments under Various Conditions of Hydrolysis with 3 N HCl

Time of hydrolysis, h	Amount of N-acetylglucosamine, %		
	IgM	$(Fc)_5$ fragment	Fab-fragment
	Hydrolysis at 110°C		
2	2.9	2.2	1.3
4	3.9	3.9	1.3
6	3.6	5.8	1.4
8	3.3	5.3	1.5
13.5	—	4.7	1.4
16.5	2.4	—	—
20.0	—	—	1.0
24.0	2.2	3.3	0.8
32.0	2.1	—	—
37.5	—	2.6	0.6
	Hydrolysis at 100°C		
1	2.3	2.2	0.6
3	—	2.3	0.9
4	2.9	—	—
6	—	2.3	1.0
8	3.1	—	—
10	—	2.4	0.9
20	—	2.6	0.9
24	—	3.6	—
31	—	5.2	—
52	—	4.1	—

Note: The figures given are the means of three determinations.

a trial hydrolysis at 120°C showed that a rise in the temperature from 110°C to 120°C with retention of the time of hydrolysis did not lead to an increase in the yield of glucosamine.

As can be seen from Fig. 1, the rates of reaching the maximum liberation of AGA during hydrolysis at 110°C differ substantially for all three preparations investigated. It is difficult to give a completely definite answer to the question of the causes of this phenomenon. It is most probably connected with conformational differences which lead to different degrees of accessibility of the AGA residues for hydrolytic liberation. If this is so, it may be thought that in this case with a rise in the dimensions of the macromolecule from IgM to the Fab fragment an increase will take place in the compactness of the three-dimensional structure. Thus, from the results of glucosamine liberated in the hydrolysis of IgM and its $(Fc)_5$ and Fab fragments with 3 N HCl the optimum temperature is 110°C and the optimum times of hydrolysis 4, 6, and 8 h, respectively. However, in this case, as well, the total amount of AGA found in the fragments was less than in the initial IgM. Furthermore, it remained unclear to what extent the values found corresponding to the actual amounts of AGA in the preparations were investigated.

In order to answer these questions we made a comparison of the maximum AGA contents that we found in IgM and its fragments and literature information on [3] the primary structure of the oligosaccharide groups (Table 2). In spite of the fact that our sample of IgM was not identical with that described in the paper cited [3], this comparison is fully permissible, since AGA forms part only of the so-called "core" or nucleus of the carbohydrate groups of IgM and this is practically the same in different IgM's.

It can be seen from Table 2 that, according to the primary structure of IgM, of the 32 AGA residues in the IgM subunit — IgM_S — eight residues are included in the two Fab fragments, 16 in the Fc fragment, and eight in the two oligosaccharide groups of the "hinge" region. The amount of AGA that we found for the initial IgM and its Fab fragment agrees very well with that given in the literature. For the $(Fc)_5$ fragment (calculated to a monomeric Fc fragment) we found seven AGA residues less than there should have been according to the primary structure. This difference does not agree badly with the number of AGA residues present in the "hinge" region. Consequently, it may be assumed that in the fragmenta-

TABLE 2. Comparison of the Amounts of N-Acetylglucosamine Found in IgM and Its Fragments after Hydrolysis with 3 N HCl at 100 and 110°C with Information on the Primary Structure of the Oligosaccharide Groups of IgM [3]

Preparation	Amount of N-acetylglucosamine (in residues)		
	calculated from the primary structure of the oligosaccharide groups of IgM	found under the optimum conditions of hydrolysis of the samples (see text)	found under the conditions usually used for the hydrolysis of the samples: 3 N HCl, 100°C, 4 h
Subunit of IgM (IgM_S)	32	32,4	24,5
Fc fragment (monomer)	24 (16 – without the "hinge" region)	17,0	8,0
Fab fragment	4	3,8	2,1

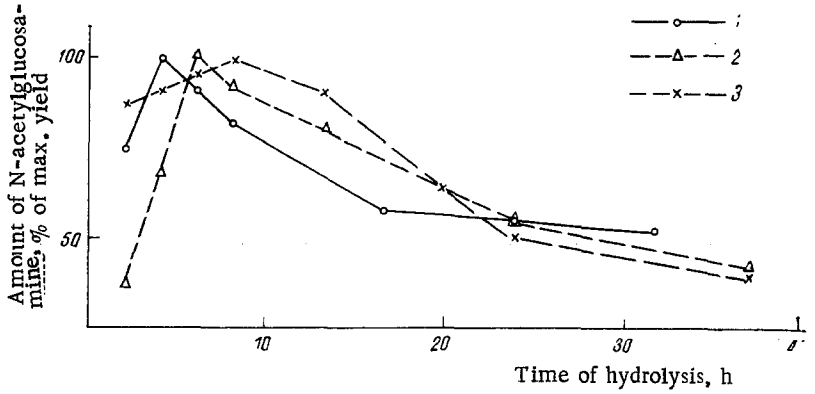

Fig. 1. Dependence of the liberation of N-acetylglucosamine on the time of hydrolysis of IgM and its $(Fc)_5$ and Fab fragments with 3 N HCl at 110°C: 1) IgM; 2) the $(Fc)_5$ fragment; 3) the Fab fragment.

tion of IgM by trypsin in our case degradation of the "hinge" region took place and the glycopeptide formed was not included in the $(Fc)_5$ fragment isolated. In fact, on the isolation of the IgM fragment we detected another carbohydrate-containing fragment, the molecular weight of which was smaller than that of the Fab fragment. In the isolation of the IgM fragments, this fraction was scarcely separated from the mixture of small peptides usually formed in the fragmentation of IgM by trypsin. The purification and analysis of this fraction therefore form an independent problem, which we shall attempt to solve.

EXPERIMENTAL

The IgM preparation was obtained by a published method [2]. To obtain the $(Fc)_5$ and Fab fragments we hydrolyzed IgM with trypsin in 0.05 M Tris-HCl buffer, pH 8.15 in the presence of 0.015 M $CaCl_2$ (buffer A) at 56°C for 2 h. The enzyme:substrate ratio was 1:25. Hydrolysis was stopped by the addition of the soya trypsin inhibitor (enzyme:inhibitor ratio 2:3) and by cooling. The $(Fc)_5$ and Fab fragments were isolated from the tryptic hydrolyzate by gel filtration on a 2 × 90 cm column of Sepharose 6B in buffer A followed by further purification initially on the same column and then on a 1.5 × 75 cm column of Bio-Gel P-30 in buffer A. The preparations of the IgM fragments obtained were dialyzed against distilled water and were freeze-dried.

The acid hydrolysis of the samples was carried out at a protein concentration of 0.1% in double-distilled 3 N HCl at 100, 110, and 120°C for up to 52 h. In the process it proved to be important to shake the tubes during hydrolysis in order to achieve the better wetting of the samples. The hydrolyzate was rapidly evaporated to dryness at 20°C in vacuum, and the residue was then evaporated twice with water under the same conditions and was analyzed on a AAA-881 amino acid analyzer (Czechoslovakia) using a 0.8 × 27 cm column and 0.35 M citrate buffer, pH 5.28. All the figures are calculated as N-acetylglucosamine.

SUMMARY

1. The process of the splitting out of N-acetylglucosamine in the hydrolysis of myelomic IgM and its $(Fc)_5$ and Fab fragments with 3 N HCl at 100, 110, and 120°C has been investigated as a function of the time.

2. The maximum splitting out of N-acetylglucosamine from IgM and the $(Fc)_5$ and Fab fragments takes place at 110°C in 4, 6, and 8 h, respectively.

3. The amounts of N-acetylglucosamine found in the preparations studied agree well with the primary structure of the oligosaccharide groups of IgM.

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THE STRUCTURE OF KENAF LIGNIN

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Previously [1, 2], by successive acidolysis three fractions of dioxane lignin (DLAK-I, DLAK-II, and DLAK-III) were isolated from the stems of kenaf of the variety "Uzbekskii 15-74," their chemical compositions were determined, their semiempirical formulae were deduced, and their molecular-weight distributions and the products of their nitrobenzene oxidation were studied.

In the present work we considered the structure of fractions of kenaf DLA isolated by the method of reductive degradation with metallic sodium in liquid ammonia.

As is well known [3-8], under the action of alkali metals in liquid ammonia on natural lignin and also on lignin isolated from wood, the alkyl-aryl ether bonds between the phenylpropane structural units (PPSEs) present in them are cleaved. This leads to the liberation of "uncondensed" structural elements.

The fractions were cleaved by the method described in the literature [3-6], and the reaction products were separated by extraction with ether from alkaline solutions (pH 8) and with ether and chloroform from acid solutions (pH 2). The yields of phenols in a single treatment of the DLAKs were 21.5, 20.7, and 19.8% (on the lignin taken), respectively. The yields of phenols from spruce LMR* [8] was 19%. As can be seen, the maximum yield of ether-soluble phenols was obtained from DLAK-I, and it fell successively on passing to DLAK-III. This agrees well with the semiempirical formulae of these lignins [1], where in DLAK-I there are 0.75 alkyl-aryl bonds per C_9 and DLAK-III only 0.20. Consequently, there are somewhat more "uncondensed" aromatic units in DLAK-I than in two other fractions of kenaf lignin.

The higher yield of low-molecular-weight cleavage products from kenaf lignin as compared with spruce LMR indicates a lower degree of condensation of the kenaf lignins. This is apparently connected with the fact that in the latter there are fairly large amounts of syringyl structural elements [2] in which the fifth position of the aromatic ring is occupied by a methoxy group and, consequently, does not take part in the condensation processes proceeding both in the formation of lignin and as the result of various chemical actions during its isolation from the plant tissue.

To investigate the complex mixture of phenols isolated after cleavage with metallic sodium in liquid ammonia we used gas–liquid chromatography. In the alkaline fraction (pH 8) among the cleavage products we succeeded in identifying phenols of the p-coumaryl, guai-

*The exact expansion of this acronym could not be ascertained. It probably means "lignin from mechanical disintegration" or the like.

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