in further step(s) and the resultant requirement of the consideration of the relative ease of cis and trans elimination reactions.

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Chemical Nature of the Receptor Site for Various Phytomitogens†

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ABSTRACT: In the inhibition assays of [^{3}H]thymidine incorporation into human peripheral lymphocytes, the stimulatory activity of four kinds of phytomitogens (concanavalin A, *Lens culinaris* hemagglutinin, PHA-M, and *Wistaria floribunda* mitogen) were found to be inhibited by glycopeptides from porcine thyroglobulin, in spite of the fact that these mitogens could be classified into two groups by the inhibition assays using simple sugars as inhibitors. In order to clarify the detailed structure with which these mitogens actually bind, sequential enzymic degradation of one of the glycopeptides with purified glycosidases was carried out, and the inhibitory activity of the residual glycopeptide at each stage of the degradation against the mitogenic as well as the hemagglutinating activities of these mitogens was tested. On the basis of the results obtained, a hypothesis that these mitogens bind to the same core region of the glycopeptide in the inhibition of lymphocyte transformation is proposed.

The phytomitogens, which are plant proteins having mitogenic activity against human peripheral lymphocytes, can generally be classified into two groups on the basis of their specificities, as shown by the inhibition assays of their mitogenic activity with simple sugars. One group, which includes *Phaseolus vulgaris* hemagglutinin, is inhibited by *N*-acetyl-D-galactosamine (Borberg *et al.*, 1968), the other, which includes concanavalin A, is inhibited most by D-mannose (Powell and Leon, 1970; Young *et al.*, 1971). These observations seem to indicate that at least two kinds of receptor sites on the lymphocyte cell surface are involved for the triggering of lymphocyte transformation. In preceding papers (Toyoshima *et al.*, 1970, 1971), we have reported the purification and the characterization of phytomitogens from *Lens culinaris* seeds and from *Wistaria floribunda* seeds. The inhibition assays of these mitogens with simple sugars have indicated the difference of their specificities (Toyoshima *et al.*, 1971). The former belongs to the same group of phytomitogens as concanavalin A, whereas the latter belongs to that including *P. vulgaris* hemagglutinin. However, we have isolated a glycopeptide from human erythrocyte stroma (Akiyama and Osawa, 1971) which has been found to exert a potent inhibitory activity against both groups of phytomitogen (Toyoshima *et al.*, 1971). Recently, Kornfeld *et al.* (1971a,b) isolated a glycopeptide from human γG-myeloma proteins which was found to be a strong hapten inhibitor for both *P. vulgaris* and *L. culinaris* hemagglutinins, and demonstrated that these phytohemagglutinins might bind to different portions of the same glycopeptide.

In this paper, we will report the strong inhibitory activities, for the hemagglutinating and mitogenic activities of both groups of phytomitogen, of a glycopeptide from porcine thyroglobulin (Fukuda and Egami, 1971a,b), and will present evidence that both groups of phytomitogen might bind to the same core region of the glycopeptide in the inhibition of lymphocyte transformation, but bind to the different sugar units in the hemagglutination inhibition reactions.

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Experimental Section

Materials. P. vulgaris hemagglutinin was a product of Difco (PHA-M) or Wellcome purified PHA (MR 69, lot K 1275; Wellcome Research Laboratories, Beckenham, U. K.). Concanavalin A was purified from jack bean (Sigma) according to the method of Agrawal and Goldstein (1967). *L. culinaris* hemagglutinin was purified from lentils (kindly provided by Dr. H. Kanai, Department of medicinal plants, Kathmandu, Nepal) by the method previously described (Toyoshima *et al.*, 1970). *W. floribunda* mitogen, having weak hemagglutinating activity, and *W. floribunda* hemagglutinin,[1] freed from mitogenic activity, were purified from *W. floribunda* seeds (purchased from F. W. Schumacher, Sandwich, Mass.) according to the method previously described (Toyoshima *et al.*, 1971). The homogeneity of concanavalin A, *L. culinaris* hemagglutinin, and *W. floribunda* mitogen used in this study was ascertained by ultracentrifugal analysis and electrophoresis on polyacrylamide gel. The glycopeptides A and B (Figure 1) from porcine thyroglobulin were prepared according to the procedure described by Fukuda and Egami (1971a).

Enzymes. Highly purified neuraminidase from *Clostridium perfringens* was kindly provided by Dr. Aoyagi, Institute of Microbial Chemistry, Kamiosaki, Tokyo. The specific activity of the neuraminidase was defined as micromoles of *N*-acetylneuraminic acid formed per minute per milligram of protein under the condition described by Cassidy *et al.* (1965). β-Galactosidase, α-fucosidase, β-*N*-acetylglucosaminidase, α-mannosidase, and β-mannosidase were purified from the liver of *Turbo cornutus*, as described by Muramatsu and Egami (1967) and Iijima *et al.* (1969). These enzymes were assayed using *p*-nitrophenyl glycosides as described by Fukuda *et al.* (1969). Each purified enzyme used in this study was found to be virtually devoid of other glycosidase activity. One unit of enzyme activity was defined as the amount of enzyme which could liberate 1.0 μmole of *p*-nitrophenol in 1 min.

Chemical Determination. Total sialic acid was measured by the periodate–resorcinol method of Jourdian *et al.* (1971) and enzymatically released sialic acid by the thiobarbituric acid method of Aminoff (1961). The total hexose content was determined by the phenol method of Dubois *et al.* (1956). Enzymatically released reducing sugars were determined by the ferricyanide method of Park and Johnson (1949). Since glycosidase preparations often released reducing endogenous sugars, control experiments without substrate were performed in the determination of enzymatically released sugars. Quantitative determination of individual neutral sugars was carried out by gas–liquid chromatography after reduction to the respective alditol, followed by trifluoroacetylation according to the method of Matsui *et al.* (1968), as described by Matsumoto and Osawa (1970) and Akiyama and Osawa (1972). Hexosamine was determined by the method of Belcher *et al.* (1954). Hydrolysis for this assay was carried out with 4 M HCl for 7 hr at 100°. *N*-Acetylglucosamine released by β-*N*-acetylglucosaminidase was measured by the method of Reissig *et al.* (1955).

Hemagglutination Assays. The titration and inhibition assays by use of human erythrocytes freshly obtained from a donor were carried out according to the method previously described (Matsumoto and Osawa, 1969).

Lymphocyte Cultures for Mitogenic Assay. Human peripheral lymphocytes were cultured by the method previously described by Toyoshima *et al.* (1970). Inhibition assays for [6-^{3}H]thymidine incorporation into the lymphocytes with sugars and glycopeptides were performed as described previously (Toyoshima *et al.*, 1971). The inhibition assays were usually carried out four times on each inhibitor against the same mitogen, and an average value of the incorporation was calculated.

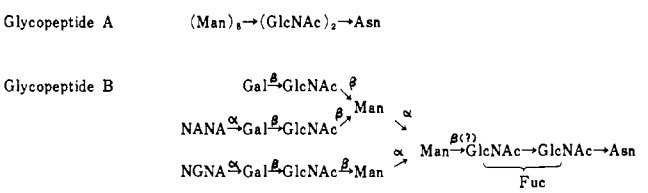

FIGURE 1: Proposed structures of glycopeptides A and B from porcine thyroglobulin.

Action of Purified Glycosidases on Glycopeptide. To 15–20 μmoles of glycopeptide in an appropriate buffer was added neuraminidase (1.5 units), β-galactosidase (10.8 units), α-fucosidase (1.0 unit), β-*N*-acetylglucosaminidase (8 units), α-mannosidase (10.5 units), or β-mannosidase (4.0 units). The buffer solutions used were as follows: 0.1 M sodium acetate buffer (pH 4.5), containing 0.03% bovine serum albumin for neuraminidase; 0.1 M sodium acetate buffer (pH 4.0), containing 0.5 M NaCl for α-fucosidase, β-galactosidase, α-mannosidase, and β-mannosidase; 0.1 M citrate–phosphate buffer (pH 4.0), containing 0.5 M NaCl for β-*N*-acetylglucosaminidase. The reaction mixture (0.5 ml) was incubated at 37° under a toluene atmosphere for either 48 hr (neuraminidase, β-galactosidase, α-fucosidase) or 72 hr (β-*N*-acetylglucosaminidase, α-mannosidase, β-mannosidase), and then applied to a Sephadex G-50 or G-25 column to separate the residual glycopeptide from the enzyme and the released sugars. After α-mannosidase treatment, it was necessary to heat the reaction mixture in a boiling-water bath for 3 min to precipitate the enzyme and then apply the supernatant to a column of Sephadex G-15 (1.0 × 8.0 cm; V_0 = 1.9 ml) to ensure a good separation of the small-core glycopeptide from the released mannose. The residual glycopeptide, which was recovered quantitatively, was lyophilized, redissolved in an appropriate buffer, and incubated with another glycosidase.

Results

Action of Glycosidases on Glycoprotein B. As shown in Figure 2, successful separations of residual glycopeptides from released sugars and enzymes were achieved by gel filtration on Sephadex columns. The sugars released from the oligosaccharide chain are listed in Table I. Although one residue of fucose had previously been suggested by Fukuda and Egami (1971b) to be linked to one of the *N*-acetylglucosamine residues penultimate to the galactose residues in the outer part of the glycopeptide, the liberation of fucose by the action of α-fucosidase could not be observed in this experiment following the β-galactosidase treatment; furthermore, about 2.5 moles of *N*-acetylglucosamine was liberated by the action of β-*N*-acetylglucosaminidase without the pretreatment with α-fucosidase. The quantitative determination of component sugars of the residual glycopeptide at each stage of the sequential enzymic degradation was carried out by gas–liquid chromatography and the results were shown in Table II. Even after prolonged treatment with α-mannosidase (Figure 3), the remaining core of the glycopeptide contained one mannose,

[1] This hemagglutinin was denoted as "fraction A" in our previous paper (Toyoshima *et al.*, 1971).

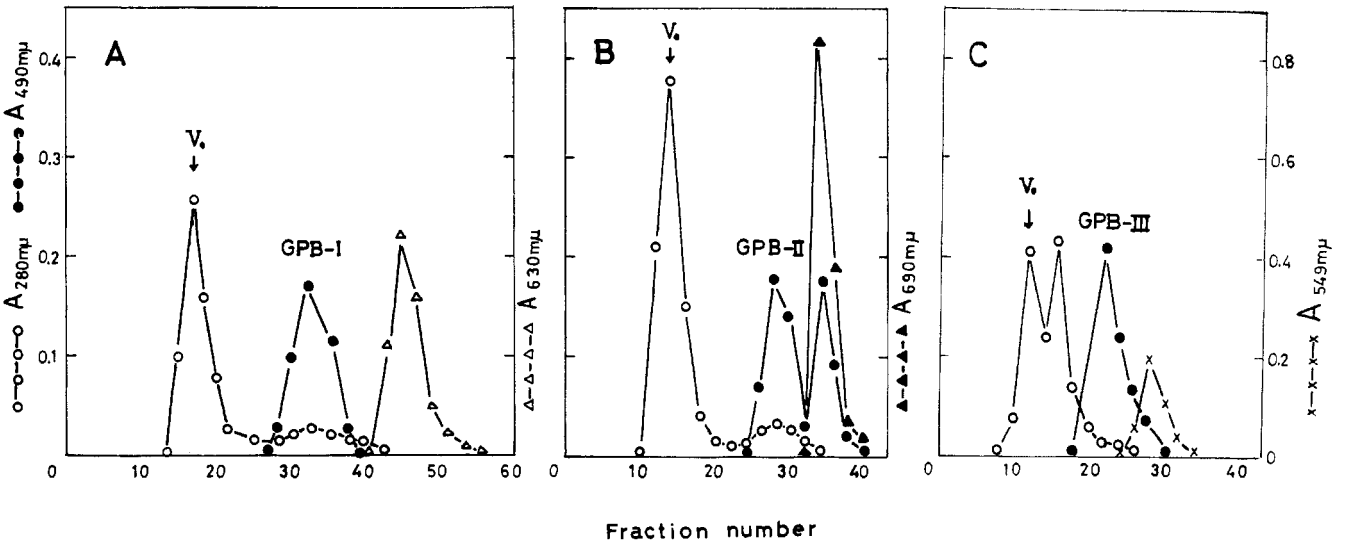

FIGURE 2: (A) Sephadex G-50 gel filtration of GPB after neuraminidase treatment. The reaction mixture was applied to a column (2.1 × 50 cm) equilibrated with distilled water. Fractions of 4 ml were collected. (B) Sephadex G-50 gel filtration of GPB-I after β-galactosidase treatment. The reaction mixture was applied to a column (1.5 × 47 cm). Fractions of 2 ml were collected. (C) Sephadex G-25 gel filtration of GPB-II after β-*N*-acetylglucosaminidase treatment. The reaction mixture was applied to a column (1.3 × 40 cm). Fractions of 2 ml were collected. Each elution was carried out with distilled water at a flow rate of 6 ml/hr at 4°. The void volumes are indicated by vertical arrows. (○) $A_{280\ m\mu}$; (●) phenol–H_2SO_4 reaction; (△) periodate–resorcinol reaction; (▲) Park–Johnson reaction; (×) Morgan–Elson reaction.

TABLE I: Enzymic Liberation of Monosaccharide from Porcine Thyroglobulin Glycopeptide B (GPB).

Enzyme Treatment	Resulting Glycopeptide	Residues Released/Mole of Glycopeptide				
		Sialic Acid	Fuc	Gal	GlcNAc	Man
GPB + neuraminidase	GPB-I	1.84				
GPB-I + β-galactosidase	GPB-II			2.76		
GPB-I + β-galactosidase + α-fucosidase	GPB-II			2.72		
GPB-II + β-*N*-acetylglucosaminidase	GPB-III				2.46	
GPB-II + β-*N*-acetylglucosaminidase + α-fucosidase	GPB-III				2.45	
GPB-III + α-mannosidase	GPB-IV					1.80
GPB-IV + β-mannosidase						0.23

TABLE II: Carbohydrate Composition of Residual Glycopeptides after Successive Treatments with Purified Glycosidases.

Glycopeptide	Residues/Mole of Glycopeptide				
	Sialic Acid	Fuc	Gal	GlcNAc	Man
GPB[a]	1.8	1.0	3.1	5.0	3.0
GPB-I[a]	n[c]	1.0	3.1	5.0	3.0
GPB-II[a]	n	1.0	n	5.0	2.9
GPB-III[b]	n	0.9	n	2.0	2.5
GPB-IV[b]	n	1.0	n	2.0	0.9

[a] Calculated on basis of five *N*-acetylglucosamine residues per mole of the glycopeptide. [b] Calculated on basis of two *N*-acetylglucosamine residues per mole of the glycopeptide. [c] Not detected.

one fucose, and two *N*-acetylglucosamine residues, and in addition, the treatment of the core with β-*N*-acetylglucosaminidase in the presence of α-fucosidase did not liberate *N*-acetylglucosamine. However, since the slow release of mannose was observed by the action of β-mannosidase on the core as shown in Table I, we have tentatively assumed the structure of the core to be *O*-β-D-Man→[*O*-α-L-Fuc→]-*O*-β-D-GlcNAc→*O*-β-D-GlcNAc-, in which the presence of the fucose residue possibly prevented the complete removal of the mannose residue by the enzyme, as in the case of the glycopeptide from γG-myeloma protein (Kornfeld *et al.*, 1971a).

Inhibition of Hemagglutination. Table III lists the hapten inhibitory activity of various sugars and glycopeptides against the hemagglutination by several phytomitogens and *W. floribunda* hemagglutinin. Whereas *W. floribunda* hemagglutinin and *W. floribunda* mitogen had been found to have almost the same specificities in the inhibition assays using simple sugars as inhibitors (Toyoshima *et al.*, 1971), a remarkable difference in specificity was observed in the inhibition experiments using

TABLE III: Inhibition of Hemagglutination with Sugars and Glycopeptides.

Sugars and Glycopeptides	Mol Wt	Minimum Amounts (μmoles/ml) Completely Inhibiting 4 Hemagglutinating Doses: PHA-M	Wellcome PHA	*W. floribunda* Hemagglutinin	*W. floribunda* Mitogen	Concanavalin A[a]	*L. culinaris* Hemagglutinin
L-Fucose		>110	ND[b]	>110	>110	>110	>110
D-Mannose		>110	ND	>110	>110	28	28
N-Acetyl-D-glucosamine		>90	>90	>90	>90	23	23
N-Acetyl-D-galactosamine		270	ND	0.36	2.9	>90	>90
N-Acetyllactosamine		>52	>52	0.33	0.66	>52	>52
Glycopeptide A	2200	>4.5	1.4	>4.5	0.55	0.28	0.28
Glycopeptide B (GPB)	3300	0.61	0.38	0.04	0.08	0.19	0.09
GPB-I (−SA)[c]	2700	0.19	ND	0.02	0.04	0.23	0.05
GPB-II (−SA, Gal)	2200	4.5	4.5	>4.5	0.45	0.07	0.04
GPB-II (−SA, Gal, GlcNAc)	1600	>6.3	>6.3	>6.3	1.6	0.19	0.19
GPB-IV (− SA, Gal, GlcNAc, Man)	1300	>7.7	>7.7	>7.7	>7.7	0.48	1.9

[a] Concanavalin A. [b] Not determined. [c] SA = sialic acid.

glycopeptide A whose structure had been shown to consist of a mannose oligomer and a core di-*N*-acetylchitobiose moiety (Arima *et al.*, 1970; M. Fukuda and F. Egami, unpublished data). The same difference in specificity was also observed between PHA-M and Wellcome PHA. The latter substance had a much weaker hemagglutinating activity (about eight times the minimum hemagglutinating dose) and a much stronger mitogenic activity (about $^1/_{20}$th of the optimal dose for maximum [^{3}H]thymidine incorporation of human peripheral lymphocytes) than the former. Thus, the glycopeptide A was inhibitory against both *W. floribunda* mitogen and Wellcome PHA, but not inhibitory against both *W. floribunda* hemagglutinin and PHA-M. In order to clarify the details of the structure with which these phytomitogens bind in their hemagglutinating reactions, the hapten inhibitory activity of the residual glycopeptides obtained by the sequential enzymic degradation of glycopeptide B, which was found to be a potent hapten inhibitor of all phytomitogens used in this study, was tested. After neuraminidase treatment (GPB-I), the hapten inhibitory activity against all proteins tested was enhanced. The following β-galactosidase treatment (GPB-II), however, gave rise to a marked loss of inhibitory activity against PHA-M, Wellcome PHA, and *W. floribunda* hemagglutinin, and an appreciable loss of inhibitory activity against *W. floribunda* mitogen was also observed, whereas the inhibitory activity against both concanavalin A and *L. culinaris* hemagglutinin was enhanced by this treatment. Removal of the next *N*-acetylglucosamine residue (GPB-III) resulted in a loss of inhibitory activity, particularly against *L. culinaris* hemagglutinin in agreement with the result already reported by Kornfeld *et al.* (1971b). Although concanavalin A and *L. culinaris* hemagglutinin had been found to have almost the same specificity in the inhibition assays that use simple sugars as inhibitors (Toyoshima *et al.*, 1970), the inhibitory activity against concanavalin A was appreciably retained even after successive treatments with β-*N*-acetylglucosaminidase and α-mannosidase (GPB-IV). In contrast the inhibitory activity against *L. culinaris* hemagglutinin was more profoundly affected, suggesting the relative importance of the inner core of the glycopeptide in determining concanavalin A hapten inhibitory activity.

Inhibition of [^{3}H]Thymidine Incorporation in Human Peripheral Lymphocyte Stimulation. Table IV summarizes the effect of various sugars and glycopeptides on [^{3}H]thymidine incorporation of human peripheral lymphocytes exposed to various phytomitogens. Whereas oligosaccharides composed of mannose residues were inhibitory against concanavalin A and *L. culinaris* hemagglutinin and, furthermore, di-*N*-acetylchitobiose showed inhibitory activity against PHA-M and *W. floribunda* mitogen, both glycopeptide A and B from porcine thyroglobulin were inhibitors of all phytomitogens tested. Since the common structural unit shared by both glycopeptides might be (Man)$_3$-GlcNAc-GlcNAc-, the inhibitory activity of the residual glycopeptides after sequential enzymic

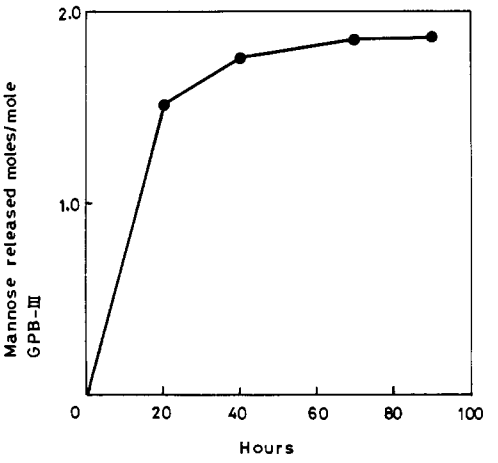

FIGURE 3: Release of mannose from GPB-III by α-mannosidase. The procedures for hydrolysis are described in the text. At 0, 40, and 70 hr, 10.5, 4.2, and 2.1 units of the enzyme were added. The reducing sugar in the reaction mixture at each time shown was determined using D-mannose as standard. Control experiments without substrate and without enzyme were run to correct the values.

TABLE IV: Effect of Sugars and Glycopeptides on [^{3}H]Thymidine Incorporation by Human Peripheral Lymphocytes Exposed to Various Phytomitogens.

Sugars and Glycopeptides	mg/ml	μmoles/ml	Incorporation (%)[a] PHA-M	*W. floribunda* Mitogen	Concanavalin A	*L. culinaris* Hemagglutinin
None[b]			100	100	100	100
αMan(1→2)Man	5	15	111	97	12	60
αMan(1→6)Man	5	15	102	72	4	19
αMan(1→6)αMan(1→6)Man	5	10	ND[c]	72	ND	25
Di-*N*-acetylchitobiose	5	12	35	26	85	80
Glycopeptide A	0.5	0.23	54	59	25	26
Glycopeptide B (GPB)	0.5	0.15	37	20	37	32
GPB-I	0.5	0.19	36	25	11	17
GPB-II	0.5	0.23	38	31	3	7
GPB-III	0.5	0.31	20	17	1	5
GPB-IV	0.5	0.38	90	83	50	25

[a] Average value of four experiments. Standard deviations were within 20%. [b] [^{3}H]Thymidine incorporations were 5000–10,000 cpm. [c] Not determined.

degradation of the glycopeptide B against [^{3}H]thymidine incorporation was tested in order to know whether the above-mentioned structural unit was important for the binding of these phytomitogens. In contrast to the effect of the glycosidase treatments on the hemagglutination inhibitory activity of the glycopeptide, the sequential treatments with neuraminidase, β-galactosidase, and β-*N*-acetylglucosaminidase did not affect the inhibitory activity against the incorporation of [^{3}H]thymidine stimulated by these phytomitogens, even in the case of PHA-M whose hemagglutinating activity could not be inhibited by the β-galactosidase-treated glycopeptide. It is of particular interest to note that the treatment with β-*N*-acetylglucosaminidase does not affect the inhibitory activity against the mitogenic activity of *L. culinaris* hemagglutinin, in spite of the fact that the hemagglutination inhibitory activity for this hemagglutinin is markedly diminished by this treatment. Subsequent removal of two residues of mannose resulted in a virtually complete loss of inhibitory activity for both PHA-M and *W. floribunda* mitogen, but the resulting inner-core residue was still a weak inhibitor of both concanavalin A and *L. culinaris* hemagglutinin.

Discussion

In the preceding paper (Toyoshima *et al.*, 1971), we have reported on the purification of a phytomitogen, that is strongly mitogenic against human peripheral lymphocytes and has relatively weak agglutinating activity, and of a phytohemagglutinin, that has strong agglutinating activity and is devoid of mitogenic activity, from *W. floribunda* seeds, and we have also shown that the two activities are not necessarily related functions. Furthermore, we have shown that four phytomitogens (PHA-M, *W. floribunda* mitogen, concanavalin A, and *L. culinaris* hemagglutinin), which can be classified into two groups on the basis of inhibition assays using simple sugars as inhibitors, are equally inhibited by a single glycopeptide obtained from human erythrocyte stroma (Akiyama and Osawa, 1971), and have suggested that these mitogens can bind to the same receptor on the cell surface in triggering the lymphocyte transformation. Kornfeld *et al.* (1971b) have presented evidence that a glycopeptide from γG-myeloma protein has a potent hapten inhibitory activity for both *P. vulgaris* and *L. culinaris* hemagglutinins and have suggested that these hemagglutinins may bind to different portions of the same oligosaccharide which is located on the surface of human erythrocytes.

In the present study, we demonstrate that both glycopeptide A and B (Figure 1), whose structures have been previously proposed (Arima *et al.*, 1970; M. Fukuda and F. Egami, 1971b, unpublished data), are inhibitors of both mitogenic and hemagglutinating activities of the four mitogens.

On the basis of the results of the sequential enzymic degradation of the glycopeptide B (Tables I and II), the linkage of the fucose residue, which had previously been assumed by Fukuda and Egami (1971b) to be linked to one of the *N*-acetylglucosamine residues penultimate to galactose residues, was tentatively modified as shown in Figure 1. The lack of reactivity of the fucose residue in the core of the glycopeptide is possibly due to the specificity of the α-fucosidase used in this study, even though this enzyme has been successfully applied to the glycopeptide from stem bromelain (Yasuda *et al.*, 1970). The definite structure of the core portion of the glycopeptide, however, requires further investigations.

The importance of the galactose residues in the binding of the glycopeptide to *P. vulgaris* and *W. floribunda* hemagglutinins was clearly shown by the finding that β-galactosidase treatment markedly abolished the inhibitory activity against these hemagglutinins. Since *N*-acetyllactosamine was found to be inactive for these hemagglutinins, the determinant sugar unit for the bindings of these hemagglutinins might have the structure Gal-GlcNAc-Man-, as previously suggested by Kornfeld and Kornfeld (1969, 1970). The glycopeptide resulting from the β-galactosidase treatment was still somewhat inhibitory against the hemagglutinating activity of *W. floribunda* mitogen, suggesting a relatively strong tendency of this mitogen for the binding to the inner part of the glycopeptide. On the other hand, the removal of the galactose residues did not affect the inhibitory activity against mitogenic activity of both PHA-M and *W. floribunda* mitogen and remarkably enhanced the hemagglutinating and mitogenic activities of

both concanavalin A and *L. culinaris* hemagglutinin. Furthermore, as shown in Table III, the importance of the next *N*-acetylglucosamine residues for the hemagglutination by *L. culinaris* hemagglutinin was also observed, in agreement with the results reported by Kornfeld *et al.* (1971b). This removal of the *N*-acetylglucosamine residues, however, does not decrease the inhibitory activity for the mitogenic activity of all phytomitogens tested, even in the case of *L. culinaris* hemagglutinin. Actually, the most potent inhibitor of these phytomitogens is the glycopeptide resulting from the β-*N*-acetylglucosaminidase treatment. Subsequent treatment with α-mannosidase, however, greatly diminished the inhibitory activity against these phytomitogens, which indicates the importance of the two outer mannose residues for the bindings of these phytomitogens. Furthermore, glycopeptide A, which had been shown to be composed of a mannose oligomer and an inner core, di-*N*-acetylchitobiose moiety, as shown in Figure 1, was a moderate inhibitor against all phytomitogens tested, even against the mitogenic activity of PHA-M whose hemagglutinating activity was not inhibited by this glycopeptide.

From the foregoing results, it might be assumed that the common determinant sugar sequence for the binding of the phytomitogens tested in this study, in the triggering of lymphocyte transformation, has the structure $(Man)_3$-GlcNAc-GlcNAc-, and differs from the determinant sugar sequence responsible for the bindings in the hemagglutinating reaction. It is conceivable that the receptor site on the lymphocyte cell surface carries such a determinant sugar sequence and is involved in transformation by various phytomitogens. Final proof must await the actual isolation of the receptor site for various mitogens from lymphocyte cell surface. Work along this line is now under progress in this laboratory.

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