A Phytomitogen from *Wistaria floribunda* Seeds and Its Interaction with Human Peripheral Lymphocytes*

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ABSTRACT: A glycoprotein, which is strongly mitogenic against human peripheral lymphocytes and has relatively weak hemagglutinating and leucoagglutinating activities, has been purified from the seeds of *Wistaria floribunda*. The preparation was homogeneous by ultracentrifugal analysis and disc electrophoresis, and had a $s_{20,w}$ value of 4.5 S. This purified mitogen was found to contain 11.4% carbohydrate in which mannose (5.8%) and glucosamine (3.5%) were the predominant sugars, with smaller amounts of arabinose, fucose, and xylose. Morphological studies on mitogen-stimulated cultures indicated that at 72 hr about 70% of the cell population had been transformed by the purified mitogen. In the inhibition assays of [^{3}H]thymidine incorporation by sugars, the stimulatory activity of the purified *W. floribunda* mitogen as well as those of the other three nonspecific mitogens (concanavalin A, *Lens culinaris* hemagglutinin, and PHA-M) were found to be equally inhibited by a single glycopeptide from human erythrocyte stroma, which had previously been disclosed to be a specific inhibitor of the hemagglutination by *L. culinaris* hemagglutinin (Akiyama, Y., and Osawa, T. (1971), *Proc. Jap. Acad. 47*, 104) in spite of the fact that these four mitogens could be classified into two groups from the inhibition assays using simple sugars such as D-mannose and *N*-acetyl-D-galactosamine as inhibitors. Further, the simultaneous stimulation of lymphocytes with two mitogens gave rise to the decrease of [^{3}H]thymidine incorporation in any combination of the above four mitogens. Based on these results, an assumption is proposed on the nature of the receptor site which is involved in the triggering of lymphocyte transformation.

Since the discovery of Nowell (1960) of mitogenic activity against human peripheral lymphocytes in the extracts of *Phaseolus vulgaris* seeds, extracts of many plant seeds have extensively been surveyed for this biological activity which serves an an interesting model for the study of the mechanism of cell transformation from resting state into growing state (Downing *et al.*, 1968; Krüpe *et al.*, 1968; Parker *et al.*, 1969). The mitogenic activity of the extracts of *Wistaria floribunda* seeds was first described by Barker and Farnes (1967).

In this paper we will present data on the purification and characterization of a strongly mitogenic glycoprotein from *W. floribunda* seeds which has relatively weak hemagglutinating and leukoagglutinating activities. Also reported in this paper are the results of inhibition assays by sugars on the mitogenic activity of the purified *W. floribunda* mitogen as well as those of the other mitogens, namely, concanavalin A, *Lens culinaris* hemagglutinin, and PHA-M. Based on those results, the nature of the receptor site for those mitogens on the cell surface is discussed.

Experimental Section

Materials. The seeds of *W. floribunda* were purchased from F. W. Schumacher, Sandwich, Mass. PHA-M was a product of Difco. Concanavalin A was purified from Jack bean (Sigma) according to the method of Agrawal and Goldstein (1967). *Lens 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). The glycopeptide (glycopeptide B-PD3) from human erythrocyte stroma was prepared according to the procedure described by Akiyama and Osawa (1971).

Purification of W. floribunda Mitogen. Finely powdered *W. floribunda* seeds (100 g) were suspended in 1 l. of 0.9% NaCl and allowed to stand overnight at 4° with occasional stirring. To the yellow clear supernatant obtained by centrifugation, solid $(NH_4)_2SO_4$ was added to give 40% and later 70 and 100% saturation. The fractions thus obtained were dialyzed against distilled water until free of NH_4^+, centrifuged, and lyophilized. Strong hemagglutinating activity was observed in the fractions precipitating with $(NH_4)_2SO_4$ at 70 and 100% saturation, whereas the mitogenic activity against human peripheral lymphocytes was detected only in the fraction precipitating at 100% saturation (crude mitogen). Further purification of the crude mitogen was achieved by chromatography successively on SE-Sephadex C-50 and Sepharose 6B as described in the legends of Figures 1 and 2.

Sedimentation Analysis. Measurements of sedimentation velocity of the mitogen were performed according to the band sedimentation method (Vinograd *et al.*, 1963) in a Spinco Model E ultracentrifuge equipped with an ultraviolet optical system at a speed of 52,640 rpm.

Disc Electrophoresis. Disc electrophoresis in polyacrylamide gels was carried out in 7.5% gels in Tris-HCl buffer at pH 8.9 according to Ornstein and Davis (1962). Staining was performed with Amido Black in 7% acetic acid, and destaining in an electric field with 7% acetic acid.

Molecular Weight Estimation by Gel Filtration. A column of Bio-Gel P-200 (1.2 × 100 cm) was equilibrated with 0.02 M phosphate buffer (pH 5.0) containing 0.15 M NaCl. The relationship between elution volume and the logarithm of the molecular weights of various proteins was established on this column according to the procedure of Andrews (1964). The

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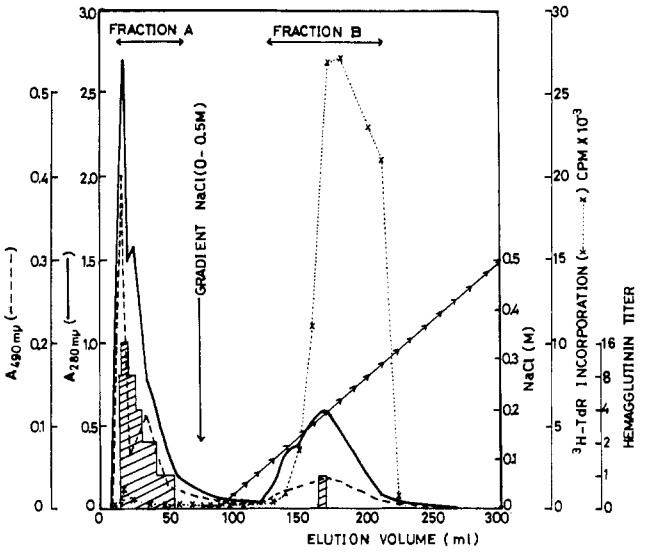

FIGURE 1: SE-Sephadex chromatography of crude mitogen. 150 mg of crude mitogen was dissolved in 5 ml of 0.05 M phosphate buffer (pH 5.0, starting buffer) and dialyzed overnight against the same buffer, and then applied to a column (2 × 30 cm) equilibrated against the same buffer. Elution was carried out with the same buffer and, after the first large peak was eluted out, gradient elution was performed with 100 ml of starting buffer in the mixing vessel and 100 ml of the same buffer containing NaCl (0.5 M) in the reservoir. Fractions of 5 ml were collected at 8 ml/hr at 4°. Hemagglutinating activity is denoted by shaded portions. Aliquots (50 μl) were used for mitogenic assay.

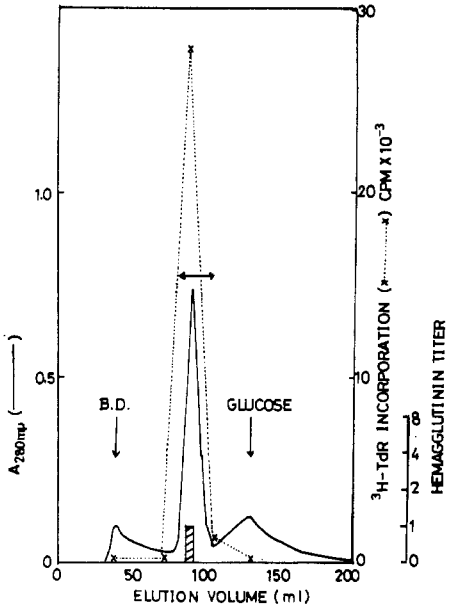

FIGURE 2: Gel filtration of fraction B on Sepharose 6B. Fraction B (15 mg) was dissolved in 1 ml of 0.02 M phosphate buffer (pH 5.0) containing NaCl (0.15 M) and applied to a column (2.5 × 30 cm) equilibrated against the same buffer. Elution was carried out with the same buffer and fractions of 2.5 ml were collected at a flow rate of 8 ml/hr at 4°. Hemagglutinating activity is denoted by shaded portions. The elution volumes of Blue Dextran and glucose were determined and are indicated by veritical arrows.

following proteins obtained from Mann Research Laboratories were used for this purpose (molecular weights shown in parentheses): bacitracin (1450), myoglobin (17,800), ovalbumin (45,000), bovine albumin (67,000), and human γ-globulin (160,000). Blue Dextran (Pharmacia) was used for determining the void volume of the column.

Amino Acid Analysis. The purified mitogen was hydrolyzed with redistilled HCl in sealed tubes for 24 and 48 hr at 110°. The amino acid content of the hydrolysates was determined on a Hitachi KLA-3D amino acid analyzer according to the method of Spackman *et al.* (1958). Values for each amino acid were calculated as previously described (Matsumoto and Osawa, 1969). Tryptophan was determined on unhydrolyzed protein samples by the spectrophotometric method of Goodwin and Morton (1946).

Sugar Analysis. Neutral sugar was determined by the phenol method of Dubois *et al.* (1956). Amino sugar was determined according to the method of Belcher *et al.* (1954). Hydrolysis for this assay was carried out with 4 M HCl for 8 hr at 100° in a sealed tube. For identification of the carbohydrates, gas–liquid chromatography was performed after reduction to the respective alditols, followed by trifluoroacetylation according to the method of Matsui *et al.* (1968), as described earlier (Matsumoto and Osawa, 1970). The trifluoroacetylated alditols derived from the purified mitogen were separated on a 1.8-m glass column packed with 2% XF-1105 on Gas Chrom Z (80–100 mesh) at 140°. The carrier gas flow was 35 cc/min.

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

Leukoagglutination Assays. Leukocyte suspensions for agglutination tests were prepared and examined according to the method of Woiwod *et al.* (1970).

Lymphocyte Cultures for Mitogenic Assay. Human peripheral lymphocytes were cultured by the method previously described (Toyoshima *et al.*, 1970).

Assay Methods. Morphological examination of lymphocyte transformation was carried out by determining the per cent transformed cells from Giemsa-stained preparations, counting approximately 1000 cells on each mitogen sample. The frequency distribution in relation to cell diameter was also calculated.

Radioactivity assay of [^{3}H]thymidine incorporation was performed by adding [^{3}H]thymidine (0.5 μCi, The Radiochemical Centre, England) to each tube (3–4 × 10^5 lymphocytes) after the appropriate incubation time. Fifteen hours later, the cells were collected and washed three times with cold 0.02 M phosphate-buffered saline (pH 7.0). To this residue was added 2 ml of cold 5% trichloroacetic acid, the precipitates were collected on glass fiber paper (Whatman GF/C), washed with 10 ml of methanol, and dried. The dried residue was then mixed with 5 ml of scintillation fluid (2,5-diphenyloxazol (3 g), 1,4-bis[2-(5-phenyloxazolyl)]benzene (0.1 g) in 1000 ml of toluene) and the counts per minutes of each sample were determined with a Packard automatic scintillation counter.

Inhibition assay with sugars was carried out as follows. To 0.05 ml of sugar solution in Eagle's minimal essential medium was added an equal volume of mitogen solution in Eagle's medium. After incubation for 10 min at room temperature, 0.9 ml of cell suspension (4 × 10^5 cells/ml) was added. The mixture was incubated for 72 hr and [^{3}H]thymidine incorporation was determined as above.

Results

Ammonium Sulfate Fractionation. Table I summarizes data pertaining to the purification of mitogen from *W. flori-*

TABLE I: Details of Purification of Crude Mitogen.

Fraction	Yield from 100 g of Seeds (mg)	Hemagglutinating Act. (μg/ml)[a]			Leuko-agglutinating Act. (μg/ml)[b]	Mitogenic Act.	Sugars (mg/ml)[c]	
		O	A	B			D-Galactose	*N*-Acetyl-D-galactosamine
$(NH_4)_2SO_4$ fractions								
0–0.4 saturation	300	>10,000	>10,000	>10,000		−		
0.4–0.7 saturation	1600	630	630	630		−	>10	>10
0.7–1.0 saturation (crude mitogen)	450	160	160	160		+	2.5	0.08
SE-Sephadex fractions								
Fraction A	160	80	80	80	320	−	2.5	0.08
Fraction B	150	5,000	5,000	5,000	2500	+	2.5	0.63
Sepharose 6B fraction	100	5,000	5,000	5,000	2500	+	2.5	0.63

[a] Minimum hemagglutinating dose against human O, A, or B cells. [b] Minimum agglutinating dose against human leukocytes. [c] Minimum amounts completely inhibiting four hemagglutinating doses.

bunda seeds. From Table I, it can be seen that there exist at least two kinds of blood-group nonspecific hemagglutinins in *W. floribunda* seeds, in which one is inhibited best by *N*-acetyl-D-galactosamine and the other is not inhibited by any sugars listed in Table III, and the mitogenic activity is observed in only one of the fractions (crude mitogen).

Purification of Crude Mitogen. The crude mitogen was applied to a column of SE-Sephadex C-50 in 0.05 M phosphate (pH 5.0, starting buffer) and, after a large peak was eluted out, gradient elution was performed as shown in Figure 1. Although the bulk of the hemagglutinating and leukoagglutinating activities were recovered in fraction A, the mitogenic activity was observed only in fraction B which had relatively weak agglutinating activity against both of erythrocytes and leukocytes (Table I). Fraction B was further purified by gel filtration on Sepharose 6B column as shown in Figure 2. The strong mitogenic activity concomitant with very weak hemagglutinating activity was eluted as a single peak (purified mitogen).

Analytical Results. Ultracentrifugation of purified mitogen yielded a single peak during the whole of the run in the densitometer chart obtained from the experiment. The sedimentation coefficient ($s_{20,w}$) calculated from the sedimentation velocity data was 4.5 S.

The electrophoretic homogeneity of the purified mitogen was confirmed by disc electrophoresis on polyacrylamide gel. A single sharp band was obtained at pH 8.9.

The amino acid composition of the purified mitogen is presented in Table II. About 87.5% of the dry weight of the purified mitogen could be accounted for as amino acid residues. If one adds the weight of carbohydrate moiety (11.4%), the recovery is 99%. This mitogen is rich in acidic and hydroxyamino acids, and low in sulfur-containing amino acids.

Carbohydrate analyses on the purified mitogen revealed the presence of 7.9% neutral sugar (w/w, phenol–sulfuric acid method with reference to mannose) and 3.5% amino sugar (w/w). Gas chromatographic investigation revealed that the major neutral sugar constituent was mannose (5.8%) and the remainder of the neutral sugar was made up of smaller amounts of fucose, arabinose, and xylose. Further, only glucosamine was detected as a component amino sugar by the amino acid analysis of the purified mitogen. Quantitative data from the foregoing carbohydrate analyses are included in Table II.

An approximate molecular weight of 70,000 was calculated on the purified mitogen from the curve (Figure 3) relating elution volume to logarithm of molecular weights of various standard proteins. However, in view of possible error in glycoprotein molecular weights estimated by gel filtration (Andrews, 1964), an accurate molecular weight of the material must wait the determination by other physicochemical methods.

Inhibition of Hemagglutination. The results of inhibition

TABLE II: Chemical Composition of Purified *W. floribunda* Mitogen.

Amino Acid	g/100 g	Carbohydrate	g/100 g
Asp	10.66	Mannose	5.80
Thr	6.05	Fucose	0.91
Ser	7.10	Arabinose	0.84
Glu	9.08	Xylose	0.33
Pro	3.48	Glucosamine	3.50
Gly	5.01	Total	11.38
Ala	3.76		
Cys	0.62		
Val	4.76		
Met	0.28		
Ile	4.92		
Leu	5.67		
Tyr	2.35		
Phe	5.82		
Try	3.05		
Lys	4.67		
His	3.68		
NH_3	1.91		
Arg	4.65		
Total	87.52		

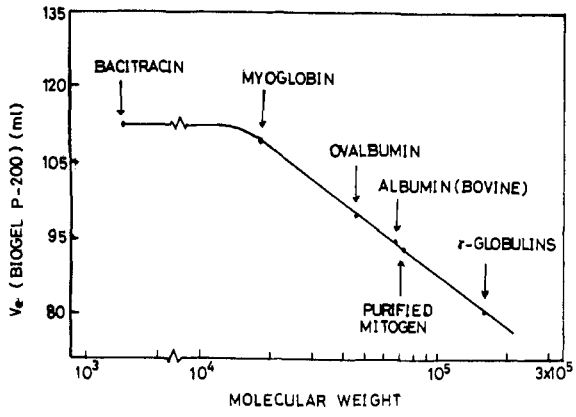

FIGURE 3: Relationship of logarithm of molecular weight to elution volume on Bio-Gel P-200 column for purified proteins. Experimental details are in the text.

tests of hemagglutination with simple sugars on the purified mitogen and the strongly hemagglutinating fraction A are given in Table III. In both cases, Mäkelä's group 2 sugars (Mäkelä, 1957), particularly *N*-acetyl-D-galactosamine, are potent inhibitors. It might be inferred from the comparison of the activity between lactose and melibiose that the sugars having β-glycosidic bond would have a greater activity than those having α-glycosidic bond. Entirely parallel results were obtained in the inhibition tests of leukoagglutination with simple sugars on the same samples (Table III).

Transformation of Lymphocytes. Figure 4 shows frequency distribution of human peripheral lymphocytes in relation to cell diameter after 72-hr culture with and without mitogens. In the culture with purified *W. floribunda* mitogen, large lymphocytes, more than 10 μ in diameter, were very abundant in comparison with a negative control. When approximately 1000 cells were counted, about 70% of the lymphocytes were found to be transformed after 72-hr culture. Mitotic cells were also observed when colchicine was added to the culture medium. This degree of transformation is substantially greater than that in the stimulatory culture with

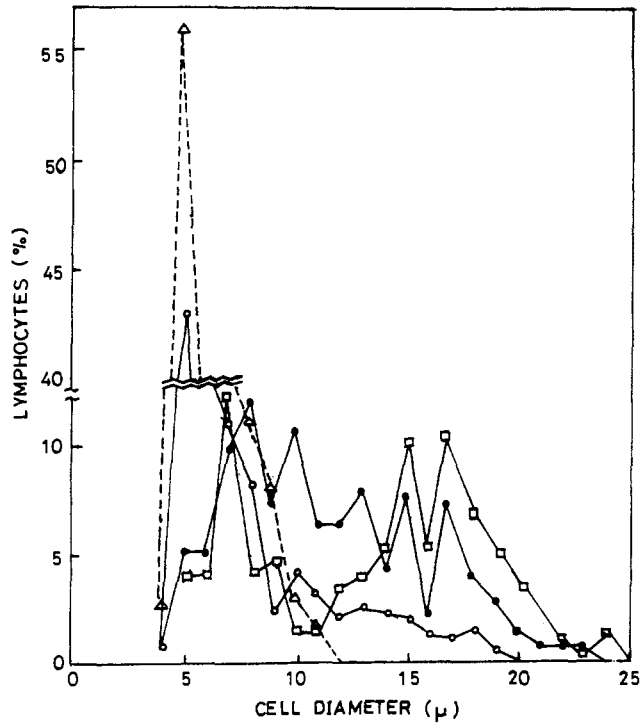

FIGURE 4: Frequency distribution of human peripheral lymphocytes in relation to cell diameter after 72-hr culture with and without mitogens. (□) Purified *W. floribunda* mitogen; (●) PHA-M; (○) *L. culinaris* hemagglutinin; (△) without mitogen. Concentration of mitogen was 0.4 mg/ml of culture medium. Each frequency was an average value of three experiments.

TABLE III: Inhibition of Hemagglutination of Fraction A and Purified Mitogen with Simple Sugars.[a]

Sugars	Min Amounts (mg/ml) Completely Inhibiting 4 Hemagglutinating Doses	
	Fraction A	Purified Mitogen
D-Galactose	2.5	2.5
L-Arabinose	10	5
N-Acetyl-D-galactosamine	0.08	0.63
Lactose	0.63	1.25
Melibiose	1.25	1.25
Raffinose	2.5	2.5
The following sugars are not inhibitory at a concentration of 20 mg/ml.		
D-Glucose	*N*-Acetyl-D-glucosamine	
L-Fucose	Maltose	
D-Mannose		

[a] Purities of sugars were checked by paper chromatography.

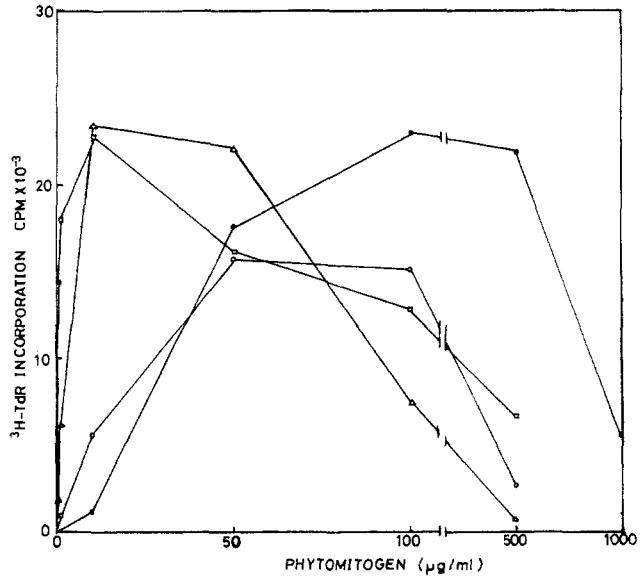

FIGURE 5: Dose response curve of various mitogens. (□) purified *W. floribunda* mitogen; (●) PHA-M; (○) *L. culinaris* hemagglutinin; (△) concanavalin A.

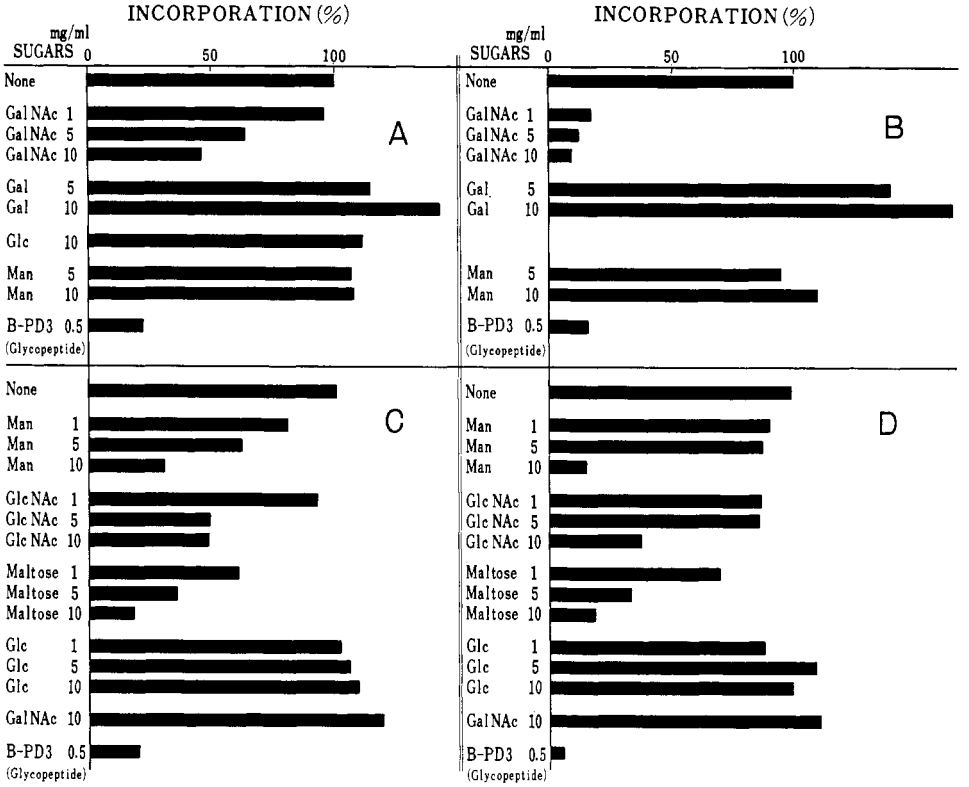

FIGURE 6: Effect of sugars on [^{3}H]thymidine incorporation by lymphocytes exposed to various mitogens. Each value represents the average of three cultures. Experimental details are in the text. (A) PHA-M; (B) purified *Wistria floribunda* mitogen; (C) concanavalin A; (D) *L. culinaris* hemagglutinin.

L. culinaris hemagglutinin (Toyoshima *et al.*, 1970), and is almost the same as in the case of PHA-M.

In order to demonstrate [^{3}H]thymidine incorporation as a function of mitogen added, lymphocytes were treated with various quantities of mitogens. The results are shown in Figure 5. Maximum incorporation after 3-days cultivation occurred at the following doses per 4×10^5 cells; 10 μg/ml for purified *W. floribunda* mitogen, 10 μg/ml for concanavalin A, 50 μg/ml for *L. culinaris* hemagglutinin, and 100 μg/ml for PHA-M.

Inhibition of [^{3}H]Thymidine Incorporation by Sugars. The effects of various sugars on [^{3}H]thymidine incorporation of human peripheral lymphocytes exposed to mitogens were examined and the results were shown in Figure 6. In the experiments utilizing purified *W. floribunda* mitogen or PHA-M, the most potent inhibitor was *N*-acetyl-D-galactosamine as in the cases of the hemagglutination–inhibition assays of both mitogens (Table III; Borberg *et al.*, 1966). On the other hand, [^{3}H]thymidine incorporation by cells stimulated with concanavalin A or *L. culinaris* hemagglutinin was best inhibited by D-mannose which has been known as the most potent inhibitor against the hemagglutination of the above two mitogens (Toyoshima *et al.*, 1970). It is interesting to note that both D-galactose and D-glucose are not inhibitory but rather stimulatory, in spite of the facts that the former is a good inhibitor of the hemagglutination of purified *W. floribunda* mitogen (Table III) and the latter is moderately inhibitory against the hemagglutination of both of concanavalin A and *L. culinaris* hemagglutinin (Toyoshima *et al.*, 1970). Another interesting point which might be of particular importance is the strong inhibitory activity of a glycopeptide (glycopeptide B-PD3) from human erythrocyte stroma against all of the above four mitogens. This glycopeptide has previously been disclosed to be a specific inhibitor of *L. culinaris* hemagglutinin and composed of sialic acid (39.1%), galactose (20.1%), mannose (4.2%), fucose (1.6%), glucosamine (7.8%), and galactosamine (5.3%) (Akiyama and Osawa, 1971).

Interaction between Various Mitogens. In order to study whether the addition of two nonspecific mitogens would act additively or competitively with regard to induction of DNA synthesis, lymphocytes were stimulated by various combinations of mitogens and incorporation of [^{3}H]thymidine was compared with the sum of the individual responses (Table IV). In general, the simultaneous stimulation of lymphocytes with two nonspecific mitogens resulted in incorporation being less than expected from the sum of both of the stimulant individually, regardless of the fact that two mitogens have different specificities in hemagglutination or leukoagglutination inhibition assays.

Discussion

In the previous papers (Matsumoto and Osawa, 1969, 1970) from this laboratory, it has been shown that *Ulex europeus* seeds contain two kinds of anti-H(O) hemagglutinins. In this paper, we have confirmed that there exist at least two kinds of blood group nonspecific hemagglutinins, differing in specificity in *W. floribunda* seeds. This series of evidence implies that care should be taken in the experiment using crude extracts of seeds.

The mitogenic protein of *W. floribunda* was separated from the strongly hemagglutinating and leukoagglutinating protein by SE-Sephadex chromatography. The former was

TABLE IV: Effect of Simultaneous Stimulation of Lymphocytes with Two Mitogens.

Mitogens (μg/ml)		[^{3}H]Thymidine Incorporation (cpm)				
A	B	A	B	Expected Response (A + B)	Obsd Response	Obsd Response as % of Expected Response
W. floribunda (5)	PHA-M (5)	14,400	200	14,600	11,500	79
W. floribunda (5)	PHA-M (50)	14,400	19,800	34,200	19,300	56
W. floribunda (5)	PHA-M (100)	14,440	17,700	32,100	29,000	90
W. floribunda (5)	*L. culinaris* (5)	14,400	2,400	16,800	22,900	136
W. floribunda (5)	*L. culinaris* (50)	14,400	10,000	24,400	16,600	68
W. floribunda (5)	*L. culinaris* (100)	14,400	12,800	27,200	16,200	60
L. culinaris (50)	*W. floribunda* (1)	10,000	5,700	15,700	7,700	49
L. culinaris (50)	*W. floribunda* (5)	10,000	14,400	24,400	16,600	68
L. culinaris (50)	*W. floribunda* (10)	10,000	12,600	22,600	17,200	76
L. culinaris (50)	Concanavalin A (1)	10,000	2,500	12,500	13,300	106
L. culinaris (50)	Concanavalin A (5)	10,000	9,600	19,600	9,600	49
L. culinaris (50)	Concanavalin A (10)	10,000	14,700	24,700	13,200	53

further purified by Sepharose-6B gel filtration to yield a glycoprotein containing 11.4% carbohydrate. Although complete separation of mitogenic activity from hemagglutinating activity could not be achieved in the present study, the purified mitogen has relatively weak hemagglutinating and leukoagglutinating activities. The fact strongly suggests that mitogenic activity is not necessarily relevant to leukoagglutination and only restricted kind of receptor sites on lymphocyte surface might be involved in triggering of lymphocyte transformation. This inference has been supported by our recent survey of many plant seeds in which certain strongly leukoagglutinating extracts have been found to be completely devoid of mitogenic activity against human peripheral lymphocytes (Osawa and Kojima, unpublished data).

In inhibition assays of stimulatory activity of various mitogens with simple sugars, the activity of concanavalin A or *L. culinaris* hemagglutinin was inhibited best by D-mannose and not by *N*-acetyl-D-galactosamine. On the contrary, the activity of *W. floribunda* mitogen or PHA-M was inhibited most by *N*-acetyl-D-galactosamine and not by D-mannose. Almost the same results have previously been reported on concanavalin A by Powell and Leon (1970) and on PHA-M by Borberg *et al.* (1968). These results seem to indicate that at least two kinds of receptor sites on lymphocyte surface are involved for the triggering of lymphocyte transformation. However, a single glycopeptide from human erythrocyte stroma, which was a good inhibitor against the hemagglutination by *L. culinaris* hemagglutinin but not significantly inhibitory against the hemagglutination by other three mitogens (Akiyama and Osawa, 1971), was found to be strongly inhibitory against the stimulatory activity of all of the four mitogens. The fact suggests that the four mitogens can recognize and bind to the same receptor site on the cell surface in the triggering of lymphocyte transformation, but the hemagglutination and the leukoagglutination need the stronger association between the agglutinin and the receptor site on the cell surface to form visible aggregates. Kornfeld and Kornfeld (1969) have also isolated a glycopeptide from human erythrocyte membrane which is strongly inhibitory against the hemagglutinating and the mitogenic properties of *Phaseolus vulgaris* hemagglutinin but apparently differs in chemical composition from our glycopeptide.

Recently, Möller has reported that the simultaneous stimulation of lymphocytes with two nonspecific mitogens in optimal concentrations gives rise to the decrease of [^{3}H]-thymidine incorporation (Möller, 1970). We have also observed an antagonistic effect of the simultaneous stimulation with two nonspecific mitogens. These results seem to imply that lymphocytes recognize the quantity of receptor sites that have been triggered regardless of the kind of mitogen, and might be another indication to support the assumption that only restricted kind of receptor sites is involved in the triggering of lymphocyte transformation. Further investigations on the structural relationship between the receptor sites of various mitogens are necessary to verify this assumption.

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Synthesis and Oncogenicity of 3-Hydroxyuric Acid*

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ABSTRACT: 3-Hydroxyuric acid has been synthesized by a method which involves unique conditions for the closure of the imidazolone ring. Administered subcutaneously it is weakly oncogenic to rats. *In vitro* xanthine oxidase reduces a small proportion of it to 3-hydroxyxanthine. The results suggest that its oncogenicity can be attributed to the reduction *in vivo* of a portion of it to 3-hydroxyxanthine.

Some chemical oncogens[1] are metabolically converted into products which are the actual initiators of the cancer process (Miller, 1970). Any metabolite of a chemical oncogen is therefore suspect of being a proximate oncogen. 3-Hydroxyxanthine and guanine 3-oxide are potent chemical oncogens (Sugiura *et al.*, 1970). Initial metabolic studies showed (Stöhrer and Brown, 1969a; Myles and Brown, 1969) that, in rats, about 95% of single doses ranging from 0.07 (Stöhrer and Brown, 1970) to 7 mg (Myles and Brown, 1969) could be accounted for in the urine within 24 hr; of this about 90% represents products which are derived *via* either oxidations or reductions catalyzed by xanthine oxidase (Stöhrer and Brown, 1969b) and by the subsequent action of uricase. From 3-hydroxyxanthine the products include: xanthine and its sequelae, uric acid and allantoin; 3-hydroxyuric acid and, from the action of uricase on it (Myles and Brown, 1969) probably *N*-hydroxyallantoins. From guanine 3-oxide the products include (Stöhrer and Brown, 1969a): a trace of 3-hydroxyxanthine; considerable guanine and its sequelae, xanthine, uric acid and allantoin, 8-hydroxyguanine 3-oxide, and 8-hydroxyguanine. Among these metabolites it was deemed that 3-hydroxyuric acid was the most important for study as a candidate for a proximate oncogen derived from 3-hydroxyxanthine.

Materials and Methods

3-Hydroxyuric Acid, Synthesis. 6-AMINO-1-BENZYLOXY-5-NITROSO- (II) AND 1-BENZYLOXY-5,6-DIAMINOURACIL (III) SULFATE. Sodium nitrite solution (3.75 g, in 50 ml of water) was slowly dropped into a suspension of finely ground 6-amino-1-benzyloxyuracil (I) (Klötzer, 1964) (12.7 g) in 0.5 N HCl (109 ml) under stirring. After stirring for 12 hr the precipitated red 6-amino-1-benzyloxy-5-nitrosouracil (14.1 g) was collected and washed thoroughly with water. The nitroso derivative was dissolved in 0.5 N sodium hydroxide (100 ml) and sodium dithionite was added in small portions (~10.2 g) until the red color faded. The gray precipitate was collected and washed with water. Stirring of the precipitate with 2 N sulfuric acid (100 ml) gave 1-benzyloxy-5,6-diaminouracil sulfate (11.1 g) as a light pink precipitate. An analytical sample was prepared by dissolving the 5,6-diaminouracil in boiling 2 N sulfuric acid and cooling immediately. It crystallized as brown needles of mp 134 (dec).

Anal. Calcd for $C_{11}H_{12}N_4O_3 \cdot 0.5H_2SO_4 \cdot H_2O$: C, 41.90; H, 4.79; N, 17.76; S, 5.08. Found: C, 42.22; H, 4.67; N, 17.65; S, 5.17.

6-AMINO-5-ETHOXYCARBONYLAMINO-1-BENZYLOXYURACIL (IV). The above diaminouracil sulfate (3.15 g), ethyl chloroformate (2.0 ml), and 1 N sodium hydroxide (30 ml) were stirred at 0° for 15 min. The ice bath was removed and the temperature was allowed to rise to 23°. The stirring was continued for about 30 min, when the acidity of the solution was pH 6. The precipitate was collected by filtration and recrystal-

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[1] The more common term "chemical carcinogen" should logically be restricted to agents that induce carcinomas. Oncogenic is an inclusive term for agents that induce any form of neoplasia (Martin, 1963; Rous, 1967).