SOLUBILIZATION AND PARTIAL CHARACTERIZATION OF A PHYTOHEMAGGLUTININ RECEPTOR SITE FROM HUMAN ERYTHROCYTES

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Abstract.—Trypsin treatment of human erythrocytes releases a soluble glycopeptide which binds to phytohemagglutinin and abolishes the erythroagglutinating and lymphocyte-stimulating properties of this molecule. The glycopeptide has been purified by alkaline borohydride treatment, proteolytic digestion, gel filtration, and DEAE-cellulose chromatography. The most highly purified glycopeptide has a molecular weight of about 2,000. The specificity for binding to phytohemagglutinin resides in the oligosaccharide portion of the molecule with the determinant sugar being a galactose residue which is penultimate to a *N*-acetylneuraminic acid in some chains and uncovered in others. The glycopeptide is about 3,000 times more potent than either *N*-acetylgalactosamine or galactose in inhibiting the mitogenic response of lymphocytes induced by phytohemagglutinin.

Phytohemagglutinin (PHA), a glycoprotein derived from the red kidney bean *Phaseolus vulgaris*, has the capacity to agglutinate erythrocytes and leukocytes and to stimulate tissue culture lymphocytes to undergo mitosis. Weber *et al.*[1] have separated PHA into two components which stimulate mitosis in lymphocytes but differ from each other in that one component has only erythroagglutinating activity while the other has only leukoagglutinating activity. Although the binding of PHA to cell surface receptor sites appears to be the initial step in the sequence of events leading to cell agglutination and mitogenesis, the nature of the cellular receptor site is unknown. In this communication we describe the solubilization and partial purification from human erythrocytes of the cellular receptor site for the erythroagglutinating PHA.

Materials and Methods.—Preparation of glycopeptides from erythrocytes: Outdated bank blood was washed 3 times with 3 vol of 0.9% NaCl–0.01 *M* $NaHCO_3$ to remove the plasma and buffy coat, and the erythrocytes were treated with trypsin (Difco) as described by Winzler *et al.*[2] for 1 hr at 37°. The cells were removed by centrifugation, the chilled supernatant fluid was brought to 5% trichloroacetic acid, and the precipitate removed by centrifugation. The supernatant fluid, containing the trypsin-released glycopeptides, was neutralized with NaOH, dialyzed overnight at 4°C, and then lyophilized.

Assays: PHA from *Phaseolus vulgaris* was obtained as Bacto-Phytohemagglutinin-P (Difco) and purified as described by Weber *et al.*[1] The unpurified PHA-P was ordinarily used for hemagglutination inhibition assays while the purified erythroagglutinin (Peak III of Weber *et al.*[1]) was used in lymphocyte culture experiments. The purified PHA which gives a single band on disc gel electrophoresis was iodinated by the method of Awai and Brown.[3] PHA receptor site activity was routinely assayed by its ability to inhibit PHA-induced hemagglutination. The standard reaction mixture contained 15 μl of 4% erythrocytes, 25 μl of 0.9% NaCl–0.01 *M* $NaHCO_3$, and 4 μl of PHA-P ($^1/_{50}$ solution prepared daily from a stock solution of 20 mg/ml). This amount of PHA produced 4+ agglutina-

tion in 3 min (1 hemagglutinating unit). One unit of inhibitory activity (IU) is defined as the amount of material necessary to inhibit completely red-cell agglutination in the standard system for 3 min.

Enzymes: Neuraminidase from *Vibrio cholerae* and Pronase were purchased from Calbiochem (Los Angeles, California). Purified β-galactosidase and β-*N*-acetylglucosaminidase were prepared from jack bean meal by a slight modification of the method of Li.[4] Galactostat was a product of Worthington (Freehold, N. J.). Fetuin was purchased from Grand Island Biological Company (Grand Island, N. Y.).

Analysis of glycopeptide composition: *N*-acetylneuraminic acid (NANA) was measured by the thiobarbituric acid method of Warren[5] following hydrolysis in 0.05 *N* H_2SO_4 for 1 hr at 80° (or 1 *N* HCl for 1 min at 100°). Total hexose was measured by the phenol-H_2SO_4 method and protein by the method of Lowry *et al.*[6] Individual neutral sugars were determined following hydrolysis in 2 *N* H_2SO_4 for 4 hr at 100° and paper chromatography in butanol:ethanol:H_2O (10:1:2). Recovery was corrected for by including ^{14}C tracer sugars in the hydrolysis. Galactose was measured with the galactostat reagent. Mannose was measured by the Park-Johnson ferricyanide method,[7] and fucose by the cysteine-H_2SO_4 method.[8] Hexosamine was determined with the amino acid analyzer or by the method of Reissig *et al.*[9] following hydrolysis in 4 *N* HCl for 4 hr at 100° *in vacuo*, lyophilization to remove HCl, and adsorption and elution from Dowex 50. Again recovery was corrected for by adding ^{14}C-glucosamine to the hydrolysis. Quantitative estimates of amino acid composition were obtained with the amino acid analyzer following hydrolysis of the glycopeptide in constant boiling HCl for 16 hr at 108° in an evacuated sealed tube. (These determinations were kindly done by Dr. Kirk Osterland.)

Results.—When intact red blood cells are incubated with trypsin, glycopeptide material is released from the cell surface into the medium.[2, 10] In order to determine whether these trypsin-released glycopeptides are involved in phytohemagglutinin binding to cell surfaces, the ability of red blood cells and lymphocytes to bind ^{131}I-PHA was determined following trypsin treatment of the cells. As shown in Figure 1, trypsin treatment reduced the binding of ^{131}I-PHA to red blood cells by about 40 per cent and to lymphocytes by about 70 per cent. With prolonged incubation there was no further reduction of PHA binding to the cells. When the released glycopeptide material was freed of trypsin by treatment with trichloroacetic acid as described in *Methods*, it was found to be a potent inhibitor of the binding of ^{131}I-PHA to untreated red cells and of the agglutination of red cells by PHA. This latter property formed the basis of a rapid and convenient assay system (see *Methods*) which was utilized in the purification of the active glycopeptide. Studies of ^{131}I-PHA binding to red cells have shown that the glycopeptide is a competitive inhibitor of PHA binding to cell surfaces (Fig. 2).[11] In order to demonstrate that the trypsin-released glycopeptides actually bind to PHA, the gel filtration study shown in Figure 3 was performed. These experiments show that PHA is capable of altering the migration of the glycopeptide on Sephadex G-75, thus providing direct evidence for the binding of the glycopeptide to PHA. This type of behavior would be predicted if the glycopeptide is a cellular receptor site for PHA. Other proteins tested, alcohol dehydrogenase (Fig. 3), albumin, and gamma globulin did not alter the migration of the glycopeptide on Sephadex G-75, showing that nonspecific binding to proteins was not occurring.

When the glycopeptide was treated with alkaline borohydride, two thirds of the *N*-acetylneuraminic acid-containing oligosaccharides (presumably those linked O-glycosidically to serine and threonine[13]) were released as shown by gel

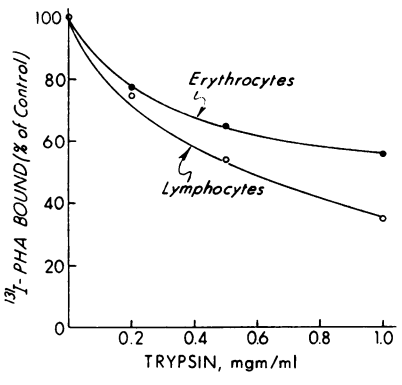

FIG. 1

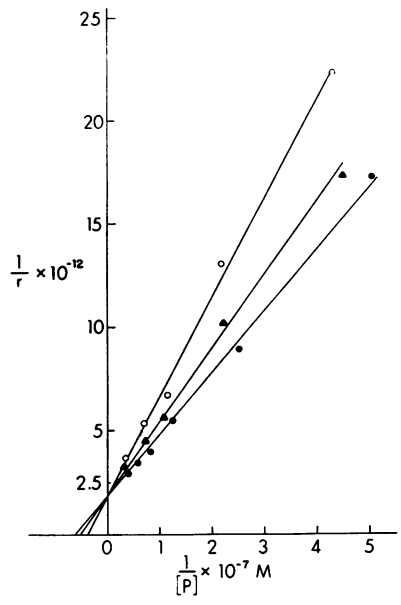

FIG. 2

FIG. 1.—Effect of trypsin treatment on ^{131}I-PHA binding to erythrocytes and lymphocytes. Erythrocytes and lymphocytes were incubated with trypsin for 20 min at 37°. The reaction mixtures were then diluted with 4 vol of normal saline and the cells collected by centrifugation. The cells were resuspended in fresh media containing saturating amounts of ^{131}I-PHA (1.8×10^{-7} *M* on the basis of molecular weight of 128,000[12]; sp. act. 6.1×10^{8} cpm/μmole) and reincubated for 30 min at room temperature. The cells were washed twice with 15 vol of saline and the amount of bound ^{131}I-PHA determined in a Packard autogamma counter.

FIG. 2—Competitive inhibition of ^{131}I-PHA binding to erythrocytes by the trypsin-released glycopeptide. The binding reactions were carried out in plastic counting tubes in a medium of 0.9% NaCl–0.01 *M* $NaHCO_3$–2% albumin which contained in 1.0 ml: from 2 to 25×10^{-8} *M* ^{131}I-PHA (sp. act. 6.1×10^{8} cpm/μmole), 4.18×10^{7} red blood cells, and the trypsin-released glycopeptide (purified through the stage of Sephadex G-25 chromatography shown in Fig. 4) as follows: ●—●, none; ▲—▲, 13 inhibitory units (IU); ○—○, 26 IU. After 15 min of incubation at 25°, the red cells were sedimented and the cell pellet was washed once. The tubes were counted to determine the ^{131}I-PHA bound. The results have been plotted as described by Steck and Wallach[12] according to the equation, $1/r = 1/Kn \cdot 1/[P] + 1/n$, where $[P]$ = molar concentration free PHA, n = no. sites per cell, $1/r = [C]/[PS]$, and $[C]$ = no. of cells and $[PS]$ = μmoles PHA bound in each reaction mixture. K is the affinity constant of PHA for erythrocytes.

filtration on Sephadex G-25 (Fig. 4).[14] The residual glycopeptide material eluting in the exclusion volume of the G-25 column retained full inhibitory activity and could be separated into two main components (*A* and *B*) upon subsequent gel filtration on Sephadex G-75 (Fig. 4).

Removal of 100 per cent of the terminal *N*-acetylneuraminic acid residues of the Sephadex G-75 peak *A* material with *Vibrio cholerae* neuraminidase did not destroy inhibitory activity (Table 1). However, β-galactosidase treatment following but not preceding neuraminidase treatment resulted in a 70–90 per cent loss of activity in various experiments. The only sugar released by this treatment was galactose, as shown by paper chromatography using as a solvent system butanol:ethanol:H_2O (10:1:2). Since fetuin, a serum glycoprotein, con-

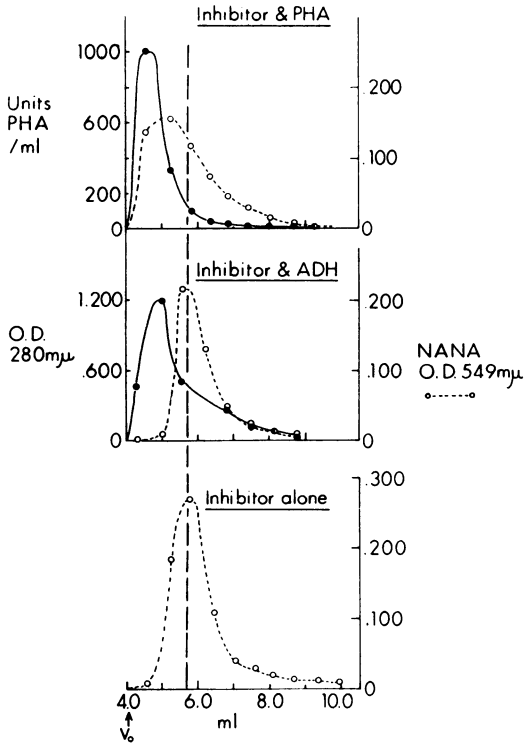

FIG. 3.—Binding of the soluble red cell trypsin fragments to PHA. Reaction mixtures (0.5 ml) containing 0.9% NaCl–0.01 *M* $NaHCO_3$, 30 IU of soluble trypsin fragments plus either 0.32 mg erythroagglutinating PHA (1600 hemagglutinating units) or 2.0 mg alcohol dehydrogenase as indicated were incubated at room temperature for 2 hr and then placed on a Sephadex G-75-40 column (1 × 12 cm) at 4°. Fractions of 0.5 ml were collected and analyzed for protein (OD 280), and NANA which indicates the location of the trypsin fragments. Since excess PHA, compared to inhibitory trypsin fragments, was present, the PHA could be measured by the usual red blood cell agglutination assay.

tains oligosaccharide units that terminate in the sequence *N*-acetylneuraminic acid → galactose → *N*-acetylglucosamine,[15] it was tested in the hemagglutination inhibition assay system and was found to be a good inhibitor of PHA agglutination of red cells. Sequential enzyme degradation of the terminal carbohydrate units of fetuin also showed that inhibitory activity was determined primarily by a galactose residue penultimate to an *N*-acetylneuraminic acid residue (Table 1). However, in this case removal of the terminal *N*-acetylneuraminic acid residues did result in a moderate loss of inhibitory activity, perhaps related to a conformation change in this high-molecular-weight glycoprotein induced by the change in the net charge of the molecule.

The partially purified glycopeptide not only inhibits red cell agglutination by PHA but is also a potent inhibitor of the mitogenic response of lymphocytes induced by the erythroagglutinating PHA (Table 2). This inhibition could be overcome by increasing the amount of PHA (expt. 2, Table 2). A number of simple sugars, particularly *N*-acetylgalactosamine, have been found to inhibit the erythroagglutinating and mitogenic properties of PHA.[17, 18] While the data in Table 2 confirm these observations, they also demonstrate that the partially purified PHA inhibitor is about 3,000 times more potent than either *N*-acetylgalactosamine or galactose on a molar basis.

The active materials in both peaks *A* and *B* (Fig. 4) have been further purified by proteolytic digestion with Pronase followed by Sephadex G-50 gel filtration and DEAE-cellulose column chromatography. Gradient elution of DEAE-

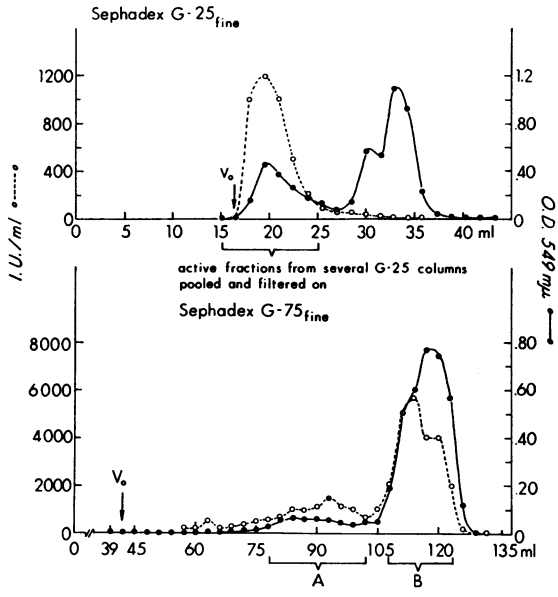

FIG. 4.—Purification of trypsin fragments by alkaline borohydride treatment and gel filtration. The desalted soluble trypsin fragments from 1,000 ml red cells were incubated with 0.1 *M* sodium borohydride in 0.2 *N* NaOH for 36 hr at room temperature, under N_2[2]. Following neutralization with 2 *N* HCl, the solution was concentrated and applied to a Sephadex G-25-80 column (1.2 × 33 cm). The column was eluted with water. The front peak, containing all the inhibitory activity, was concentrated and combined with two other identical preparations and applied to a G-75-40 column (1.5 × 80 cm). Elution was carried out with water. NANA is shown by its absorption at 549 mμ in the thiobarbituric acid test.

cellulose columns with phosphate buffer, pH 6.8 (3 to 100 mM), separated the active glycopeptides from hexose-rich oligosaccharide material which did not adsorb and from inactive *N*-acetylneuraminic acid-rich material which adsorbed more strongly to the DEAE-cellulose. Table 3 shows the composition of the most highly purified DEAE-cellulose fraction derived from peak B. Assuming that there is a single oligosaccharide chain with a glycosylamine-type linkage between the aspartic acid and *N*-acetylglucosamine, the molecular weight of the glycopeptide would be approximately 2,000. In contrast to the peak *A*

TABLE 1. *Effect of glycosidase treatment on PHA inhibitory activity.*

Treatment	Red cell inhibitor (total inhibitory units)	Fetuin
Buffer alone	250	1,000
Neuraminidase alone*	250	575
β-Galactosidase alone†	250	1,000
Neuraminidase followed by β-galactosidase	60	80

* The reaction was carried out under a toluene atmosphere in 0.06 *M* phosphate citrate buffer, pH 6.2, containing 0.001 *M* $CaCl_2$ and 50 units/ml neuraminidase. The release of free *N*-acetylneuraminic acid was determined by the thiobarbituric acid test.[5] Total release of *N*-acetyl neuraminic acid occurred by 30 hr at 37°.

† The reaction was carried out under a toluene atmosphere in 0.04 *M* citrate buffer, pH 4.6, containing 0.2 mg/ml of purified β-galactosidase (0.25 μmole O-nitrophenylgalactoside hydrolyzed/min/mg protein). The incubation time was 20 hr at 37°.

TABLE 2. *Effect of inhibitors on incorporation of ^{3}H-thymidine into DNA by lymphocytes.*

	^{3}H-thymidine incorporated (cpm/4 hr/culture)	Inhibition (%)*
Expt. 1		
Control	1,630	
PHA (3.5 μg/ml)	7,586	
+ inhibitor (I.U./ml)		
10	7,400	3
30	6,600	17
60	5,600	33.5
100	4,950	44.5
150 (= 0.015 mM)†	4,540	51
+ *N*-acetylgalactosamine, 45.5 mM	3,470	69
+ *N*-acetylglucosamine, 45.5 mM	5,930	28
+ galactose, 55.5 mM	4,100	58.5
Expt. 2		
Control	573	
PHA (7.0 μg/ml)	19,367	
+ inhibitor, 100 I.U./ml	14,310	28
PHA (14.0 μg/ml)	26,535	
+ inhibitor, 100 I.U./ml	25,270	5
PHA (26.0 μg/ml)	34,817	
+ inhibitor, 100 I.U./ml	32,320	7

I.U. = inhibitory units.

Red cells were allowed to settle from a blood-Dextran mixture, and lymphocytes recovered from the supernatant fluid were freed of granulocytes and platelets by passage through a column of nylon fibers packed into a 50-ml glass syringe. The filtrate was sedimented twice at 400 × *g* for 7 min and the pellet (>90% lymphocytes) was suspended in Eagle's minimal essential medium (MEM). Each culture tube contained in 2 ml: 7% fetal calf serum in MEM, 4 mM glutamine, 100 units penicillin, 100 μg streptomycin, 2 × 10^6 lymphocytes, and, as indicated, erythroagglutinating PHA and the red cell inhibitor (peak *A* from Sephadex G-75) at the concentration shown.

After incubation at 37° for 72 hr in 5% CO_2–95% air, 3 μc of ^{3}H-thymidine were added to each culture for 4 hr. The cells were washed with saline, suspended in cold 5% trichloroacetic acid, and sonicated to rupture the cells. The precipitate was washed with cold trichloroacetic acid and methanol, dissolved in 0.5 ml NCS (Nuclear-Chicago), and counted in a Packard Tri-Carb scintillation counter in Bray's solution.[16]

* The cpm incorporated by the control cultures (endogenous DNA synthesis) were subtracted from the other values before calculating the % inhibition.

† Assuming one terminal *N*-acetylneuraminic acid residue per oligosaccharide.

material tested in Table 1, which had a *N*-acetylneuraminic acid residue covering each galactose residue, the glycopeptide purified from peak *B* contained approximately one *N*-acetylneuraminic acid residue for every two galactose residues. The glycopeptide lost half its galactose upon treatment with β-galactosidase alone, and all of its galactose and 90 per cent of its inhibitory activity after treatment with neuraminidase and β-galactosidase. When that treatment was followed by the action of β-*N*-acetylglucosaminidase, two of the three *N*-acetylglucosamine residues were liberated. These results suggest that the glycopeptide contains a branched oligosaccharide with the following structure:

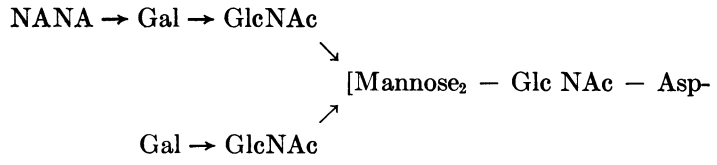

TABLE 3. *Composition of a purified red blood cell glycopeptide.*

	Molar ratio
N-acetylneuraminic acid	1.1
Total hexose	3.3
Galactose	1.8
Mannose	1.8
N-acetylglucosamine	2.9
N-acetylgalactosamine	<0.1
Fucose	0.05
Aspartic acid	1.34
Serine	1.03
Threonine	0.84
Glutamic acid	0.43
Estimated molecular weight	2000

The glycopeptide material analyzed was obtained by DEAE-cellulose chromatography of Pronase-digested peak *B* material (Fig. 4). Carbohydrate and amino acid analyses were carried out as described in *Methods*.

Discussion.—These studies demonstrate that trypsin treatment of human erythrocytes releases from the cell membrane glycopeptide fragments which retain their capacity to bind to PHA. Winzler *et al.*[2] have found that such glycopeptides, while heterogeneous, fall into a single size range with a molecular weight of about 10,000. These glycopeptides have multiple oligosaccharide chains, of which only a portion are capable of binding to PHA to inhibit its action. This is clearly shown by the separation of the active material away from inactive oligosaccharide material at virtually every step of its isolation. Since the oligosaccharide chains involved in PHA binding are resistant to alkaline borohydride treatment, they most likely are not linked to the peptide chain through O-glycosidic bonds involving the hydroxyl groups of serine and threonine,[13] but rather form glycosylamine linkages between aspartic acid and *N*-acetylglucosamine (Table 3).

Treatment of the glycopeptide with glycosidases has shown that the specificity for binding to the erythroagglutinating PHA resides in the oligosaccharide portion of the glycopeptide, with the determinant sugar being a galactose residue covered by a terminal *N*-acetylneuraminic acid. This finding was somewhat unexpected in view of previous reports stating that *N*-acetylgalactosamine was involved in PHA binding to both red cells and lymphocytes.[17, 18] We find that while both *N*-acetylgalactosamine and galactose can inhibit the lymphocyte mitogenic response to PHA, the red-cell glycopeptide is about 3,000 times more potent than either sugar. Furthermore, the active glycopeptide obtained by DEAE-cellulose chromatography (Table 3) has a very low content of *N*-acetylgalactosamine (less than 1% of the total hexosamine). These data taken together with the glycosidase experiments (Table 1) strongly argue against *N*-acetylgalactosamine's being the determinant sugar in the binding of the erythroagglutinating PHA to cells.

The fact that the partially purified red-cell glycopeptide is capable of inhibiting the PHA-induced lymphocyte mitogenic response suggests that the erythroagglutinating PHA may bind to a similar oligosaccharide on both the lymphocyte and erythrocyte cell surfaces. This finding is of particular interest since it indicates the presence of a biologically active oligosaccharide on the lymphocyte

cell surface. It is now well established that the surfaces of mammalian cells contain many different oligosaccharides in the form of either glycoproteins or glycolipids. Some of these, such as A, B, H blood group substances, have been well characterized.[19, 20] However, very little is known about the biologic role that these surface oligosaccharides may have. In the system investigated here, the oligosaccharide on the lymphocyte surface acts as the receptor site for PHA binding to the cell and that interaction between PHA and the cell leads to a profound alteration in the cell's metabolism resulting in mitogenesis.

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Abbreviations: PHA, phytohemagglutinin; Gal, galactose; GlcNAc, *N*-acetyl-D-glucosamine; NANA, *N*-acetylneuraminic acid.

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[11] The affinity constant K for PHA binding to erythrocytes was determined from the $\frac{1}{[P]}$ intercept of the control set in Fig. 2 ($5.7 \times 10^6\ M^{-1}$) and the number of binding sites per red cell, n, was determined from the $1/r$ intercept (3.4×10^5 per cell). Steck and Wallach,[12] using very different experimental conditions, obtained $K = 8.4 \times 10^7\ M^{-1}$ and $n = 5 \times 10^5$.

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