Specific Leukocyte Receptors for Small Endogenous Hormones

DETECTION BY CELL BINDING TO INSOLUBILIZED HORMONE PREPARATIONS

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ABSTRACT Receptors for small endogenous hormones on human leukocytes were studied by insolubilizing the hormones and incubating them with the cells. Histamine, norepinephrine, and prostaglandin E_2 (PGE_2) were conjugated to either of two types of carrier: (bovine or rabbit) serum albumin or a random copolymer of DL-alanine and L-tyrosine. The conjugates were linked to agarose beads (Sepharose) and the resultant drug-conjugate-beads were incubated with leukocytes. Norepinephrine (when linked to its carrier via glutaraldehyde) and histamine preparations bound the majority of leukocytes. The binding appeared to be specific for the hormones tested. For example, the binding by histamine-rabbit serum albumin-Sepharose was prevented or reversed by high concentrations of histamine and histamine antagonists, but not by catecholamines or their pharmacologic antagonists. Similarly, binding of cells to the norepinephrine conjugate was inhibited by some catecholamines and propranolol, but not by histamine or histamine antagonists. Conjugates of norepinephrine linked via carbodiimide did not bind cells. The protein or copolymer carriers did not contribute to binding per se. The hormone-protein-conjugates bound more cells than the hormone-polymer conjugates. The former (unlike the free amines) failed to stimulate accumulation of cyclic AMP in leukocytes. The norepinephrine linked to polymer via glutaraldehyde, however, did stimulate leukocyte cyclic AMP accumulation, possibly because of the flexibility of the polymer. Columns of the various Sepharoses were used to determine the distribution of receptors to each hormone in mixed leukocyte populations. The majority of cells appeared to have receptors for both histamine and norepinephrine (bound through glutaraldehyde). Receptors to prostaglandins may have been detected by the column procedure, but their distribution could not be quantitated. The approach described provides a means to separate leukocytes on the basis of what are likely to be preformed receptors to small endogenous hormones, and to study the physiologic importance and function of the receptors.

INTRODUCTION

Basic methods have been devised to detect and chemically characterize cell surface receptors to hormones or antigens. One method involves insolubilization of the hormone, antigen, or hapten, preventing its entrance into the cell and allowing study of the function of the cell membrane or the intact cell when it makes contact with the chemical. Thus either the pharmacologic effect of the insoluble hormone agonist (1–3), the detection of a receptor to antigen on selected cells of a suspension (4, 5), or the distribution of the contact points of an antigen for its receptor on the cell can be studied (6). A second and complementary approach has been to interact the radio-labeled substance with a cell containing the receptor and to determine the influence of pH, temperature, and varying concentrations of ions or un-

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labeled substances on the binding property of the labeled chemical. The second approach has been used with perfusions of tissues, suspensions of cells or membranes and solubilized cell walls containing the receptors (7–11).

We have used aspects of both approaches in an attempt to study receptors for a series of small endogenous hormones on human leukocytes. In the past, using pharmacologic techniques, we have established the presence on human leukocytes of receptors for histamine, beta adrenergic catecholamines and the E series of prostaglandins. Each category of drug stimulated adenyl cyclase in mixed leukocytes. Antagonists of one class of agonist inhibited the effects of that class alone. Thus it was deduced that separate receptors existed for each group of agonists (12–15). Additional studies located receptors for histamine on the leukocyte surface (16). To do this, histamine was first conjugated to a carrier (rabbit serum albumin [RSA],[1] bovine serum albumin [BSA], or a synthetic random copolymer of alanine and tyrosine [P]), and the multivalent conjugate was attached to agarose beads (Sepharose [S]) (16). When incubated with cells, the drug-conjugate-bead bound human leukocytes and leukocytes from several animal sources. The attraction appeared to be specific for histamine, because it was prevented by histamine antagonists and high concentrations of free histamine, but not by comparable concentrations of catecholamines or prostaglandins.

The purposes of this report are: (*a*) to describe the methods for synthesizing a random copolymer carrier and hormone-carrier conjugates, as well as the chemical manipulation that preserves the basic function of the bound primary amines; (*b*) to describe the pattern and evidence for specificity of binding of human peripheral blood leukocytes by various catecholamines or prostaglandin E_2 conjugated to carriers and then bound to Sepharose; (*c*) to compare the binding of cells to agonists with the stimulation of leukocyte cyclic AMP accumulation; and (*d*) to describe the techniques employing columns of drug-conjugate-Sepharose that can be used to separate cells predominantly on the basis of a physiologic property—their preformed plasma membrane receptors for endogenous hormones. The process of separation may make it possible to analyze the distribution of the receptors on the cells for histamine and catecholamines.

[1] *Abbreviations used in this paper:* BSA, or B, bovine serum albumin; cyclic AMP, cyclic 3′-5′-adenosine monophosphate; ECDI, 1-ethyl-3(3-dimethylaminopropyl)carbodiimide HCl; H, histamine; MEM, Eagle's minimal essential medium; N, norepinephrine; P, copolymer of D-L-alanine and L-tyrosine; PBS, 0.15 M NaCl and 0.01 M sodium phosphate, pH 7.4; PGE_1, PGE_2, $PGF_{2\alpha}$, prostaglandin E_1, E_2, and $F_{2\alpha}$, respectively; RSA or R, rabbit serum albumin; S, Sepharose beads; H-B, H-P, H-R, conjugate of histamine with BSA, P, and RSA, respectively; Iso-R, conjugate of isoproterenol with RSA; N-B, N-R, conjugate of norepinephrine with BSA and RSA, respectively; PGE_1-R, conjugate of prostaglandin E_1 with RSA; PGE_2-R, conjugate of prostaglandin E_2 with RSA; $PGF_{2\alpha}$-R, conjugate of prostaglandin $F_{2\alpha}$ with RSA; H-R-S, N-R-S, PGE_1-R-S, etc., and H-P-S, N-P-S, PGE_1-P-S, etc., drug preparations linked by ECDI to carrier and then to S; H-P-S(GA), H-R-S(GA), drug preparations of histamine linked to P-Sepharose via glutaraldehyde and to RSA-Sepharose via glutaraldehyde, respectively; N-P-S(GA), N-R-S(GA), drug preparations of norepinephrine linked to P-Sepharose via glutaraldehyde and to RSA-Sepharose via glutaraldehyde, respectively.

METHODS

Synthesis and characterization of a random copolymer of alanine-tyrosine

A linear copolymer of DL-alanine with L-tyrosine (P) was prepared by random copolymerization of *N*-carboxy-α-amino acid anhydrides according to the method described by Sela and Fuchs (17). The principal steps of the synthesis were to mix 14.5 g *N*-carboxy-DL-alanine anhydride (125 mmol) in 250 ml anhydrous dioxane with 5.15 g *N*-carboxy-L-tyrosine anhydride (25 mmol) in 150 ml dioxane and to initiate the polymerization by adding 0.6 g (5.1 mmol) of diaminohexane. The mixture was stirred vigorously at room temperature for 24 h. 800 ml of ether was added to complete precipitation of the polymer which had continuously and spontaneously precipitated in the dioxane. The polymer was then filtered and washed with 1 liter of ether. The yield of polymer in different batches varied from 13 to 15 g.

Amino acid analysis after acid hydrolysis revealed that the polymer had a residue molar ratio of DL alanine to L-tyrosine of 3:1, and an average molecular weight of 1840, as established by ultracentrifugation using the method of Yphantis ($\bar{v} = 0.73$) (18).

Low molecular weight and a random structure were properties desired for the polymer synthesized. The initiation of polymerization with diamine results in a copolymer with amines at each end. The general structure of the polymer is shown below:

$$H_2N{-}\underset{\displaystyle R}{\overset{|}{C}}H{-}CO{-}[NH{-}\underset{\displaystyle R}{\overset{|}{C}}H{-}CO]_m$$

$$-NH-(CH_2)_6-NH-[CO-CH(R)-NH]_n$$

$$-CO-CH(R)-NH_2$$

where R represents the side chain of either tyrosine or alanine. Thus one terminal can be used to bind the polymer to Sepharose, while the other end is attached to the hormone. The distance between the two terminals is about 20 amino acids which was thought to provide a long enough flexible arm to accommodate to the stress of the receptor-hormone interaction.

Activation of Sepharose and coupling to protein or polymer

The activation of the agarose was according to the method of Porath, Axen, and Ernback (19). 4½ g (wet weight) agarose beads (Sepharose 4B, Pharmacia, Uppsala, Sweden [S]) were washed with water and suspended in 15 ml deionized water to which 0.5 g CNBr (Eastman Chemicals, Rochester, N. Y.) was added. The pH of the suspension was adjusted to 11, and maintained there for 8 min by periodic dropwise addition of 1 N NaOH. The reaction was terminated by filtration of the Sepharose, and the activated Sepharose was washed three times with 100 ml of deionized water. Then the activated Sepharose in 10 ml of 0.2 M $NaHCO_3$ was added to solutions which contained either 10 or 40 mg of either rabbit serum albumin (RSA) (Mann Research Labs Inc., New York), bovine serum albumin (BSA) (Mann), or the copolymer (P). The suspension was placed in plastic sealed containers, and gently but continuously inverted for 16 h at 4°C. The suspension was then washed with at least 100 vol of water, and finally stored as a 25% wt/vol suspension in 0.15 M NaCl and 0.01 M sodium phosphate, pH 7.1 (PBS). The amount of either protein or polymer attached to Sepharose was determined by measuring the decrease in optical density of the supernatant solution (at 280 nm) caused by the binding process. More than 90% of the proteins, polymers, or drug conjugates with the carriers (see below) were bound in all preparations.

Synthesis of drug-protein or polymer conjugate-Sepharose: conjugates made with carbodiimide

Histamine-RSA (H-R) was prepared by dissolving histamine dihydrochloride (700 mg, Fluka AG, Basel, Switzerland), RSA (100 mg) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl (ECDI) (2 g, Ott Chemical Co., Muskegon, Mich.) in 10 ml of water. The histamine was conjugated via its amino function to the carbodiimide-activated carboxyl groups of the RSA. The solution stood for 2 h at room temperature, and was then dialyzed against 300 vol of water changed three times over 24 h at 4°C. The H-R was characterized by one of two methods. The first was to hydrolyze a portion in 6 N HCl at 108°C for 24 h, placing it on Whatman 3 MM paper and separating the histamine from amino acids by high voltage electrophoresis (15 min at 3,500 V at pH 3.5). Then the amount of histamine per mg of the conjugate-H-R was determined by a quantitative ninhydrin method (20). With this method the unknown quantity of histamine was compared with standards treated identically. The second method was to add a ^3H tracer of histamine (New England Nuclear Corp., Boston, Mass.) throughout the procedure and calculate the percentage of the total on the carrier. Both methods produced comparable results. The various batches contained between 30 and 40 mol of histamine/mol of RSA. Batches of H-BSA (denoted H-B) and H-copolymer (H-P) were synthesized and characterized in the same manner. There were 1–2 mol of histamine/mol of polymer linked to Sepharose, and 30–40 mol of histamine/mol of BSA. 4 mg of H-R, H-B, or polymer were linked to 1 g Sepharose as described above.

Norepinephrine-RSA (N-R). The free base of norepinephrine (350 mg, Calbiochem, Los Angeles, Calif.) was dissolved in HCl, (the pH readjusted to 3.0) and coupled to RSA (50 mg) with 1 g of ECDI in a final volume of 10 ml. The solution remained at room temperature for 2 h and was then dialyzed at 4°C against 1 liter of 0.02 M acetic acid changed three times in 24 h. The resultant solution was characterized by using trace amounts of [^{14}C]norepinephrine (Amersham/Searle Corp., Arlington Heights, Ill.) as described for histamine conjugates. 70 mol of norepinephrine were bound to 1 mol of protein carrier (BSA or RSA). 2 mg of either N-R or N-BSA (denoted as N-B) were attached to 1 g of activated Sepharose.

Isoproterenol-RSA (Iso-R). DL-isoproterenol-HCl (80 mg—Calbiochem) to which 12 μCi [^3H]isoproterenol (New England Nuclear) had been added, was dissolved in 5 ml water and reacted with 100 mg of succinic anhydride (BDH Chemicals Ltd., Poole, England). The succinic anhydride was added in 20-mg portions at 2–3 min-intervals while the pH was maintained between 7 and 9 by dropwise addition of 1 N NaOH. To the resultant reaction mixture, 110 mg of RSA and 500 mg ECDI were added. The coupling mixture was kept for 4 h at room temperature, and was then dialyzed at 4°C against 1 liter of water changed three times in 24 h. The isoproterenol-RSA was characterized by determining the amount of radioactivity in a portion of the conjugate. Between 10 and 15% of the original isoproterenol was bound to RSA, or 23–25 mol of isoproterenol/mol RSA. Isoproterenol was the only secondary amine used in this series. The carboxyl group of the succinic acid may have been involved in linkage of the drug to RSA. We assume that the linkage was through the secondary amine or less likely through an ester in linkage with one of the hydroxyl groups. 2 mg Iso-R were reacted with 1 g of activated Sepharose.

Prostaglandin E_2-RSA (PGE_2-R). Prostaglandin E_2 (a gift from Alza Corporation, Palo Alto, Calif.), 5 mg, was dissolved in 0.5 ml 20% ethanol/water and added to 60 mg RSA in 2 ml water. 12 mg ECDI were added to the mixture and the coupling allowed to proceed at room temperature for 24 h. The mixture was then dialyzed against two portions of 2,000 vol of water at 4°C. The amount of PGE_2 coupled to RSA was determined by following the binding of ^3H-labeled prostaglandin (New England Nuclear). About 20% was bound in the average batch, or 2.7 mol PGE_2/mol RSA. 2 mg of the conjugate were linked to 1 g Sepharose. Prostaglandin was linked to the polymer using similar techniques.

Prostaglandin E_1-RSA (PGE_1-R) and prostaglandin $F_{2\alpha}$-RSA ($PGF_{2\alpha}$-R). These were prepared precisely the same way as PGE_2-R.

Prostaglandin E_2-polymer Sepharose (PGE_2-P-S). PGE_2 (0.75 mg) was reacted for 5 min with 1.5 mg ECDI in 0.4 ml of 25% dioxane in water. The solution was mixed with 1 ml polymer-Sepharose (75% wt/vol, 4 mg polymer bound to 1 g Sepharose). The reaction was stopped after 12 h by washing the Sepharose with 200 vol of water.

Synthesis of drug-protein or polymer conjugate-Sepharose: conjugate made with glutaraldehyde

Histamine-RSA-Sepharose (GA) (H-R-S (GA)). Sepharose to which RSA was already linked (8 mg RSA/1 g Sepharose) was the starting material. 2 ml of the RSA-Sepharose (50% suspension wt/vol) were reacted with 50 mg histamine dihydrochloride and 0.2 ml 50% glutaraldehyde (GA) (Fluka). The mixture remained at room temperature for 16–20 h and was then washed on a sintered glass filter with 4 × 10 ml of water. The washed H-R-S was resuspended in 2 ml of water to which 50 mg $NaBH_4$ (BDH Chemicals) were added. The total reduction of C = N bonds was associated with changing of the color of

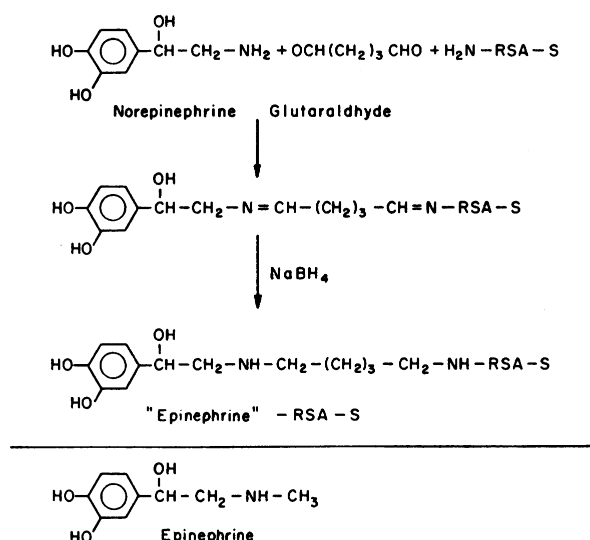

FIGURE 1 Schematic representation of the conversion of norepinephrine to a molecule resembling epinephrine during the conjugation of the amine with either protein or polymer via glutaraldehyde. The end result is *N*-alkylated norepinephrine.

the mixture from yellow to white and was completed after 1 h when the Sepharose was washed with 2×10 ml of water. By this procedure 7.35 μmol histamine was bound to 1 g Sepharose (wet).

Histamine-Polymer-Sepharose (GA) (H-P-S(GA)). Histamine dihydrochloride (50 mg) was mixed with 2 ml polymer-Sepharose (4 mg polymer/1 g Sepharose) suspension (50% wt/vol), and 0.2 ml 50% glutaraldehyde was added. The subsequent steps were as described above for H-R-S(GA).

Norepinephrine-RSA-Sepharose (GA) (N-R-S (GA)). Norepinephrine free base (25 mg) was dissolved in HCl. The pH was adjusted to 3.0. The solution was mixed with 2 ml (50% wt/vol) RSA-Sepharose (8 mg RSA/1 g Sepharose) to which 0.2 ml of 50% glutaraldehyde was added. Subsequent steps were identical to H-R-S (GA). The most likely chemical configuration of the resultant drug-conjugate-Sepharose is shown in Fig. 1. As shown in the figure, the norepinephrine is converted to a secondary amine, like that of epinephrine.

Norepinephrine-Polymer-Sepharose (GA)N-P-S(GA)). The same amount of the free base of norepinephrine (as described above) was interacted with 2 ml (50% wt/vol) of polymer-Sepharose (4 mg polymer/1 g Sepharose) and glutaraldehyde (0.2 ml of 50% solution). Subsequent steps were identical to those described for H-R-S (GA).

Handling of human leukocytes

Venous blood of normal volunteers was subjected to sedimentation with 3% Dextran 250 (Pharmacia) in PBS, and the leukocytes were isolated as previously described (12, 21). Plastic venipuncture tubes contained heparin (5 U/ml of blood) as an anticoagulant. After separation and one wash in cold 0.32 M sucrose, the cells were suspended in a convenient volume of either PBS or Eagle's Minimal Essential Medium (MEM) without glutamine (Microbiological Associates, Jerusalem). The differential count of cells prepared in this fashion was similar to that of stained smears from peripheral venous blood of the same subject. Contamination by platelets was of the order of 1 platelet per 15–20 leukocytes (12).

Binding of leukocytes to Sepharose. Different batches of Sepharose beads (0.05 ml of a 25% suspension of agarose in PBS) were incubated with $5\text{–}10 \times 10^6$ leukocytes for 15 min with gentle shaking at 37°C. After incubation the mixture was gently resuspended and a portion of cells and the beads were examined under the microscope. 100 consecutive beads were examined from each specimen and scored as binding if they held 20 cells or more per bead, and as negative if fewer cells were bound. Two additional types of experiments were conducted. In one, the cells were incubated with each of several drugs at various concentration for 10 min at 37° before the Sepharose-drug conjugate was added, and then incubated for an additional 15 min. The binding of the beads was assessed. In the second type of experiment, the cells were incubated with a Sepharose-drug conjugate, and the free drugs were added after 15 min of incubation. The mixture was incubated for an additional 15 min and the binding was assessed. These latter experiments tested the ability of the drugs to displace cells after they had been bound to Sepharose.

Preparation of columns. 24×10^6 leukocytes were incubated with various Sepharoses (0.6 ml of a 25% suspension of agarose/PBS) for 15 min at 37°C. The suspension was then poured into a plastic column (made from the lower 3 cm of 10 ml pipettes [Falcon Plastics, Los Angeles, Calif.]) containing a small sponge rubber plug at its tip. The columns were washed with 0.5 to 1.0 ml portions of PBS until the unbound cells were separated from the column. In some experiments the bound cells were eluted from the columns by adding 1 ml portions of various drugs, and gently shaking and resuspending the column contents with a Pasteur pipette (Fig. 5). In the experiments described in Table IV, sufficient numbers of columns of either R-S, H-R-S, or N-R-S(GA) were run to allow collection of 36×10^6 unbound cells that were divided into three portions. 1 portion was incubated with 0.3 ml of either R-S, H-R-S, or N-R-S(GA) suspensions. The incubates were then poured into columns as above and washed with PBS until the unbound cells were separated from the column. The unbound cells were counted and appropriate calculations made.

Leukocyte cyclic AMP concentrations. Leukocytes in PBS or MEM (6×10^7 cells/ml) were exposed to drugs or 0.6 ml of various Sepharoses or 0.5 ml of the supernatant from the Sepharose mixture for 15 min. At the end of the incubation, cells were separated from the medium by centrifugation at 800 *g* for 10 min at 0°C. The cell pellet was resuspended in 1.2 ml, 5% trichloroacetic acid. Cyclic AMP was extracted and measured by the competition binding assay of Gilman (22), slightly modified as previously described (15).

RESULTS

Interactions of Sepharose preparations with leukocytes

Binding. By visual assessment, none of the control Sepharoses bound cells. Neither Sepharose alone, activated Sepharose, nor sepharose to which RSA, BSA, or the copolymer was attached, appeared to bind cells. When either histamine or norepinephrine were linked

directly to activated Sepharose, they were no more capable of binding cells than the controls (16). When histamine was conjugated to RSA and attached to Sepharose by either the ECDI or glutaraldehyde (GA) method, the majority of beads were coated with leukocytes but not platelets or erythrocytes. The binding of leukocytes seemed proportional to their incidence in the incubation mixture. The majority of binding took place within 12 min and was virtually complete in 15 min (Fig. 2), whether the incubation was conducted at 37° or 4°C. The same time-course of binding was observed for the other drug-Sepharose conjugates.

Iso-R-S did not bind as well (28±7% [SD] beads with greater than 20 cells/bead) as H-R-S (87±7%), H-R-S(GA) (71±10%), or N-R-S(GA) (86±6%) (Tables I and II). Only 23% of H-P-S beads bound cells. All preparations of drugs linked to polymer and Sepharose bound significantly fewer cells than the corresponding drug-RSA-Sepharose preparation. Of interest was the remarkable difference between the binding affected by N-R-S, which never exceeded 5% of the beads binding more than 20 cells/bead, and the binding of cells by N-R-S(GA), which was equivalent to H-R-S. Prostaglandin E_2 attached to RSA and Sepharose did not appear to attract cells during the incubation. The amount of each type of Sepharose and the approximate molarity of protein and drug (histamine, PGE_2, norepinephrine, or isoproterenol) in the incubation mixtures are recorded in Table I. The differences in binding between these Sepharose-drug-protein conjugates and the polymer-drug conjugates could not be attributed to differences in opportunity of the cells to make contact with the agonist. In fact, the number of moles of norepinephrine in the ECDI conjugates was

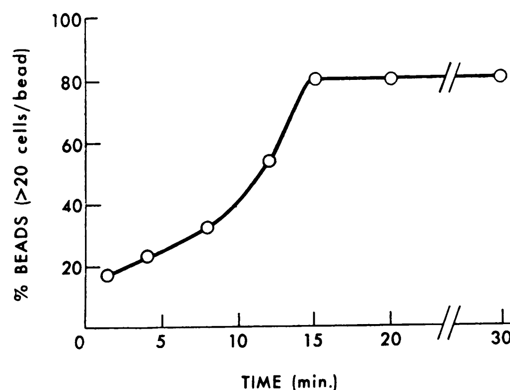

FIGURE 2 Time course of binding of leukocytes by histamine-RSA-Sepharose. Each point represents the mean of four points obtained on duplicate samples from two consecutive experiments.

TABLE I

Chemical Composition and Cell Binding Activity of Sepharose Preparations

Preparation*	Drug	Carrier	Ratio drug/carrier	% Beads binding >20 cells
	nanomoles‡			
Histamine-R-S	135	4.4	30.5	87±7
Histamine-R-S(GA)	460	8.7	51	71±10
Histamine-P-S	320	162	1.98	23±12
Isoproterenol-R-S	65.1	2.2	30	28±7
Norepinephrine-R-S	154	2.2	70	3±2
Norepinephrine-R-S(GA)	287	8.7	33	86±6
Norepinephrine-P-S(GA)	52	162	0.33	0
Prostaglandin E_2-R-S	6.06	2.2	2.7	3±5
Prostaglandin E_2-P-S	40	162	0.25	0

* R = rabbit serum albumin; P = copolymer of DL-alanine and L-tyrosine; S = Sepharose.

‡ Represents nanomoles in 0.3 ml of 25% suspension (wt/vol) of the Sepharose preparation in PBS. This amount was used in experiments to characterize binding and to determine the pharmacologic properties of the preparation.

TABLE II

Characteristics of Binding of Human Leukocytes by Drug-RSA-Sepharose

Drug in pre-incubation	Conc.‡ M	Percent of beads binding more than 20 cells*		
		H-R-S	H-R-S(GA)	N-R-S(GA)
Control		87±7	71±10	86±6
Antihistamines				
Diphenhydramine	10^{-3}	28±22§	5	52±6
	10^{-4}	33±7§	30	85±7
Antazoline	10^{-3}	21±13‖	12	79±5
	10^{-4}	57	37	—
Tripelennamine	10^{-3}	31±7‖	—	85±10
	10^{-4}	38	56	—
Pyrilamine	10^{-3}	24±4‖	2±7§	69±7
	10^{-4}	44	30±10	87
Adrenergic blocking agents				
Propranolol	10^{-3}	84±7	76±11	11±8§
	10^{-4}	—	—	37±6§
Phentolamine	10^{-3}	45	—	53±38
	10^{-4}	95±1	95±4	71±10
Agonists				
Histamine	10^{-3}	64±29‖	40	86±5
	10^{-5}	91±2	—	—
Epinephrine	10^{-3}	84±13	70±26	60±27
D, L-isoproterenol	10^{-3}	86±16	83	56±19‖
D-isoproterenol	10^{-3}	87	50	39±6§
L-isoproterenol	10^{-3}	81	70	23±12§
Norepinephrine	10^{-3}	82±21	88	84±6
Phenylephrine	10^{-3}	87±6	82	75±12
Prostaglandin E_2	10^{-3}	85±12	72±6	90±7

* Data are expressed as mean or mean ±SD. Standard deviation was calculated only when 7 or more points were obtained. Means without SD are from 3 points. Each point represents the mean of triplicates. Variance between duplicate samples is <6%. Statistical comparisons were only determined between controls and groups with more than 7 points.

‡ 5 × 10^6 cells were preincubated for 10 min at 37°C with drug as indicated in 0.45 ml Minimal Essential Medium, pH 7.4. 0.05 ml of 25% wt/vol mixture of drug-Sepharose were then added and incubated for 10 min with gentle shaking.

§ P value <0.001 compared with control (paired t test).

‖ P value <0.01 compared with control (paired t test).

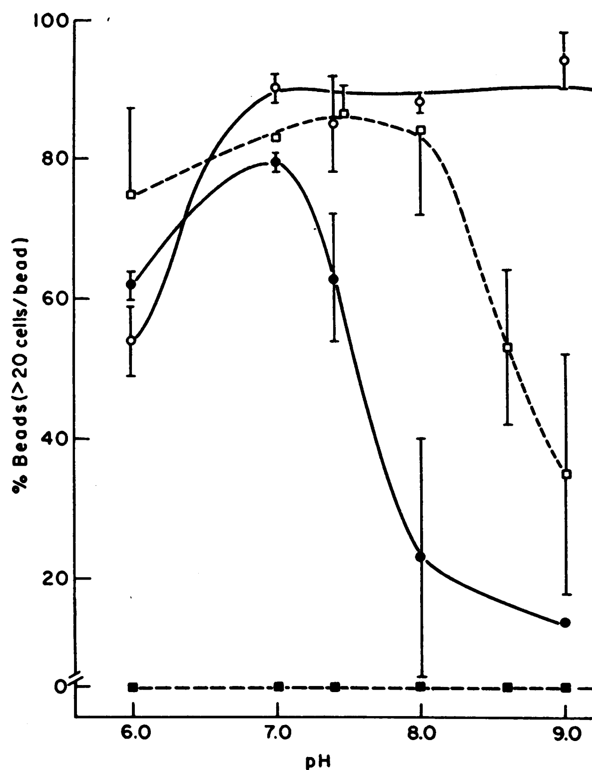

FIGURE 3 The influence of pH on binding of cells by four types of drug-conjugate-Sepharose incubated with leukocytes. Open symbols (○, □) indicate preparations made with glutaraldehyde. Closed symbols (●, ■) indicate preparations made with ECDI. Solid line (——) indicates histamine as the hormone; ---- indicates norepinephrine as the hormone. Thus the ■----■ represents N-R-S; ●——● represents H-R-S; □----□ represents N-R-S(GA); ○——○ represents H-R-S(GA). Bars represent the range of three or more determinations.

more than twice that in the GA conjugates, yet only the latter (N-R-S[GA]) bound cells. However, the approximate molarity of the prostaglandin E_2 RSA-Sepharose preparation was lower than that of the other drug conjugates. This may have been responsible in part for the lack of apparent binding of cells to the prostaglandin E_2 (Table I).

The scoring of beads as positive or negative for binding is arbitrary and could be somewhat misleading. Several instances of binding of some cells were consistently seen with isoproterenol and PGE_2 conjugates to RSA and Sepharose. However, because the number of cells was less than 20/bead, they were scored as negative.

Further characteristics of binding were studied using those Sepharose-drug conjugates that readily bound large numbers of cells, i.e., H-R-S, H-R-S(GA), and N-R-S(GA) (Table I). Binding of cells was pH dependent (Fig. 3). Binding was maximal at about pH 7, and sharply decreased at higher pH values for both H-R-S and N-R-S(GA). Binding of H-R-S differed from H-R-S(GA) in that the latter was able to continue binding cells as the pH rose above 8. N-R-S did not bind cells over the whole range of pH values tested.

Drug conjugate-Sepharose preparations using carriers of BSA or RSA had essentially similar properties. H-R-S had 87±5% of beads with more than 20 cells/bead; 89±9% of H-B-S beads had more than 20 cells. Similar curves of pH effects on binding, and prevention of binding or displacement of bound cells by drugs, were obtained with either protein carrier. Clumping of cells did not occur under the circumstances of our incubations and therefore could not have contributed to the results of the binding experiments.

Specificity of cell binding. Experiments that sought to determine whether binding of cells by H-R-S, H-R-S-(GA), and N-R-S(GA) was specific are summarized in Fig. 4 and Tables II and III. Binding of cells by H-R-S was significantly inhibited by preincubation of the cells with histamine, each of four antihistamines, and histamine-RSA alone. The inhibition was dose-dependent and was not duplicated by comparable concentrations of catecholamines, prostaglandins, or competitive an-

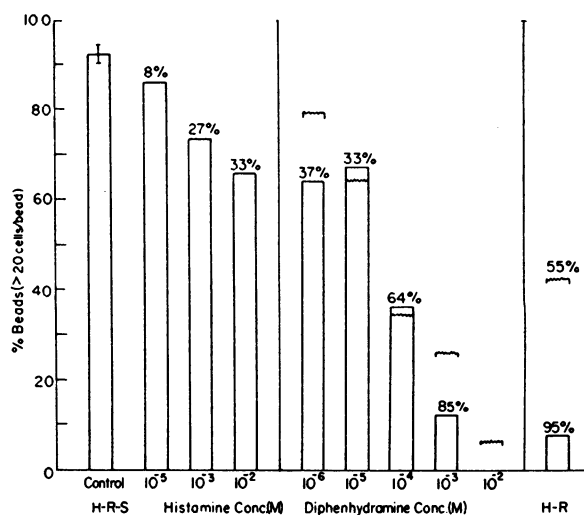

FIGURE 4 Illustration of experiments testing the ability of free histamine, diphenhydramine, and H-R to interfere with binding of cells by H-R-S. The open bars represent experiments in which the drugs were preincubated with cells before the H-R-S was added. The ⌐¬ represents experiments in which the drugs were added after the H-R-S had been incubated with the cells. Percentage markers above the bars or ⌐¬ (in H-R) represent the per cent change from control. Values from the controls represent the mean ±SD of 26 separate experiments. Each point in the remaining figure represents the mean of duplicates from three separate experiments.

TABLE III

*Leukocyte Cyclic AMP after Exposure to Sepharose Preparations**

Drug or Sepharose preparation	Experiment number					
	1	2	3	4	5	6
Control	27±5‡	40	40±3‡	25±5‡	29±5‡	34±4‡
Histamine 1×10^{-4} M	94	114	—	—	—	—
Isoproterenol 1×10^{-4} M	90	135	119	96	—	—
Epinephrine 1×10^{-4} M	—	—	—	—	86±9	95±5
Prostaglandin E_1 1×10^{-5} M	267	430	251	—	—	—
R-S	32	40	39	—	—	—
P-S	22	30	26±5	20±6	—	—
H-R-S	25	25	—	—	—	—
H-R-S(GA)	30	—	—	—	—	—
H-P-S	23	—	—	—	—	—
N-R-S	23	39	—	—	—	—
N-R-S(GA)	—	—	—	—	28±3	30±6
N-P-S(GA)	36	59	28±1	12±1	49±6§	49±7§
N-P-S(GA) + propranolol 10^{-4}	—	—	—	—	24±7	30±6
Supernatant of N-P-S(GA)‖	—	—	38±0	17	27±5	32±5
Iso-R-S	28	—	—	—	—	35±2
PGE_2-R-S	36	32	38	—	35±7	40±6
PGE_2-P-S	—	—	—	—	86±3§	83±8§

* Each tube contained drug at the stated molarity or 0.3 ml 25% (wt/vol) suspension of the indicated Sepharose preparation in 1 ml PBS containing 1×10^7 leukocytes. Theophylline, 1×10^{-2} M, was present in all tubes. Cyclic AMP was measured after 15 min incubation at 37°C, and is expressed as picomoles per 1×10^7 cells.

‡ Mean ±SD of four determinations. Values shown without standard deviation are the means of duplicate determinations, differing by not more than 6%.

§ Significantly different from control ($P < 0.01$, standard t test).

‖ In experiments 3 and 5, cells were incubated with 0.3 ml supernatant from N-P-S(GA) incubation of experiment 2. In experiments 4 and 6, cells were incubated with 0.3 ml supernatant of N-P-S(GA) obtained by centrifugation of the Sepharose preparation used in the same experiment.

tagonists of catecholamines. The general pattern of prevention of binding seen in H-R-S was also seen when using H-R-S(GA) or H-P-S. Once bound, cells could be displaced from the H-R-S by high concentrations of diphenhydramine or histamine-RSA (0.25 mg/ml incubate) (Fig. 4). The displacement of H-R was not as complete as when the chemical was preincubated with cells before adding H-R-S (Fig. 4). Prevention of binding by H-R was caused by clumping of the leukocytes by H-R.

The binding of leukocytes to the Sepharose drug conjugate constructed by linking norepinephrine to RSA via glutaraldehyde conformed with that of a beta adrenergic agonist, and was clearly separable from the binding behavior of the histamine conjugate (Table II). The binding of cells to N-R-S(GA) was not prevented by comparable concentrations of histamine or histamine antagonists, but was significantly inhibited by either D- or L-isoproterenol and the beta adrenergic blocking agent, propranolol. Epinephrine produced slight inhibition of binding. Phentolamine, an alpha adrenergic blocking agent, interfered with binding in some experiments, but not consistently. Norepinephrine and phenylephrine, predominantly alpha adrenergic agonists, did not inhibit binding of cells by N-R-S(GA).

These results are parallel to our previous experiments measuring the effects of histamine and adrenergic amines on leukocyte cyclic AMP accumulation (13–15), in which specific amine antagonists clearly separated the effects of histamine and catecholamines, presumably by preventing interaction with separate receptors. In those experiments also, the rank order of potency of adrenergic agonists [isoproterenol and epinephrine > norepinephrine >> phenylephrine (= 0)] was characteristic of actions on beta rather than alpha adrenergic receptors.

Effects of insolubilized drugs on leukocyte cyclic AMP. All the Sepharose preparations were tested for

ability to increase the cyclic AMP content of leukocytes. All failed to do so, except the N-P-S(GA), which increased the cyclic AMP in four of six experiments (Table III). To be certain that minor amounts of catecholamine were not being dissociated from the Sepharose, both the supernatant of N-P-S(GA) and the supernatant from incubations of the same preparation with leukocytes were tested, and neither altered cyclic AMP content of the cells. Furthermore, the stimulation by N-P-S(GA) was almost completely inhibited by propranolol (10^{-4} M), minimally inhibited by phentolamine and unaffected by four antihistamines all at 10^{-4} M. None of four batches of Sepharose, activated Sepharose, R-S, P-S, H-R-S, H-R-S(GA), H-P-S, H-P-S(GA), Iso-R-S, or N-P-S stimulated cyclic AMP accumulation. Two preparations of PGE_2-P-S but not PGE_2-R-S stimulated cyclic AMP accumulation. Some of these results are shown in Table III.

Although the results with various batches of N-P-S-(GA) were not consistent, the unique augmentation of cyclic AMP accumulation by N-P-S(GA) suggests that the glutaraldehyde linking procedure had produced chemical and pharmacologic properties different from those resulting from the ECDI procedure. The glutaraldehyde procedure may have preserved some of the reactivity of the amino group, which in other systems is essential for adrenergic agonist activity. The likely structure of N-P-S(GA) (Fig. 1) probably preserves the basicity of the amine group. In addition, alkyl substitution of norepinephrine (producing a secondary amine) clearly enhances beta rather than alpha agonist activity in other systems (23). The proposed structure (Fig. 1) for N-P-S(GA) provides just such an alkyl substitution, producing a catecholamine similar to epinephrine. Incidentally, further substitution (with ECDI or glutaraldehyde) of the amine group of isoproterenol and epinephrine (which are already secondary amines) would be expected to decrease agonist activity, by decreasing the basicity of the nitrogen atom (ECDI) or introducing steric hindrance (GA) at a site which is critical for intrinsic activity of the agonist (23). Iso-R-S and H-R-S both failed to stimulate leukocyte cyclic AMP accumulation (Table III).

Sepharose-drug columns

Column experiments were carried out for three purposes: (*a*) to further define the character of cell binding and the distribution of what might be specific receptors in the total population of cells; (*b*) to try to quantitate the number of cells bound to any Sepharose

TABLE IV

Determination of Distribution of Receptors to Catecholamine and Histamine

Sequential drug-R-S or R-S columns		Mean % cells subtracted by column				Mean % over nonspecific (R-S binding)	% Cells in second column with single receptors (i.e. Δ% between specific H-R-S and N-R-S(GA) binding)
Column 1	Column 2						
R-S*	—	51±13				—	—
H-R-S	—	82±7‡				31	—
N-R-S(GA)	—	79±2§				28	—
Experiment:		1	2	3	4		
R-S	R-S	42	21	20		—	
	H-R-S	80	63	65		40±2‖	
	N-R-S(GA)	75	59	52		34±3¶	
H-R-S	R-S	0	16	0	0	—	—
	H-R-S	42	26	13	40	26±14	—
	N-R-S(GA)	74	56	42	53	56±13¶	30
N-R-S(GA)	R-S	18	36	25	30	—	—
	H-R-S	73	70	87	85	51±12**	21
	N-R-S(GA)	40	49	65	75	30±15	—

See Methods section for details of experimental procedures.
* Mean ±SD in single column experiments are taken from 10 separate experiments.
‡ *P* values <0.001 vs. control (calculated by Student's *t* test).
§ *P* values <0.01 vs. control (calculated by Student's *t* test).
‖ *P* values <0.001 vs. control (calculated by paired *t* test).
¶ *P* values <0.01 vs. control (calculated by paired *t* test).
** *P* values <0.02 vs. control (calculated by paired *t* test).

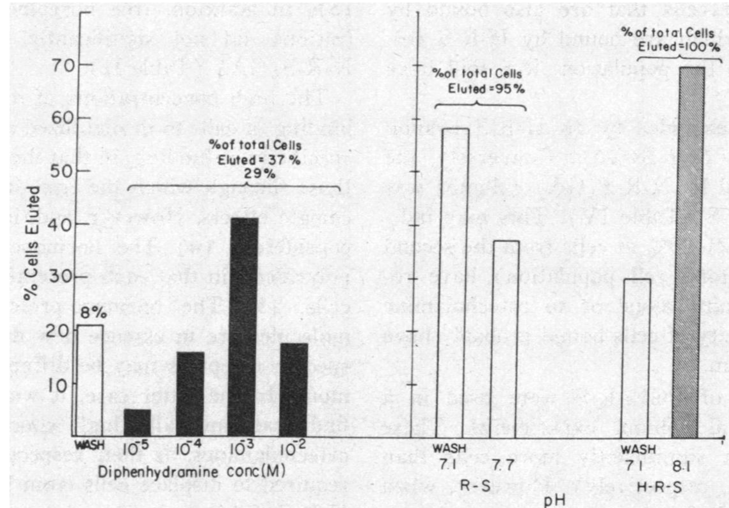

FIGURE 5 Columns constructed of H-R-S and R-S. On the left, diphenhydramine was used in successive 1 ml portions to elute a maximum of 29% of the total number of cells added to a H-R-S column. The beads were resuspended several times in a Pasteur pipette between portions. In the right panel, 60% of the cells were eluted from R-S simply by washing the PBS pH 7.1. When the pH of the eluting fluid was raised to 8.1, a total of 95% of the cells were eluted before the pH of the eluate reached 8.0. On the far right are cells eluted from H-R-S by PBS pH 7.1 □ vs. pH 8.1 (cross-hatched column). Cells in this column were counted after the eluate fluid reached pH 8.1. The column contents were always resuspended. Each point represents the mean of duplicates from two experiments.

rather than to depend on visual scoring of binding; and (*c*) to devise a method by which cells with specific receptors could be recovered.

Using H-R-S we found that the columns retained 82±7% of the cells, or 31% more than was retained by the R-S (Table IV). Further, when the H-R-S column was washed extensively with PBS, pH 8.1, almost all of the retained cells were eluted from the column. More than 95% of the eluted cells excluded methylene blue dye and therefore were presumably viable. The recovery from H-R-S columns by elution with an antihistamine, diphenhydramine, was 29% (Fig. 5). We have no way at present to determine whether some of the cells bound by R-S had histamine receptors or whether the great majority of cells with histamine receptors were specifically eluted by diphenhydramine. However, the similarity of 31% additional retention by H-R-S over R-S and the 29% elution of cells from H-R-S by an antihistamine is interesting. Propranolol (1×10^{-3} M) did not displace cells from the H-R-S columns.

When columns were constructed from H-R-S(GA), pH changes did not increase elution of cells over that obtained by washing at pH 7.1. Diphenhydramine did elute about the same percentage of cells from H-R-S as from H-R-S(GA). Adrenergic antagonists had no effect, and because histamine was relatively inefficient in displacement of cells from beads, it was not tried in the experiments with columns.

N-R-S(GA) columns retained significantly more cells than R-S and about the same number as H-R-S. Only 15% of the total number were eluted by a gradient elution of 10^{-5} to 10^{-2} M propranolol. Surprisingly, only 8% of the total were eluted by washing with PBS at pH 8.5. Likewise there was no effect of the antihistamine pyrilamine on elution of cells from these columns.

Determination of the "distribution of receptors" in the population of cells was approached by sequential column experiments in which the unbound cells of the first column were passed (recycled) through a second column (Table IV). About 50% of cells were retained by R-S. We consider this retention nonspecific because retention by Sepharose, activated Sepharose and R-S columns was equivalent. We have not been able to determine precisely how much retention is due to the RSA, the contiguous surface of beads, or the sponges or column material, or is a function of the volumes of fluid we use on the columns. When cells passed through the R-S were subsequently re-exposed to new batches of R-S, substantially fewer cells were retained. Regardless of which Sepharose-drug conjugate was used first, a lower percentage of cells was retained when the non-bound cells were rechromatographed on fresh batches of the same substance or R-S. In the four experiments in which the cells excluded from columns of H-R-S were chromatographed on R-S, practically no additional retention was seen. This seems to indicate that RSA may

contribute to binding of cells that are also bound by H-R-S, but once cells which are bound by H-R-S columns are removed from the population, R-S will have little more effect.

The binding of cells excluded by an H-R-S column was only significant on N-R-S(GA). Conversely, the binding of cells excluded by N-R-S(GA) columns was only significant on H-R-S (Table IV). This may indicate that a maximum of 21–30% of cells from the second column (4–6% of the total cell population) have receptors either to histamine alone or to catecholamine alone, but that the majority of cells bound probably have multiple receptors on them.

Columns constructed of PGE_2-R-S were used in a similar set of sequential column experiments. These columns did not retain significantly more cells than R-S (57±5 vs. 51±2%, respectively). However, when cells excluded from an R-S column were exposed to a second column composed of PGE_2-R-S, there was a small increase of binding on the PGE_2-R-S compared with recycling on R-S. Preliminary experiments indicated that when PGE_2-R-S served as the first column, a second PGE_2-R-S column retained only a small additional percentage of cells (about 18%), whereas an H-R-S column retained 65% of the cells which were not bound by the prostaglandin column. These experiments suggest that the column procedure (in contrast to direct visualization, Table I) may detect binding of cells to beads coated with prostaglandin. Since there is presently no specific procedure for displacing cells from PGE_2-R-S, the presence of receptors to prostaglandins on cell surfaces must be established by more direct methods.

DISCUSSION

We describe methods which can be used to insolubilize vasoactive hormones. We intended to determine whether these preparations might be useful in detecting hormone receptors on a heterogeneous population of leukocytes. Studies with the hormone-carrier-bead preparations indicate they can bind cells. Whether or not the binding is via receptors for the free hormones on the plasma membrane remains to be seen. In one sense, the binding is "specific": the protein and polymer carriers alone attached to S did not bind or aggregate cells; binding was seen only when the hormones were attached to the carrier-bead preparation. However, the cells did not bind to hormone-carrier-bead preparations precisely as they would be expected to bind free hormone. The cells were displaced from hormone-containing beads, and binding to beads was prevented by the corresponding amine or relatively specific amine antagonists, but at concentrations higher than required for the agonists to stimulate cyclic AMP accumulation, or the antagonists to prevent it (13–15). In addition, free norepinephrine at high concentrations did not significantly displace cells from the N-R-S(GA) (Table II).

The high concentrations of drugs required to prevent binding of cells to insolubilized amines may indicate nonspecificity of binding, in that the binding sites may not be those through which the free drugs exert their pharmacologic effects. However, additional possibilities must be considered: (*a*) The hormone-protein molecules were polyvalent, in that each presented many hormones to the cells. (*b*) The hormone-protein or hormone-polymer molecules are in essence new drugs, whose affinities for specific receptors may be different from the parent hormone. In the latter case, it would not be surprising to find that unusually high concentrations of histamine, catecholamines, or their respective antagonists would be required to displace cells from histamine-carrier-bead or N-R-S(GA). (*c*) The character of the hormone may have been changed by the procedure of coupling it to the carrier, so that the molecule presented to the cells is relatively unlike the corresponding free amine. Future experiments, using free and bound enantiomers and congeners of these hormones, will help determine to what degree the binding is a function of the hormone's structure versus effects caused by the coupling procedure. (*d*) Finally, although we might expect that antagonists of biogenic amines work by displacing the amines from receptor sites on cells, these antagonists have a variety of pharmacologic effects, and we cannot be sure that an agonist must be displaced from a cell before its actions are inhibited. Future experiments, comparing the pharmacologic stimulation of cyclic AMP accumulation by hormone-carrier preparations to their binding properties, and the effects of specific antagonists on each process, will help to determine whether the binding described in this paper depended in any way on the natural hormone receptors of the cells.

The binding phenomena we have described are interesting in several other ways. Binding was not affected by temperature, indicating that cell metabolism was not required for the interaction; if the binding were indeed related to hormone receptors, the receptors were probably present on the cell surface prior to the time of incubation.

Drugs bound directly to Sepharose did not bind cells; the carrier arm that intervened between the bead and the drug was necessary to allow binding. The "arms" contributed to the ability of the hormone-Sepharose preparations to bind cells, but the BSA, RSA, or copolymer did not produce binding or pharmacologic effects (cyclic AMP accumulation). The necessity to separate the small hormone from the bead before binding occurs is analogous to the findings of experiments designed to characterize the receptors to larger polypeptide hormones or

haptens (24, 25). These too had to be separated from the bead surface before binding to receptors occurred.

When drugs were attached to the protein carriers, their binding properties were much more pronounced than when the copolymer was used (Fig. 6). The most likely explanation for the difference in cell binding potency of the protein vs. polymer preparations is that the former has many drug molecules per mole and serves as a polyvalent attractant. In contrast, the copolymer has only 1 mol of drug per mole of polymer, so that the "density" of drug presented to the cell would be low. The fact that polyvalent attractants are more successful in holding a cell than univalent attractants also has its counterpart in immunologic experiments where multivalent carriers of hapten or antibody were more capable than univalent carriers of binding cells by their receptors (25).

Studies using the copolymer as the carrier add to the understanding of the binding process. There is no qualitative difference in the characteristics of binding by histamine-polymer-Sepharose or histamine-protein-Sepharose. Yet the polymer is random. Therefore, the data provided by the polymer indicate that the length of the arm is probably its most important characteristic in allowing binding of cells to drug.

For some kinds of experiments, the polymer preparations may prove to have distinct advantages over the protein carriers. Only the N-P-S(GA) stimulated cyclic AMP accumulation in leukocytes, although the total number of moles of norepinephrine was much greater in N-R-S and N-R-S(GA) (See Table I). Perhaps the polymer's simpler structure made it more flexible than a complex protein, thus allowing a better fit of hormone into the receptor site than could occur with the protein carrier. Accordingly, the polymer-conjugate could stimulate cell function but would not bind well, because it lacked polyvalent attractants. The protein conjugates, for the same reasons, would bind well but would fail to exert pharmacologic activity (elevation of cellular cyclic AMP). Although this explanation is attractive, it remains unproved, and does not explain the absence of pharmacologic activity of H-P-S or H-P-S (GA) preparations.

The polymer carrier has at least one additional advantage over the protein-carrier-drug conjugate. Because the polymer is short and has a simple structure and can be constructed with different functional groups on each terminal, it might be used in studies for mapping of receptors on individual cells. Thus, the carboxyl end of a polymer (synthesized but not reported here) could be attached to ferritin, dansyl, or other "indicator" molecules, and the amino end to the drug in question. Such studies are presently in progress. Finally, it is conceivable that in studies designed to purify receptors to the agonist drug by affinity chromatography, the multivalent protein carrier could be used to attract as much receptor material as possible, while the pharmacologically active polymer drug conjugate could be used if activation of an enzyme marker is advantageous.

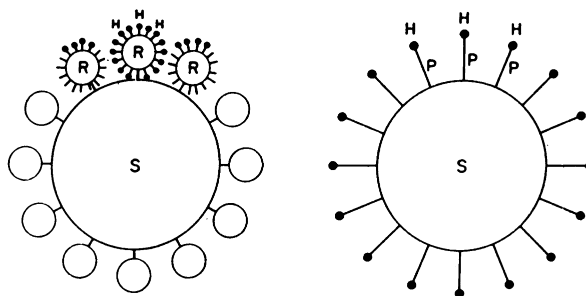

FIGURE 6 Schematic representation of the difference between the polyvalent H-RSA-S (left) and the univalent H-polymer-S (right).

Some advantages to systematic insolubilization of small hormones for the study of cell receptors are indicated. One advantage over the study of the interaction of free soluble radio-labeled drugs on cells or portions of cells is that only the surface properties of the cell are involved in the binding of the insolubilized drug. The experiments show that binding can be studied independent of the pharmacologic action of the drug.

Another advantage to studies with insolubilized drugs is that further characteristics of the drug-cell interaction can be deduced. The importance of the functional amino group of catecholamines or histamine is indicated by the contrast between the binding properties of norepinephrine or histamine conjugated to carrier by ECDI, and the properties of the same amines conjugated to the carrier via glutaraldehyde. Since isoproterenol is a potent stimulator of leukocyte adenyl cyclase, we would expect it to bind well to leukocytes. But when isoproterenol, a secondary amine, was coupled to succinic acid, either an hydroxyl group was changed or isoproterenol was converted to a tertiary amine.

With both histamine and norepinephrine, the primary amine is probably the functional group bound to carrier. Coupling with ECDI produces an amide bond between the amino group of the hormone and a carboxyl residue of either aspartic acid or glutamic acid of the protein (26); The binding process drastically reduces the basicity of the amino groups (27). In contrast, coupling of the amino group of the hormone via glutaraldehyde (to free amino groups of the carrier) preserves the basic function of the hormonal amino group, since the imino ($CH=N$) bonds formed by interaction of the aldehyde with the amine group are completely reduced to alkyl amines by sodium borohydride (Fig. 1).

Consequently, preservation of the basic amino function of norepinephrine in N-R-S(GA) may explain the im-

pressive increase in its binding ability over that of N-R-S (an amide). In addition (as noted under Results), alkyl substitution of the amino group of norepinephrine in N-P-S(GA) may have increased its pharmacologic activity (ability to stimulate cyclic AMP production) by making it "look like" free epinephrine (an alkylated derivative of norepinephrine), which is a potent beta adrenergic agonist in leukocytes (13, 15).

Our results may shed some light on the mechanism of binding of histamine to its receptor. Antihistamines are capable of preventing binding by, or displacing bound cells from, both histamine preparations (H-R-S and H-R-S(GA)). The fact that the leukocyte binding to insolubilized histamine does not depend on whether ECDI or glutaraldehyde is used as a linking agent may indicate that the imidazole portion of the molecule is of critical importance for recognition by a receptor, at least at pH 7.0 (Table II). H-R-S, prepared with ECDI, would lack basicity at its side-chain nitrogen (*27*), but would retain a basic nitrogen on the imidazole ring. The relatively low pK_a of the imidazole nitrogen (5.97) would account for sharp reduction in the binding ability of H-R-S (Fig. 3) since, as pH is raised to 8, the positive charge would be lost. The glutaraldehyde linkage (H-R-S(GA)), however, would preserve positive charge (at pH 8 or 9) of the side chain amino group of histamine (pK_a 9.8), and consequently the molecule's binding ability at higher pH values (Fig. 3).

The basic function of the amino group of norepinephrine may be more critical to binding and pharmacologic effect than that of histamine, since histamine possesses alternate basic nitrogen groups in the imidazole portion which may add to the function of the primary amine: hence the failure of N-R-S to bind cells (Fig. 3), since ECDI linkage has destroyed the amine's basicity. The pH dependence of binding to N-R-S(GA) (Fig. 3) could reflect preservation of the basic amino group. These observations may indicate that receptors to both histamine and norepinephrine contain ionized acidic groups, as have been found in the active site of acetylcholinesterase (28, 29).

The experiments with columns may add useful dimensions to the approach we are describing. Although a drug-carrier-Sepharose preparation may not show binding of cells when examined under the microscope, the binding potential is increased when the columns are used. Further discriminatory power may be added to the detection of receptors when sequential columns are used. The distribution of receptors on a cell suspension was assessed. The majority of bound cells may contain both histamine and epinephrine receptors, while a small percentage of cells may have only one or the other receptor. Limitations of the method are also apparent, because if different cell groups have a spectrum of affinity for the hormone, it might be interpreted that the receptors did not exist on some cells only because cells were not bound by the drug-carrier-Sepharose preparation. At present another limitation of the method is that equivalent conjugation of all drugs on carriers has not been accomplished, and some negative experiments using Sepharose with a low drug/carrier molar ratio do not exclude the presence of receptors.

Finally, the columns may be able to be used to separate cells on the basis of their physiologic receptors even though the cells have relatively similar morphology. Subsequent studies of the unbound cells and cells eluted from the column may begin to define the function of both cells and their receptors (30). It is also logical to assume that the same approach might be used in other systems with dispersed cells from other origins (31).

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