Distribution of Free and Antibody-Bound Peptide Hormones in Two-Phase Aqueous Polymer Systems

By BERNARD DESBUQUOIS and G. D. AURBACH
*Section on Mineral Metabolism, Metabolic Diseases Branch,
National Institute of Arthritis and Metabolic Diseases,
National Institutes of Health, Bethesda, Md. 20014, U.S.A.*

(Received 1 November 1971)

Peptide hormones labelled with radioactive iodine were partitioned into the aqueous two-phase polymer systems developed by Albertsson (1960) and the conditions required for separation of free from antibody-bound hormone have been worked out. Hormones studied included insulin, growth hormone, parathyroid hormone and [arginine]-vasopressin. Free and antibody-bound hormones show different distribution coefficients in a number of systems tested; two systems, the dextran-polyethylene glycol and dextran sulphate-polyethylene glycol system, give optimum separation. Free hormones distribute readily into the upper phase of these systems, whereas hormone-antibody complexes, as well as uncombined antibody, are found almost completely in the lower phase. Various factors including the polymer concentration, the ionic composition of the system, the nature of the hormone and the nature of added serum protein differentially affect the distribution coefficients for free and antibody-bound hormone. These factors can be adequately controlled so as to improve separation. The two-phase partition method has been successfully applied to measure binding of labelled hormone to antibody under standard radioimmunoassay conditions. It exhibits several advantages over the method of equilibration dialysis and can be applied to the study of non-immunological interactions.

Earlier work showed that binding to antibody markedly affects the distribution of certain antigens in the aqueous polymer two-phase systems developed by Albertsson (1960). This phenomenon was first investigated with the proteins phycoerythrin and serum albumin and antibodies against them (Albertsson, 1960; Albertsson & Philipson, 1960). Since changes in distribution were observed under conditions in which antigen-antibody complexes remained soluble, it was suggested that the polymer systems might be useful to detect binding of antigen to antibody in immunological reactions that do not give rise to a precipitate. This possibility was exploited by several investigators to study the interaction between poliovirus and immunoglobulins, to detect virus antibodies in human serum (Philipson, 1966; Philipson *et al.*, 1966), and to develop an assay for antibodies against *Escherichia coli* alkaline phosphatase (Reese & Treffers, 1969).

We have studied the distribution of peptide hormones labelled with radioactive iodine in the aqueous polymer two-phase systems, and worked out the conditions required for optimum separation of free from antibody-bound hormone. Based on these findings, a method of separation has been developed and used in the radioimmunoassays of insulin, para-

thyroid hormone, growth hormone and [arginine]-vasopressin. A preliminary report of this work has been presented (Desbuquois, 1970).

Materials and Methods

Polypeptide hormones and antisera

Crystalline porcine insulin (lot PJ 5682) was obtained from Eli Lilly and Co., Indianapolis, Ind., U.S.A. Purified bovine parathyroid hormone was prepared as previously described (Potts & Aurbach, 1965). Purified human growth hormone and [arginine]-vasopressin were gifts of Dr. Jesse Roth and Dr. Philip Gorden, National Institute of Arthritis and Metabolic Diseases. Guinea-pig antiserum to bovine insulin (lot 469) was obtained from Dr. Peter H. Wright, Department of Pharmacology, Indiana University School of Medicine, Indianapolis, Ind., U.S.A. Guinea-pig and rabbit antisera to bovine parathyroid hormone were developed as previously described (Berson *et al.*, 1963). Guinea-pig antiserum to human growth hormone and rabbit antiserum to vasopressin were gifts of Dr. Jesse Roth and Dr. Gary Robertson, National Institute of Arthritis and Metabolic Diseases.

Polymers

Dextrans 2000, 500, 250 and 70, sodium dextran sulphate 2000 and DEAE-dextran 2000 were purchased from Pharmacia Fine Chemicals Inc., Piscataway, N.J., U.S.A.; methylcellulose 4000 (Methocel) was from Dow-Corning Corporation, Midland, Mich., U.S.A.; polyethylene glycol 6000 (Carbowax 6000) was from Union Carbide Corp., Baltimore, Md., U.S.A.; polyvinylpyrrolidone K-40, polyvinyl alcohol and sodium CM-cellulose were from Matheson, Coleman and Bell, East Rutherford, N.J., U.S.A. Polymers were dissolved in water or the buffer specified and solutions were kept at 4°C until used. The bulk commercial powders of dextran, dextran sulphate, methylcellulose and polyvinylpyrrolidone contained some water, determined by heating at 110°C to constant weight. The concentrations of polymers cited in this study have been corrected for water content of the bulk material.

Other materials

[¹²⁵I]iodide and [¹³¹I]iodide were purchased as sodium iodide from Tracerlab, Waltham, Mass., U.S.A., or Union Carbide Corp.; only fresh preparations were used. Crystalline human serum albumin and rabbit immunoglobulins (fraction II) were obtained from Pentex Biochemical Corp., Kankakee, Ill., U.S.A.; bovine serum albumin (fraction V) was from Armour Pharmaceutical Company, Kankakee, Ill., U.S.A.; human immunoglobulins (fraction II) were from Hyland Laboratories, Costa Mesa, Calif., U.S.A.; cellulose powder was from Fisher Scientific Co., Silver Spring, Md., U.S.A.; micro-precipitated silica (QUSO G-32) was from Philadelphia Quartz Company, Philadelphia, Pa., U.S.A. Other chemicals were of analytical grade.

Iodination of polypeptide hormones and human immunoglobulins

Polypeptide hormones were labelled with ¹²⁵I or ¹³¹I as described by Hunter & Greenwood (1962) with minor modifications. The metabisulphite step was omitted in the iodination of [arginine]-vasopressin (Roth *et al.*, 1966; Robertson *et al.*, 1970). Specific radioactivities (100–600 μ Ci/ μ g) did not exceed the maximum values theoretically attainable for one atom of iodine per molecule of hormone; the latter were calculated by assuming an isotopic abundance of 100% for ¹²⁵I and 30% for ¹³¹I. Iodination mixtures were freed of 'damaged' components and unchanged iodide by previously described procedures: insulin was adsorbed on a cellulose column and eluted by iodoacetamide-treated plasma (Yalow & Berson, 1964); parathyroid hormone was adsorbed on QUSO G-32 and eluted by acetone-acetic acid (Yalow & Berson, 1966); growth hormone and vaso-

pressin were purified by gel filtration on Sephadex G-75 (Hunter & Greenwood, 1962) and G-25 (Robertson *et al.*, 1970) respectively. Efficiency of the purification was assessed by paper chromatoelectrophoresis (Yalow & Berson, 1964) or ability to bind to excess of antibody or both; the tests showed that the radioiodinated preparations used were 90–95% undamaged hormones.

Human immunoglobulins labelled with radioactive iodine were purchased from E. R. Squibb and Sons, New York, N.Y., U.S.A., or obtained from Dr. Stephen E. Mergenhagen, National Institute of Dental Research. Iodination was carried out as described by McFarlane (1956) to specific radioactivities of 100–200 μ Ci/mg.

Incubations

Incubations were carried out in buffers containing serum albumin, immunoglobulins or human plasma; the nature of these is indicated under each experiment. Labelled hormones (0.005–0.050 ng/ml) were incubated at 4°C, (1) without antibody, (2) with excess of antibody to bind almost all the tracer, and (3) with antibody diluted to bind part (usually 30–50%) of the tracer and increasing amounts (0.005–2.000 ng/ml) of unlabelled hormones. Radioiodinated immunoglobulins were included in the incubation mixtures containing antibody-bound hormones. After incubation, the polymer solutions were added and the partition coefficient of radioactive hormone was determined. Parallel samples were analysed for free or antibody-bound radioactive hormone or both by using paper chromatoelectrophoresis (Yalow & Berson, 1964) as a reference method for insulin, parathyroid hormone and growth hormone, and precipitation by an anti-immunoglobulin serum (Feinberg, 1956) for [arginine]-vasopressin. These samples were handled identically with those submitted to two-phase polymer partition with respect to dilution, pH, ions and temperature.

Partition experiments

To 0.5–1 ml of incubation mixture were added separately aqueous solutions of two polymers cooled to 4°C. The nature and the final composition of the polymer systems are indicated under each experiment; final weight of polymers in each tube was 2 g. The contents of the tubes were mixed with a Vortex mixer and centrifuged at 1000g for 15 min in a refrigerated centrifuge. After separation, samples of each phase or upper phase alone were withdrawn with disposable pipettes, weighed and analysed for radioactivity by crystal scintillation detection. Occasionally, sodium [¹²⁵I]iodide was used as a marker instead of weighing; the latter shows a constant distribution coefficient at a given polymer concentration, pH and ionic composition. Com-

parison of the behaviour of radioactive hormone in the two-phase system and in the reference separation methods, as well as analysis of each phase by these methods, showed that addition of the polymers did not affect the antigen-antibody equilibrium that existed in the original incubation mixture.

Results were expressed as partition (distribution) coefficient of radioactive hormone. The latter was calculated either from the concentration of radioactive hormone in each phase, or from the upper phase and the total in the whole system:

$$k = \frac{u}{l} \quad (1)$$

$$k = \frac{u}{t(l+k_v) - k_v} \quad (2)$$

where k is the distribution coefficient, u , l and t are the concentrations of radioactive hormone in the upper phase, lower phase and total system respectively, and k_v (k_w) is the ratio volume (weight) of upper phase/volume (weight) of lower phase. The partition coefficient was calculated with a desk-top computer or determined graphically by using a logit-log transformation; it was shown (eqn. 2) that when t and k_v are constant, $\log k$ is a linear function of $\log u$. Results were the same by either method of analysis provided that the hormone was uniformly distributed in each phase without adsorption on to glassware or precipitation at the interface. The coefficient of variation of k in the dextran-polyethylene glycol system for 17 determinations was 0.8% for radioiodide and 2.2% for radioiodinated insulin.

The distribution of labelled hormone which had been incubated with antibody at a concentration permitting only partial binding depended on the proportion of antibody-bound hormone. The latter was calculated by using eqn. (3):

$$B/F = \frac{(l+k_v k_b)(k_t - k_x)}{(l+k_v k_t)(k_x - k_b)} \quad (3)$$

where B/F is the ratio of antibody-bound radioactive hormone to free radioactive hormone, k_x the apparent partition coefficient of radioactive hormone in the incubation mixture, k_t and k_b the respective partition coefficients of free and antibody-bound radioactive hormone, and k_v (k_w) the phase volume (weight) ratio.

Results

Distribution of free and antibody-bound labelled peptide hormone in various two-phase polymer systems

A number of two-phase solvent systems were tested to determine conditions for optimum separation of free from antibody-bound radioactive hor-

none. The nature and composition of these systems and the corresponding partition coefficients are listed in Table 1. Polymer concentrations were selected to give phase compositions fairly remote from that of the critical point (Albertsson, 1960) and a phase-volume ratio (volume of upper phase/volume of lower phase) close to unity. In fact, separation can be improved by varying the phase-volume ratio. From mathematical considerations, which will not be developed here, it can be shown that completeness of the separation is a function both of the separation factor (ratio partition coefficient of free hormone/partition coefficient of antibody-bound hormone) and of the phase-volume ratio. The higher the partition coefficients, the higher the phase-volume ratio required for optimum separation.

Antibody-bound hormones and immunoglobulins show more unilateral distributions than do free hormones; respective distributions depend on the phase system and on the individual hormone. Free hormones generally distribute equally between phases in systems made of uncharged polymers, and favour the upper phase of those containing charged polymers. Antibody-bound hormones and immunoglobulins are found in the lower phase of all systems except those containing two ionic polymers. The dextran- and the dextran sulphate-polyethylene glycol systems provide the most sensitive separation between free and antibody-bound hormone. Free [arginine]-vasopressin shows partition coefficients close to unity in almost all systems. Partition coefficients of other hormones generally increase in the order parathyroid hormone < growth hormone < insulin. Distribution of antibody-bound hormones is fairly similar to that for immunoglobulin, but varies somewhat from one hormone-antibody system to another.

Factors affecting the distribution of labelled peptide hormones in the dextran-polyethylene glycol system

The dextran-polyethylene glycol was selected for more extensive investigation. Several factors were found to affect differentially the distribution of free and antibody-bound radioactive hormone in this system. Independent separation methods showed that none of the factors to be described affected the antigen-antibody equilibrium in those samples in which excess of antibody was present.

Type of dextran. As shown in Table 2, partition coefficients of free and antibody-bound insulin and immunoglobulins depend on the molecular weight range of dextran used: the higher the molecular weight, the more unilateral the distribution. Although dextran 2000 provided a wider separation, the high viscosity of this polymer made it too cumbersome to use as a routine. Subsequent studies were carried out by using dextran 500.

Table 1. *Partition coefficients of peptide hormones and of immunoglobulins labelled with radioactive iodine in aqueous polymer two-phase systems*

The buffer was sodium phosphate, 0.02M, pH 7.4, containing 2.5mg of serum albumin/ml for insulin, growth hormone, vasopressin and immunoglobulins, or human plasma (1:10, v/v) for parathyroid hormone. Abbreviations: CMC, carboxymethylcellulose; D, dextran; DEAE-D, DEAE-dextran; DS, dextran sulphate; MC, methylcellulose; PEG, polyethylene glycol; PVA, polyvinyl alcohol; PVP, polyvinylpyrrolidone; F, free hormone; B, antibody-bound hormone. All partition coefficients were determined by using eqn. 1 developed in the Materials and Methods section except those in the DS-PEG and DS-PVP systems, which were calculated from eqn. 2. Hormone-antibody complexes were partially precipitated at the interface of the latter systems.

All percentages are w/w.

Two-phase solvent systems	Insulin		Parathyroid hormone		Growth hormone		[Arginine]-vasopressin		Immuno-globulins
	F	B	F	B	F	B	F	B	
Non-ionic polymer/non-ionic polymer in water									
D 500, 12%; PEG, 6%	1.80	0.01	0.30	0.04	0.80	0.04	0.98	0.05	0.04
D 2000, 1.6%; MC, 0.8%	1.04	0.20	1.18	0.28	1.00	0.38	1.02	0.58	0.52
D 500, 3%; PVA, 2.6%	1.10	0.04	0.58	0.10	0.72	0.22			0.20
D 500, 10%; PVP, 10%	5.17	0.06	0.90	0.08	3.86	0.59			0.50
PEG, 13.8%; PVP, 19%	0.27	0.01	0.02	0.01	0.14	0.01	0.32	0.03	0.03
Ionic polymer/non-ionic polymer/NaCl in water									
DS 2000, 15%; PEG, 6%; 0.3M-NaCl	6.10	0.03	1.00	0.06	3.30	0.08	1.15	0.17	0.08
DS 2000, 1.6%; MC, 0.7%, 0.3M-NaCl	1.00	0.10	0.56	0.17	1.05	0.28	0.62	0.38	0.37
DS 2000, 2.4%; PVA, 2.6%; 0.3M-NaCl	1.00	0.04	0.35	0.06	0.70	0.15			0.18
DS 2000, 10.5%; D 500, 4.5%; 1M-NaCl	3.40	37.4	10.0	21.3	6.62	48.8	1.15	35.6	24.3
DS 2000, 12%; PVP, 8%; 0.3M-NaCl	4.60	0.70	1.40	0.25	3.17	2.53	2.30	7.40	1.95
Ionic polymer/ionic polymer/NaCl in water									
DS 2000, 10%; DEAE-D, 2%; 1.6M-NaCl	4.12	1.17	7.70	38.0	3.63	45.1	1.72	25.4	22.4
DS 2000, 5%, CMC, 1.6%; 0.6M-NaCl	1.50	2.90	1.40	5.90	1.11	4.06	0.66	2.64	2.07

Concentrations of the polymers. Varying the concentrations of polymers affected the distribution of insulin, parathyroid hormone and immunoglobulins as illustrated in Fig. 1. As dextran and polyethylene glycol concentrations are increased at a constant ratio, the distribution coefficient for hormones as well as for antibody deviates further from unity. Above 10% (w/v) dextran and 5% (w/v) polyethylene glycol, almost all hormone-antibody complexes and immunoglobulins are found in the lower phase, whereas free hormones distribute between both phases. Insulin-antibody complexes show higher distribution coefficients than other hormone-antibody complexes and immunoglobulins alone at low

polymer concentration; the opposite is characteristic for high polymer concentrations.

pH and ions. pH and ions were allowed to vary within limits that did not affect the antigen-antibody equilibrium. As shown in Fig. 2, distribution of free insulin is markedly affected by the nature and the ionic strength of the buffer. Partition coefficients differ in barbital, tris and phosphate buffers and are affected by pH in the two last-named buffers; the more alkaline the system, the higher the partition coefficient. Ionic strength of the phosphate buffer also affects distribution; the higher the ionic strength, the higher the partition coefficient. Distribution of free parathyroid hormone and growth hormone, but not

Table 2. *Effect of the molecular weight of dextran on the distribution of radioiodinated insulin and immunoglobulins in the dextran-polyethylene glycol system*

Polymer concentrations were: dextran, 12% (w/w); polyethylene glycol, 6% (w/w). The buffer was 0.02M-sodium phosphate, pH 7.4, containing human serum albumin (2.5mg/ml).

Weight-average mol.wt. of dextran ($\bar{M}_w$)	Partition coefficients		
	Free insulin	Antibody-bound insulin	Immunoglobulins
70000	1.60	0.04	0.10
250000	1.70	0.02	0.06
500000	1.80	0.01	0.03
2000000	1.90	0.01	0.03

Fig. 1. *Effect of polymer concentration on the distribution of radioiodinated hormones and immunoglobulins*

Radioiodinated insulin (●, ○), growth hormone (▲, △), parathyroid hormone (■, □) and immunoglobulins (◆) were examined in the dextran 500-polyethylene glycol system. The buffer was 0.02M-sodium phosphate, pH 7.4, containing human serum albumin (2.5mg/ml) for insulin, growth hormone and immunoglobulins, and human plasma (1:10, v/v) for parathyroid hormone. Filled-in symbols, free hormones; open symbols, antibody-bound hormones.

vasopressin, is similarly affected by pH in phosphate buffer (Fig. 3). On the other hand, ionic strength and pH show little effect on the distribution of antibody-bound hormones and immunoglobulins. Therefore separation is best when partition coefficients of free hormones are high.

Halides, which have been shown to affect the behaviour of several proteins in the dextran-polyethylene glycol system (Albertsson, 1960, 1970), also influence the distribution of labelled hormones. As shown in Table 3, these salts at a concentration of 0.5M increase the partition coefficient of free as well as antibody-bound parathyroid hormone. Their effectiveness increases in the order $\text{Cl}^- < \text{Br}^- < \text{I}^-$, and potassium salts show greater effect than sodium salts. The partition coefficient of free hormone is more affected than that of the hormone-antibody complex; this allows for a more efficient separation. Halides did not improve the separation for other peptide hormones. As shown for other proteins (Albertsson, 1960), the effect of sodium chloride on the distribution coefficient of labelled hormones depends on the concentration of this salt. Sodium chloride at low concentration decreases the distribution coefficient for free insulin, but does not affect that for hormone-antibody complex; high concentrations increase the distribution coefficients for free as well as antibody-bound hormone (Fig. 4). In addition, high concentrations of sodium chloride increase the partition coefficient for immunoglobulin alone to a greater extent than that for insulin-antibody complex (Fig. 4).

Urea. As shown in Fig. 5, urea increases the partition coefficients of insulin and parathyroid hormone, whether bound to antibody or not. The partition coefficient of free parathyroid hormone is more affected than that of antibody-bound hormone; this results in a slightly more efficient separation.

Added serum proteins. As shown in Table 4, distribution coefficients of labelled free hormones depend on the nature of added serum proteins. Partition coefficients are lower when plasma is added than with serum albumin alone. At high concentrations of plasma, part of the effect is due to salts, since dialysed

Fig. 2. Effect of the ionic composition of the phase system on the distribution of radiiodinated insulin in the dextran 500-polyethylene glycol system

The polymer concentrations were: dextran, 12% (w/w); polyethylene glycol, 6% (w/w). Buffers contained human serum albumin (2.5 mg/ml). (a), Partition coefficient as a function of pH with sodium phosphate (●, ○), sodium barbital (▼, ▽) or tris-HCl (▲, △) buffers, 0.02M. (b), Partition coefficient as a function of pH with sodium phosphate buffer, 0.02M (●, ○), 0.12M (▲, △) and 0.20M (▼, ▽). Filled-in symbols, free insulin; open symbols, antibody-bound insulin.

plasma does not cause as much effect as undialysed plasma (experiments not shown). Immunoglobulins, which are almost completely precipitated from the upper phase, cause a still greater decrease in the partition coefficients. The distribution of growth hormone is more affected by plasma or immunoglobulins than that of insulin or parathyroid hormone. Protein environment does not influence the distribution of antibody-bound hormones.

Altered or 'damaged' hormone. The influence of 'damage' to labelled hormones was studied by partitioning unpurified iodination mixtures that had been freed of iodide as well as purified preparations left at room temperature in plasma. Incubations with insulin or parathyroid hormone were carried out for periods up to 24 h at room temperature. Polymers were then added and the phases separated. Analysis of each phase by chromatoelectrophoresis (Yalow & Berson, 1964) or talc adsorption (Rosselin *et al.*, 1966) showed that 'damaged' components, identified as labelled material not binding to these absorbents,

Fig. 3. Partition coefficients for radiiodinated peptide hormones in the dextran 500-polyethylene glycol system with 0.02M-sodium phosphate buffers of different pH values

The polymer concentrations were: dextran, 12% (w/w); polyethylene glycol, 6% (w/w). Buffer contained human serum albumin (2.5 mg/ml) for insulin, growth hormone and [arginine]-vasopressin, and human plasma (1:10, v/v) for parathyroid hormone. ●, ○, Insulin; ■, □, parathyroid hormone; ▲, △, growth hormone; ▼, ▽, [arginine]-vasopressin. Filled-in symbols, free hormones; open symbols, antibody-bound hormones.

give partition coefficients different from those of the corresponding hormone. Partition coefficients for the 'damaged' components varied from one labelled preparation to another, differed with iodination 'damage' and incubation 'damage', and were affected by pH and the nature of serum proteins present in the system (experiments not shown).

Standard curves for peptide hormones

The two-phase polymer systems can be used for separation of free and antibody-bound labelled

Table 3. *Effect of sodium and potassium halides on the distribution of radioiodinated parathyroid hormone in the dextran 500–polyethylene glycol system*

Polymer concentrations were: dextran 500, 12% (w/w); polyethylene glycol, 6% (w/w). The buffer was 0.02M-sodium phosphate, pH7.4, containing human plasma (1:10, v/v). The halide concentration was 0.5M.

Halide	Partition coefficient	
	Free hormone	Antibody-bound hormone
None	0.30	0.04
NaCl	0.70	0.05
NaBr	0.90	0.06
NaI	1.25	0.07
KCl	1.10	0.06
KBr	1.50	0.07
KI	1.80	0.08

hormone under standard radioimmunoassay conditions. Fig. 6 shows standard curves prepared by plotting the partition coefficient of radioactive hormone in the two-phase system against the concentration of unlabelled hormone. Phase system and ions were selected so as to give the best separation. As the concentration of unlabelled hormone rose, there was a progressive increase in the partition coefficient of labelled hormone. Shapes of the curves were somewhat different because of differences in the partition coefficients of the individual hormones, degree of binding and affinity of the individual antisera. However, bound/free ratios as calculated from the partition coefficients were in good agreement with those obtained by paper chromatoelectrophoresis or the double-antibody method, and the sensitivities of these various methods were equivalent.

Discussion

Molecules of different size or surface properties or both can be separated by distribution into aqueous two-phase polymer systems (Albertsson, 1960, 1970) and it was known that antigen–antibody complexes distribute differently from free antigen in these systems. Albertsson (1960) and Albertsson & Philipson (1960) showed that binding to specific antibody causes a marked decrease in the partition coefficients of phycoerythrin and serum albumin in the dextran–polyethylene glycol and dextran–methylcellulose systems, both being transferred either to the lower phase or to the interface of the system. These changes were observed in the region of antigen excess, where in the absence of polymers no precipitation of antigen–antibody complexes occurs.

Fig. 4. *Effect of sodium chloride on the distribution of radioiodinated insulin and immunoglobulins in the dextran 500–polyethylene glycol system*

The polymer concentrations were: dextran, 12% (w/w); polyethylene glycol, 6% (w/w). The buffer was 0.02M-sodium phosphate, pH7.4, containing human serum albumin (2.5mg/ml). ●, Free insulin; ○, antibody-bound insulin; ◆, immunoglobulins.

Basically similar observations were made by these investigators (Philipson, 1966; Philipson *et al.*, 1966) in a study on the interaction between poliovirus and antibody in the dextran–polyethylene glycol system. Complete separation of free virus from virus–antibody complex could also be achieved by using the aqueous polymer system in countercurrent distribution (Philipson, 1966).

The experiments described in the present paper show that the polymer two-phase systems can also be used to detect and measure binding to antibody of peptide hormones at the very low concentration of reactants under which radioimmunoassays are generally carried out. Free and antibody-bound hormones exhibited different distribution coefficients in a

Fig. 5. Effect of urea on the distribution of radioiodinated hormones and immunoglobulins

Radioiodinated insulin (●, ○), parathyroid hormone (■, □) and immunoglobulins (◆) were partitioned in the dextran 500–polyethylene glycol system. The polymer concentrations were: dextran, 12% (w/w); polyethylene glycol, 6% (w/w). Other conditions were as described in Fig. 2. Filled-in symbols, free hormones; open symbols, antibody-bound hormones.

number of systems tested; two of them, the dextran–polyethylene glycol and the dextran sulphate–polyethylene glycol systems, gave optimum separation. In the absence of antibody, most labelled hormones studied distributed in favour of the upper (polyethylene glycol-rich) phase. After incubation with excess of antibody, almost all the radioactivity was found in the lower (dextran-rich) phase or at the interface. After incubation with antibody at a concentration permitting partial binding, partition coefficients were lower than those of free hormones, but higher than those of antibody-bound hormones. Bound/free ratios, as calculated from the partition coefficients, were in good agreement with those obtained by using independent separation methods

such as paper chromatoelectrophoresis or precipitation by an anti-immunoglobulin serum. Analysis of the phases of the system by paper chromatoelectrophoresis (Yalow & Berson, 1964), talc (Rosselin *et al.*, 1966), or dextran-coated charcoal (Herbert *et al.*, 1965) also showed that free and antibody-bound hormone distributed as expected. This suggested that addition of the polymer mixture did not modify the antigen–antibody equilibrium that existed before fractionation. Other investigators (Philipson, 1966) also found no dissociation of poliovirus immunocomplexes under the influence of the polymers.

A number of factors such as the concentration of the polymers, the ionic composition of the system, and the size of the partitioned molecule have been shown to affect the distribution of proteins in the dextran–polyethylene glycol system (Albertsson, 1960, 1970). It was expected that these factors would differentially affect the respective distributions of free and antibody-bound hormones, and thereby influence the separations. The concentration of the polymers was an important factor whenever free and antibody-bound hormones favoured opposite phases; the more widely the composition of the system changed from that at the critical point (Albertsson, 1960) the more unilateral was the distribution and the better the separation. However, marked increases in concentration of polymers were limited for practical purposes owing to increased viscosity. The ionic composition of the system was also of importance, partition coefficients of free hormones being higher and separation more effective with phosphate ions than with other electrolytes. The nature of the hormone affected distribution; free insulin showed a higher partition coefficient than growth hormone, parathyroid hormone and [arginine]-vasopressin, whereas the opposite was characteristic of hormone–antibody complexes. Therefore separation was more sensitive for insulin than for other hormones. The presence of immunoglobulins, which are almost completely precipitated out of the upper phase, and to a lesser extent of plasma, resulted in a decrease in the partition coefficients of free hormones, but did not affect those of antibody-bound hormones. 'Damage' to labelled hormones also caused a change in the partition coefficient, but to a variable degree; it was not therefore possible to estimate the amount of 'damage' present in an unknown sample without using an independent method such as paper chromatoelectrophoresis. The behaviour of free labelled hormones as well as of unlabelled hormones in the dextran–polyethylene glycol system is being further investigated.

The behaviour of labelled hormones in the dextran sulphate–polyethylene glycol system was basically the same as in the dextran–polyethylene glycol system, and separation was comparable. Several differences, however, were observed. Partition co-

Table 4. *Partition coefficients of free insulin, parathyroid hormone and growth hormone labelled with radioactive iodine in the dextran 500–polyethylene glycol system in the presence of various serum proteins*

Polymer concentrations were: dextran, 12% (w/w); polyethylene glycol, 6% (w/w). Proteins were dissolved in or dialysed against sodium phosphate, final concentration 0.02M, pH 7.4.

Proteins	Partition coefficients		
	Insulin	Growth hormone	Parathyroid hormone
Human serum albumin (2.5mg/ml)	1.85	0.90	
Bovine serum albumin (10mg/ml)	1.82	0.88	0.28
Rabbit immunoglobulins (10mg/ml)	0.80	0.20	0.16
Human plasma (1:20, v/v)	1.84		
Human plasma (1:10, v/v)	1.85	0.53	
Human plasma (1:5, v/v)	1.70		
Human plasma (1:2.5, v/v)	1.45		0.25

Fig. 6. *Standard curves for labelled peptide hormones*

Incubations of labelled hormones with antibody and unlabelled hormones were done as described in the Materials and Methods section. Insulin (●) and vasopressin (▼) were partitioned in the dextran 500–polyethylene glycol system [dextran, 12% (w/w); polyethylene glycol, 6% (w/w)] with 0.02M-sodium phosphate, pH 8.0, containing human or bovine serum albumin (2.5 mg/ml). Growth hormone (▲) and parathyroid hormone (■) were distributed in the dextran sulphate 2000–polyethylene glycol system [dextran sulphate, 15% (w/v); polyethylene glycol, 6% (w/v)] with 0.02M-sodium barbital, pH 8.6, containing human serum albumin (2.5mg/ml) for growth hormone and human plasma (1:12.5, v/v) for parathyroid hormone.

efficients of both free and antibody-bound hormones were about fivefold higher than in the dextran–polyethylene glycol system; antibody-bound hormones were partially precipitated at the interface; separation was less critically affected by the ionic environment. The dextran sulphate–polyethylene glycol system was found to be a more suitable one for

the separation of free and antibody-bound parathyroid hormone.

It has been shown (Helsing, 1969) that the decreases in solubility of immunoglobulin–anti-immunoglobulin G complexes observed in aqueous solutions of dextran is higher for immunoglobulin G polymers than for immunoglobulin G monomers, but no information is available on the effect of antigen size on the distribution of immunocomplexes in the two-phase polymer systems. The results reported here indicate that the distribution coefficients for hormone–antibody complexes vary somewhat from one hormone to another, and also differ significantly from that for immunoglobulin alone. This would suggest that the molecular size and surface properties of the complex are not identical for the individual hormones and differ from those of immunoglobulin alone. Results obtained by other investigators using ultracentrifugation (Berson & Yalow, 1962) and polyacrylamide gel electrophoresis (Heideman, 1964) would also suggest that the physical properties of the insulin–antibody complex are different from those of uncombined anti-insulin antibody.

The method of equilibrium dialysis (Marrack & Smith, 1932; Eisen & Karush, 1949), which has been developed to measure binding of low-molecular-weight haptens to antibody, may be regarded as a particular liquid–liquid partition method in which free haptens distribute equally between two phases, whereas antibody-bound hapten is found exclusively in one phase. Although thermodynamically sound, the procedure shows certain disadvantages. Equilibration is relatively slow; the usual pore size of membranes does not allow studies of binding with large molecules; there may be effects of the membrane itself. The two-phase aqueous polymer systems provide a separation on a similar thermodynamic basis with almost instantaneous equilibrium, and can be used with larger molecules than haptens.

Other aqueous or water-rich two-phase systems

have been developed by different investigators to distribute high-molecular-weight proteins. Some of these systems are formed by water, an organic solvent miscible with water, and a third component such as a salt, a sugar or a polyhydric alcohol (Von Tavel & Signer, 1956). Others are obtained by mixing water, a water-soluble polymer and a low-molecular-weight component such as a salt (Albertsson, 1960). The finding that serum albumin and immunoglobulins favour opposite phases in the system diethylcarbitol- α -glycerophosphate-L-sorbitol-water (Von Tavel & Signer, 1956) suggests that such a system could be used to separate free and antibody-bound protein antigens. We found the system polyethylene glycol-potassium phosphate developed by Albertsson (1960) satisfactory for separation of free and antibody-bound [arginine]-vasopressin (B. Desbuquois & G. D. Aurbach, unpublished work).

Two-phase partition analysis has also been used to study non-immunological molecular interactions, such as binding of dyes (Karush, 1951) and steroid hormones (Schellman *et al.*, 1954) to serum albumin. However, because the phase systems used in these studies are made of organic solvents immiscible with water, the method is limited by the risk of protein denaturation at the interface and is applicable only to low-molecular-weight ligands soluble in the organic phase. In contrast, the high water content and the low interfacial forces in the polymer two-phase systems make it possible to distribute biological substances without denaturation (Albertsson, 1960), and partition is also feasible with higher-molecular-weight ligands insoluble in organic solvents. The dextran-polyethylene glycol system has successfully been used to study the interaction between adenine nucleotides and the enzyme formyltetrahydrofolate synthetase (Curthoys & Rabinowitz, 1970). We have found that this system also allows one to detect binding of calcium, thyroxine and steroid hormones to serum proteins (B. Desbuquois & G. D. Aurbach, unpublished work).

We found the two-phase polymer partition method too cumbersome for routine application to radioimmunoassay of peptide hormones in plasma. However, it may prove helpful in exploring new radioimmunoassay systems and whenever routine methods of separation are unsatisfactory. In the course of this work we observed that aqueous polyethylene glycol alone causes precipitation of hormone-antibody complexes whereas free hormones remain in solution. This observation has led to the development of a more convenient method for separating free from antibody-bound hormone in radioimmunoassays (Desbuquois & Aurbach, 1971).

We acknowledge the help of Dr. G. L. Robertson, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Md., U.S.A., who

labelled [arginine]-vasopressin and carried out the double-antibody precipitation tests with this hormone, and are indebted to Dr. Stephan E. Mergenhagen, National Institute of Dental Research, for his gift of labelled immunoglobulin. This work was supported in part by U.S. Public Health Service International Postdoctoral Research Fellowship no. F05 TW1188-01.

References

- Albertsson, P. A. (1960) *Partition of Cell Particles and Macromolecules*, John Wiley and Sons, New York
- Albertsson, P. A. (1970) *Advan. Protein Chem.* **24**, 309
- Albertsson, P. A. & Philipson, L. (1960) *Nature (London)* **184**, 38
- Berson, S. A. & Yalow, R. S. (1962) *Trans. N.Y. Acad. Sci.* **24**, 487
- Berson, S. A., Yalow, R. S., Aurbach, G. D. & Potts, J. T., Jr. (1963) *Proc. Nat. Acad. Sci. U.S.* **49**, 613
- Curthoys, N. P. & Rabinowitz, J. C. (1970) *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **29**, 531
- Desbuquois, B. (1970) *Program Endocrine Soc.* **52nd Meet.** Abstract no. 215, p. 144
- Desbuquois, B. & Aurbach, G. D. (1971) *J. Clin. Endocrinol.* **33**, 732
- Eisen, H. N. & Karush, F. K. (1949) *J. Amer. Chem. Soc.* **71**, 363
- Feinberg, R. (1956) *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **15**, 586
- Heideman, M. L. (1964) *Biochemistry* **3**, 1108
- Hellsing, K. (1969) *Biochem. J.* **114**, 145
- Herbert, V., Lau, K. S., Gottlieb, C. W. & Bleicher, S. J. (1965) *J. Clin. Endocrinol.* **25**, 1375
- Hunter, W. M. & Greenwood, F. C. (1962) *Nature (London)* **194**, 495
- Karush, F. K. (1951) *J. Amer. Chem. Soc.* **73**, 1246
- Marrack, J. & Smith, F. C. (1932) *Brit. J. Exp. Pathol.* **13**, 394
- McFarlane, J. (1956) *Biochem. J.* **62**, 135
- Philipson, L. (1966) *Virology* **28**, 35
- Philipson, L., Killander, J. & Albertsson, P. A. (1966) *Virology* **28**, 22
- Potts, J. T., Jr. & Aurbach, G. D. (1965) in *The Parathyroid Glands: Ultrastructure, Secretion, and Function* (Gaillard, P. J., Talmage, R. V. & Budy, A. M., eds.), pp. 53-67, University of Chicago Press, Chicago
- Reese, R. T. & Treffers, H. P. (1969) *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **28**, 308
- Robertson, G. L., Klein, L. A., Roth, J. & Gorden, P. (1970) *Proc. Nat. Acad. Sci. U.S.* **66**, 1298
- Rosselin, G., Assan, R., Yalow, R. S. & Berson, S. A. (1966) *Nature (London)* **212**, 355
- Roth, J. S., Glick, S. M., Klein, L. A. & Petersen, M. J. (1966) *J. Clin. Endocrinol.* **26**, 671
- Schellman, J. A., Lumry, R. & Samuels, L. T. (1954) *J. Amer. Chem. Soc.* **76**, 2808
- Von Tavel, P. & Signer, R. (1956) *Advan. Protein Chem.* **11**, 237
- Yalow, R. S. & Berson, S. A. (1964) *Methods Biochem. Anal.* **12**, 69
- Yalow, R. S. & Berson, S. A. (1966) *Nature (London)* **212**, 357