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Effects of Iodination on the Distribution of Peptide Hormones in Aqueous Two-Phase Polymer Systems

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1. The effect of iodination on the distribution of peptide hormones into the aqueous two-phase dextran–polyethylene glycol system and on the solubility of these hormones in aqueous polyethylene glycol and in water was assessed. Hormones that were studied included insulin, glucagon and parathyroid hormone. 2. The partition coefficient of native insulin in the dextran–polyethylene glycol system showed a minimum (about 1) near the isoelectric point of the hormone (pH 5). Partial iodination of insulin (one atom per molecule) caused little change in the distribution of the hormone. More extensive iodination markedly decreased the partition coefficient in the region of the isoelectric point and displaced the pH value at which the partition coefficient was a minimum towards lower values. 3. The solubility of native insulin in aqueous polyethylene glycol and in water showed a pH-dependence similar to that observed for the distribution in the dextran–polyethylene glycol system. Iodination of insulin decreased the solubility of the hormone in polyethylene glycol and in water in parallel, and decreased the pH value at which solubility was a minimum. The changes in solubility correlated with the degree of iodination and accounted for the changes in distribution observed at high concentrations of insulin. 4. Comparable effects of iodination on distribution and solubility were also observed with glucagon. 5. At concentrations of insulin below its maximum solubility, serum proteins caused a decrease in the partition coefficient of iodinated hormone, but not of native hormone. These effects correlated with the degree of iodination and resulted from a co-precipitation of iodinated insulin with serum proteins.

In a recent study, we found that free and antibody-bound peptide hormones labelled with radioactive iodide can be separated by differential distribution into aqueous two-phase polymer systems (Desbuquois & Aurbach, 1972) and solubility in aqueous polyethylene glycol (Desbuquois & Aurbach, 1971). The peptide hormones that were studied included insulin, parathyroid hormone, growth hormone and [arginine]vasopressin. We also observed that the distribution and solubility properties of free hormones were dependent on the degree of iodination. In the present studies, the effects of iodination on the distribution of insulin and glucagon in the dextran–polyethylene glycol system as well as on the solubility of these hormones in aqueous polyethylene glycol and in water have been characterized in detail.

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Experimental

Materials

Crystalline porcine insulin and bovine–porcine glucagon were gifts from Dr. R. E. Chance, Eli Lilly and Co., Indianapolis, Ind., U.S.A. The sources of bovine parathyroid hormone and specific antisera to bovine insulin and bovine parathyroid hormone were given previously (Desbuquois & Aurbach, 1972). Guinea-pig antiserum to glucagon was a gift from Dr. P. Cuatrecasas, Department of Pharmacology, Johns Hopkins Medical School, Baltimore, Md., U.S.A. Dextran 500 was obtained from Pharmacia Fine Chemicals, Piscataway, N.J., U.S.A. Polyethylene glycol (Carbowax 6000) was obtained from Union Carbide Corporation, Baltimore, Md., U.S.A. Other materials were obtained as described by Desbuquois & Aurbach (1972).

Iodination of polypeptide hormones

Insulin and glucagon were iodinated both in milligram (method A) and microgram (method B) quantities. Parathyroid hormone was iodinated only in microgram quantities (method B). In both methods, iodination was performed with NaI and the oxidizing agent was chloramine-T.

Method A. About 5–10mg of peptide, 2–5mg of stable NaI and 20–40μCi of $Na^{125}I$ or $Na^{131}I$ were dissolved in 3–6ml of 0.1M-Tris–HCl buffer, pH8.5. Since glucagon shows a low solubility in buffer alone, urea (7M) was included in the iodination mixture for this hormone. In molar equivalents, the ratio of stable iodide to peptide was 16:1 for insulin and 8:1 for glucagon. An aqueous solution of chloramine-T (10–20mg/ml) was added to the reaction mixture in successive fractions (about 0.3mol/mol of peptide for each fraction). After each addition, incorporation of iodine into hormone was estimated by treating 1–2μl samples of the reaction mixture with 5% (w/v) trichloroacetic acid in the presence of 1% (w/v) albumin. Chloramine-T additions were continued until the incorporation of iodine into hormone reached 8–9 atoms of iodine/molecule for insulin and 4 atoms/molecule for glucagon. At appropriate intervals, samples of the reaction mixtures were withdrawn and mixed with 10μl of a solution of sodium metabilsulphite (0.5M). For each sample, labelled hormone was separated from unchanged iodide by dialysis or by gel filtration on Sephadex G-25 for insulin or G-15 for glucagon. After purification, more than 95% of the labelled material was precipitable by 5% trichloroacetic acid. The iodine content and the concentration of the iodinated preparations were determined from the starting ratios of stable and radioactive iodide to hormone and the fractional incorporation of radioactivity into trichloroacetic acid-precipitable material.

Method B. Iodination of hormones in microgram quantities was done by the method of Hunter & Greenwood (1962), as described by Desbuquois & Aurbach (1972). When a low iodine content was required, iodination was done only with radioactive iodide. The specific radioactivities achieved (20–100 mCi/mg) corresponded to a theoretical iodine content of 0.05–0.3 atom/molecule of hormone. When a high iodine content was required, the reaction mixtures were supplemented with stable iodide. For the latter, the iodine content was calculated from the starting ratio of stable iodide to hormone and the fractional incorporation of radioactive iodide into peptide as determined by paper chromatoelectrophoresis (Yalow & Berson, 1964). Immediately after iodination, the reaction mixtures were freed of 'damaged' components and free iodide. Procedures for the purification of insulin and parathyroid hormone have been described previously (Desbuquois & Aurbach, 1972). Glucagon was purified by adsorption on a cellulose column and elution with human plasma, as described for insulin by Yalow & Berson (1964). The efficiency of the various purification procedures was assessed as described by Desbuquois & Aurbach (1972).

Partition experiments

The peptides iodinated in milligram quantities (method A) were partitioned at high concentrations (1μg/ml–1mg/ml) without carrier proteins. The peptides iodinated in microgram quantities (method B) were partitioned at low concentrations (0.001–0.1μg/ml) in the presence of unlabelled hormone (10–20μg/ml) or carrier serum proteins (2.5–10mg/ml). Partition experiments were also performed with unlabelled peptides with and without carrier serum proteins. Adsorption on glassware in either type of experiment did not exceed 5%.

Two-phase systems were prepared at 4°C by mixing into 13mm×75mm test tubes 960mg of a 25% (w/w) solution of dextran, 240mg of a 50% (w/w) solution of polyethylene glycol, and 0.8ml of peptide dissolved into buffer; the nature of the latter is indicated for each experiment. Final concentrations of the polymers were 12% (w/w) for dextran and 6% (w/w) for polyethylene glycol. Phases were separated as described by Desbuquois & Aurbach (1972) and analysed for their hormone content. The concentration of labelled peptides was determined by crystal-scintillation detection. The concentration of unlabelled peptides was determined by specific radioimmunoassay (for concentrations below 50–100μg/ml) or u.v. absorbance (for concentrations above 50–100μg/ml).

Radioimmunoassays for insulin, glucagon and parathyroid hormone were done by using specific antisera and the corresponding radioiodinated hormone. For each individual partition experiment, phases were diluted into buffers containing serum albumin or human plasma and assayed at several dilutions. After incubation, free and antibody-bound radioactive hormones were separated by dextran-coated charcoal (Herbert *et al.*, 1965). Appropriate controls showed that the polymers did not affect the binding of hormone to antibody and did not interfere with the separation step. The data were analysed as described by Rodbard *et al.* (1968).

To measure the absorbance at 280nm, phases were diluted into 0.01M-HCl and analysed against blanks of the same polymer composition. It was shown that the polymers did not affect the u.v. spectra of the hormones.

Partition (distribution) coefficients were calculated from the concentration of hormone in each phase or occasionally from concentrations in the upper (polyethylene glycol-rich) phase and in the total

mixture of both phases. Details of the calculations were given by Desbuquois & Aurbach (1972).

Solubility experiments

The solubility of the peptide hormones in aqueous polyethylene glycol and in water was determined under conditions (e.g. temperature, buffer and pH) basically similar to those used in the partition experiments. The concentration of polyethylene glycol selected (12.5%, w/w) was closely similar to that in the upper (polyethylene glycol-rich) phase of the dextran–polyethylene glycol system. In experiments to determine the maximum solubility of insulin and glucagon in polyethylene glycol and in water, concentrations of these peptides (0.1–2mg/ml) were selected so as to exceed their expected maximum solubility, and carrier proteins were omitted. In experiments with low concentrations of peptides (less than 0.1μg/ml), human plasma (1:10, v/v, final concentration) was added as carrier. After incubation for 30min at 4°C, peptide and serum protein precipitates were collected by centrifugation for 30min at 40000*g* and 2000*g* respectively, and the concentration of peptides in the supernatant fluid was determined in each case. Results were expressed as solubility (experiments without carrier proteins) or as the percentage of hormone precipitated (experiments with carrier proteins).

Results

Distribution of insulin in the dextran–polyethylene glycol system at various pH values

The distribution of insulin in the dextran–polyethylene glycol system, like that of other proteins (Albertsson, 1970, 1971), is pH-dependent (Fig. 1). In buffers containing phosphate ions, insulin favours the upper (polyethylene glycol-rich) phase; the partition coefficient of the hormone shows a minimum (about 1) near the isoelectric point (pH5). Iodination of insulin at an average of one atom per molecule causes little change in the partition coefficient of the hormone over the pH range tested (Fig. 1). More extensive iodination markedly decreases the partition coefficient in the pH range 3–6, the effect being maximal in the region of the isoelectric point (Fig. 1). In addition, high degrees of iodination of insulin cause an increase in the partition coefficient at low (below 3) and high (above 9) pH (Fig. 1), and displaces the pH of minimum partition coefficient towards lower values (not shown).

Distribution of insulin at various hormone concentration

The partition coefficient of unlabelled insulin in the dextran–polyethylene glycol system is independent of the concentration of this hormone below 10μg/ml, but shows a rapid decrease at higher concentrations in the region of the isoelectric point (Fig. 2). The apparent fall in the partition coefficient observed under these conditions is almost completely attributable to the low solubility of the hormone in the upper (polyethylene glycol-rich) phase (see below). Partially iodinated and native insulin show closely similar partition coefficients at concentrations of 25μg/ml or less, but the former shows a distinctly lower partition coefficient than the latter at concentrations above this value. More extensive iodination markedly decreases the partition coefficient at all insulin concentrations tested. However, the relative decrease in the partition coefficient caused by increasing degrees of iodination is less at low (0.01μg/ml) than at high (10 and 100μg/ml) concentrations of insulin.

Solubility of insulin in aqueous polyethylene glycol and in water

It has been shown in earlier studies that polyethylene glycol causes proteins and other biological macromolecules to precipitate from solution (reviewed by Fried & Chun, 1971). We investigated the possibility that the changes in the distribution of insulin caused by the iodination might result from changes in the solubility of this hormone in aqueous polyethylene glycol and in water. The solubility of unlabelled insulin in buffer alone shows a minimum (about 250μg/ml) near the isoelectric point of this hormone (Fig. 3). Addition of polyethylene glycol lowers the solubility of insulin independently of pH, in agreement with observations made with other proteins (Juckes, 1971). The pH of minimum solubility in water and in polyethylene glycol is virtually identical with that of the minimum partition coefficient in the dextran–polyethylene glycol system. Partially iodinated insulin shows distinctly less solubility and a lower pH of minimum solubility in polyethylene glycol and in water than does native insulin (Fig. 3). Iodination of insulin to a greater extent than 1 atom per molecule causes a further decrease in solubility and in the pH of minimum solubility (Fig. 3). A linear dependence is found when the logarithm of solubility is plotted against the iodine content, and the slopes of the curves of solubility in polyethylene glycol and in buffer are virtually identical. The effect of iodination on solubility almost completely accounts for the decrease in the partition coefficient of insulin observed at the high concentrations (10 and 100μg/ml) of hormone.

Distribution and solubility properties of glucagon

The distribution of glucagon in the dextran–polyethylene glycol system and the solubility of this

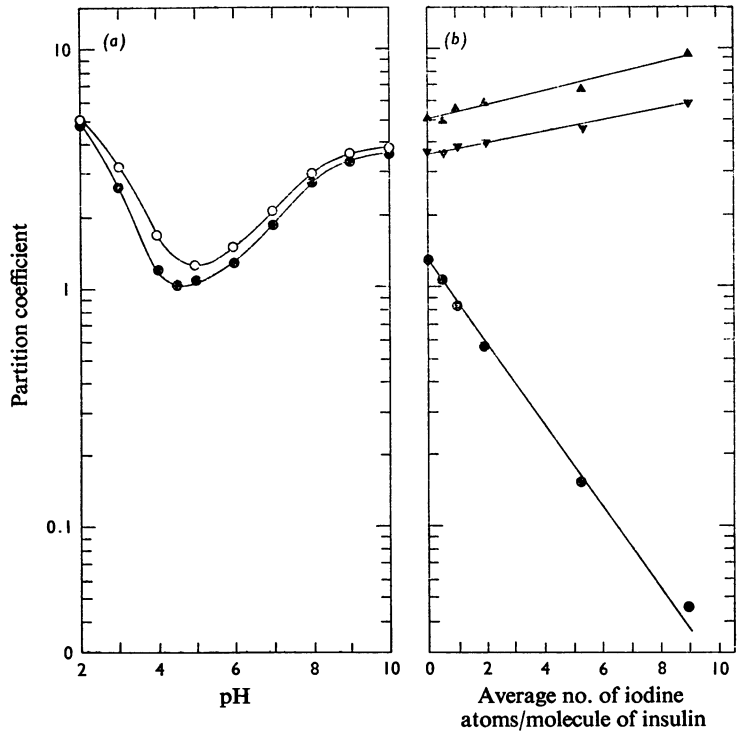

Fig. 1. *Effect of iodine content on the distribution of iodinated insulin in the dextran–polyethylene glycol system at various pH values*

Insulin was iodinated to various degrees by method A described in the Experimental section. Distribution experiments were performed in the presence of sodium phosphate (pH 6–8), citrate–sodium phosphate (pH 2–6) and Tris–sodium phosphate (pH 8–10) buffers. The concentration of sodium phosphate was 0.02 M. (*a*) Partition coefficient of native insulin (○) and of insulin labelled at an average of 0.9 atom of iodine/molecule (●) shown as a function of pH. (*b*) Partition coefficient of iodinated insulin at pH 2 (▲), pH 5 (●) or pH 10 (▼) shown as a function of iodine content.

hormone in polyethylene glycol and in water both show a pH-dependence somewhat similar to that observed with insulin (not shown). However, glucagon differs from insulin in several ways: (1) the individual values for the partition coefficients at high and low pH are lower than those observed with insulin (about 3 and 2.5 respectively); (2) the solubility of glucagon in buffer and the relative decrease in solubility of the hormone caused by the addition of polyethylene glycol (about 45 and 15 μg/ml at the pH of minimum solubility respectively) are less than with insulin; (3) the pH of minimum partition coefficient and minimum solubility (about 4) is lower than that for insulin. Increasing iodination of glucagon results in modification of the distribution properties of this hormone qualitatively similar to those found for insulin (Table 1), and such changes are also accompanied by changes in solubility properties (not shown).

Effects of serum proteins on the distribution of insulin in the dextran–polyethylene glycol system

The partition coefficients of iodinated hormones in the dextran–polyethylene glycol system depend on the nature of added serum proteins (Desbuquois & Aurbach, 1972). We investigated the possibility that serum proteins might affect differently the partition coefficients of native and iodinated insulin. The distribution of native insulin in buffers containing serum albumin shows a pH-dependence somewhat similar to that observed in buffers without carrier proteins (Fig. 4). Iodination of insulin at an average of less than one atom per molecule markedly decreases the partition coefficient of the hormone in the pH range 4–5 (Fig. 4). More extensive iodination further decreases the partition coefficient; the effect extends through a broad pH range towards the alkaline region (Fig. 4). In addition, high degrees of

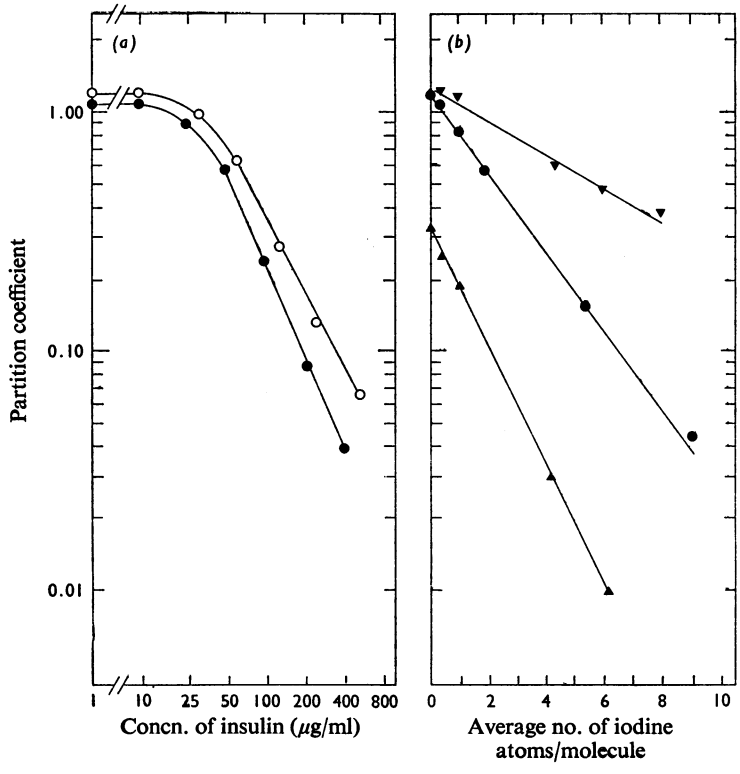

Fig. 2. *Effect of iodine content on the distribution of iodinated insulin in the dextran–polyethylene glycol system at various hormone concentrations*

Insulin was iodinated to various degrees by method A (concentrations above 1 μg/ml) or method B (concentrations below 1 μg/ml) described in the Experimental section. Distribution experiments were performed in 0.02 M-citrate–phosphate buffer, pH 5. (*a*) Partition coefficient of native insulin (○) or insulin labelled at an average of 0.9 atom of iodine per molecule (●) shown as a function of insulin concentration. (*b*) Partition coefficient of iodinated insulin at hormone concentrations of 100 μg/ml (▲), 10 μg/ml (●) and 0.01 μg/ml (▼) shown as a function of iodine content. In experiments with concentrations of iodinated insulin of less than 1 μg/ml, unlabelled insulin (10 μg/ml) was added as a carrier.

iodination increase the partition coefficient of insulin at low pH (Fig. 4). γ-Globulin and whole serum show effects comparable with those observed with serum albumin, although occurring in a somewhat broader pH range (not shown).

Effects of serum proteins on the solubility of insulin in aqueous polyethylene glycol

Aqueous polyethylene glycol causes precipitation of serum proteins out of solution in the middle pH range (4–5 for serum albumin; 4–9 for γ-globulin and whole serum). We investigated the possibility that native and iodinated insulin might co-precipitate with serum proteins to a different degree (Fig. 5). At concentrations below 0.1 μg/ml, unlabelled insulin does not co-precipitate within the entire range of pH tested (Fig. 5). By contrast, a marked co-precipitation of iodinated insulin occurs at pH values below 7, with a maximum effect at pH 4–5. About 50% of lightly iodinated insulin co-precipitates with serum proteins at pH 4; more extensive iodination increases the fraction of hormone which co-precipitates at this pH up to 95%. Polyethylene glycol also causes iodinated glucagon and parathyroid hormone to co-precipitate with serum proteins at the middle pH range, whereas unlabelled hormones remain fully soluble at these pH values (not shown). Thus co-precipitation of iodinated hormones with serum proteins accounts, at least in part, for the effects of these proteins on the distribution of the hormones in the dextran–polyethylene glycol system.

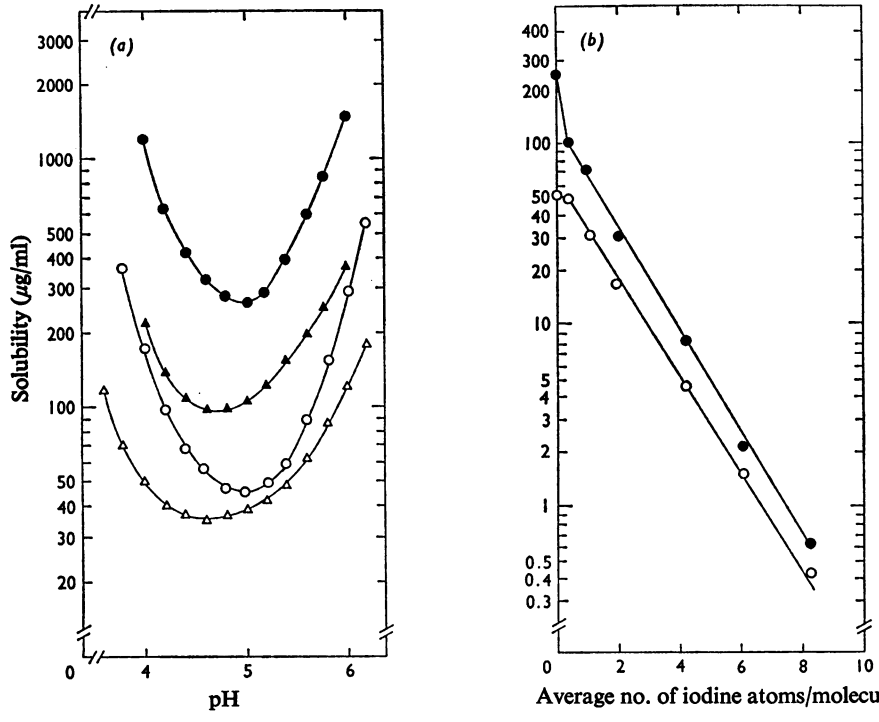

Fig. 3. *Effect of iodine content on the solubility of native and iodinated insulin in aqueous polyethylene glycol and in water*

(*a*) Solubility of native and partially iodinated insulin as a function of pH. Insulin was iodinated to an average iodine content of 0.9 atom per molecule by method A described in the Experimental section. Unlabelled (●, ○) or iodinated (▲, △) insulin was diluted into 0.02M-citrate–phosphate buffers of various pH values with (○, △) and without (●, ▲) 12.5% (w/w) polyethylene glycol. (*b*) Solubility of iodinated insulin at pH5 as a function of iodine content. Insulin was labelled to various iodine contents by method A described in the Experimental section. Unlabelled and iodinated insulin were diluted into 0.02M-citrate–phosphate buffer, pH5.0, with (○) and without (●) 12.5% (w/w) polyethylene glycol.

Table 1. *Partition coefficient of iodinated glucagon in the dextran–polyethylene glycol system as a function of iodine content*

Glucagon was labelled with various amounts of iodine according to method A described in the Experimental section. Distribution experiments were performed under conditions similar to those used with insulin, as described in the legend to Fig. 1. The values for the partition coefficient are the means of duplicate determinations.

Average no. of iodine atoms/ molecule of glucagon	Partition coefficient		
	pH5	pH2	pH10
0	1.15	2.65	3.00
0.7	0.72	2.68	3.05
1.0	0.45	3.75	3.30
2.1	0.24	4.70	3.30
2.9	0.17	4.90	3.42
3.6	0.10	5.00	4.40

Discussion

The experiments described in the present paper show that iodination of insulin and glucagon markedly alters the distribution of these hormones in the two-phase aqueous dextran–polyethylene glycol system and also decreases their solubility in aqueous polyethylene glycol and in water. These changes are modest for hormones labelled at an average of less than one atom of iodine per molecule, but become more pronounced as the iodine content is raised. Since the preparations of iodinated hormones used in this study were mixtures of populations of molecules containing various numbers of iodine atoms per molecule the true distribution and solubility properties of each individual population cannot be ascertained. However, results obtained with preparations of partially iodinated insulin, where the iodinated species is predominantly mono-

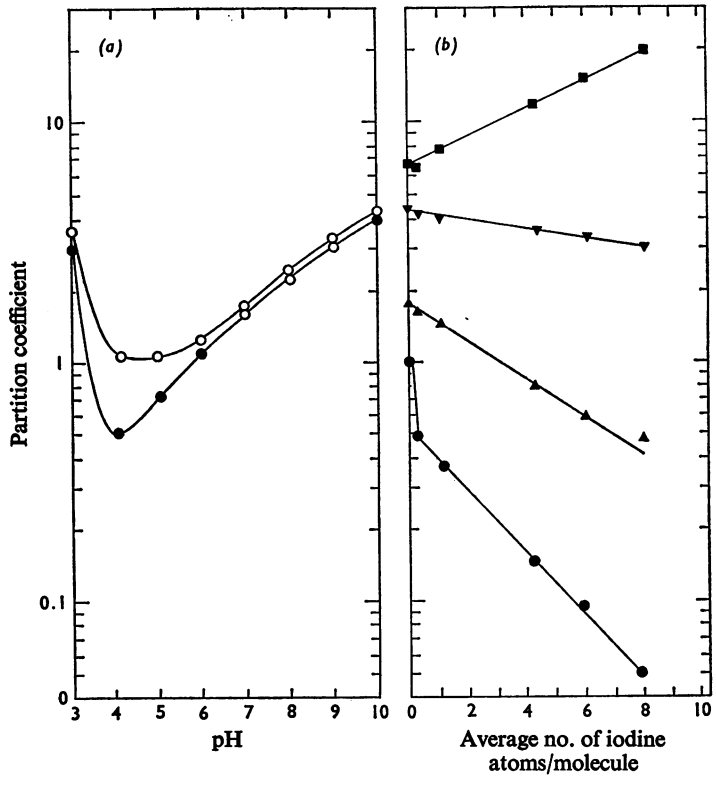

Fig. 4. *Effect of iodine content on the distribution of iodinated insulin in the dextran–polyethylene glycol system in buffers containing serum albumin*

Insulin was labelled to various degrees by method B described in the Experimental section. Buffers were as indicated in the legend to Fig. 1 and contained 2.5mg of bovine serum albumin/ml. Insulin concentration was 0.01–0.1 μg/ml. (*a*) Partition coefficient of native insulin (○) or insulin labelled at an average of 0.1 atom of iodine per molecule (●) shown as a function of pH. (*b*) Partition coefficient of iodinated insulin at pH3 (■), pH5 (●), pH7 (▲) and pH9 (▼) shown as a function of iodine content.

iodoinsulin, would suggest that the latter already shows slight, but distinctly decreased, solubility in polyethylene glycol and in water, relative to native insulin. The lower solubility of partially iodinated insulin in aqueous polyethylene glycol is more apparent if serum proteins are present.

Although many proteins and polypeptides have been iodinated and used as analytical tools, only proteins of high molecular weight such as albumin (Perlman & Edelhoch, 1967), γ-globulin (Edelhoch & Schlaff, 1963), carboxypeptidase (Simpson & Vallee, 1966) and thyroglobulin (Edelhoch & Lippoldt, 1962) have been extensively characterized in terms of their physical properties. It has been shown that iodination increases the electrophoretic mobility of insulin (Berson & Yalow, 1966) and the sedimentation velocity of serum albumin (Perlman & Edelhoch, 1967). The changes in these physical properties, which correlate well with the degree of iodination, appear to reflect increased negative charge and increased mass and density respectively. In addition, a high degree of iodination may result in conformational changes, as suggested by modifications of parameters including viscosity, optical rotation, solubility and biological activity. It is unknown which mechanism(s) account(s) for the changes in the distribution and solubility properties of the hormones caused by iodination. The shift towards the lower values of the pH of minimum partition coefficient and solubility probably reflects a decrease in the isoelectric point; the decrease in partition coefficient and solubility may result from structural changes. It is of interest that extensive iodination of insulin and glucagon, which results in a loss of the biological activity of the former (reviewed by Randle, 1964), but not of the latter (Bromer *et al.*, 1973), causes similar changes in distribution and solubility properties of both hormones. This finding

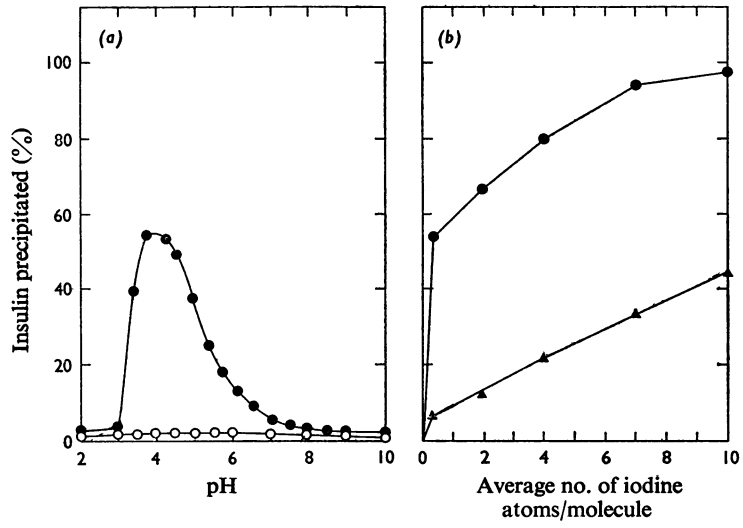

Fig. 5. *Effect of iodine content on the solubility of iodinated insulin in aqueous polyethylene glycol in buffers containing serum proteins*

Insulin was labelled with various amounts of iodine by method B described in the Experimental section. Buffers were 0.02M-sodium citrate (pH3–6), 0.02M-sodium phosphate (pH6–8) and 0.02M-sodium barbital (pH8–10), and contained human plasma, 1:10 (v/v) final concentration ratio. Concentration of insulin was 0.01–0.1 μg/ml. (*a*) Solubility of native insulin (○) or insulin labelled at an average of 0.1 atom of iodine per molecule (●) shown as a function of pH. (*b*) Solubility of iodinated insulin at pH4 (●) and pH7 (▲) shown as a function of iodine content.

would suggest that such changes probably do not result from a loss of the native configuration of hormone. Another factor which might account for the changes in distribution and solubility is the increased hydrophobicity of the hormone molecules caused by the substitution of iodine into the tyrosine residues.

Although a number of proteins have been characterized in aqueous two-phase polymer systems (Albertsson, 1970), few studies have been performed with low-molecular-weight and relatively structureless proteins such as polypeptide hormones. The results of this study, as well as those reported by Desbuquois & Aurbach (1972), show that the influence of pH, buffers and salts on the partition behaviour of peptide hormones is qualitatively similar to that observed with other proteins. A number of mechanisms have been proposed to explain the dependence of the partition coefficient of proteins on ions and pH. In particular, it has been suggested that the effects of pH on distribution might result from conformational changes of the proteins (Albertsson, 1970). The finding that peptide hormones such as glucagon, ACTH and parathyroid hormone, whose structures are not affected by pH in dilute aqueous solution (Edelhoch & Lippoldt, 1969), nevertheless show partition characteristics that are pH-dependent does not support this contention.

Several investigators have shown that non-ionic water-soluble polymers, such as dextran and polyethylene glycol, precipitate proteins from solution (reviewed by Fried & Chun, 1971). However, little attention has been paid to the relationships between the distribution of proteins in aqueous two-phase polymer systems and their solubility in the phase components of such systems. In studies with albumin and caeruloplasmin, Albertsson (1971) found a correlation between their distribution in dextran–polyethylene glycol and their solubility in the polyethylene glycol-rich phase. The results of the present study clearly indicate that the apparent fall in the partition coefficient of insulin caused by increasing degrees of iodination, at least at high hormone concentrations, results from a decrease in the solubility of insulin in the upper phase. Likewise, co-precipitation of iodinated insulin with serum proteins accounts for the effects of these proteins on the partition coefficient of the hormone in the dextran–polyethylene glycol system. These results suggest that in certain circumstances, precipitation of a solute by polyethylene glycol may account for the low apparent partition coefficient of this solute in the dextran–polyethylene glycol system.

In most studies on the distribution of proteins in aqueous two-phase polymer systems, relatively high

concentrations of proteins were used and the protein concentration in each phase was determined by using spectroscopic or colorimetric methods. These methods are of limited sensitivity and are not free from interference by the polymers. In addition, many proteins show a low solubility in the dextran–polyethylene glycol system in the middle pH range. The results of the present study suggest that the use of partially iodinated and adequately purified peptide or protein derivatives may be advantageous in partition studies, provided that these derivatives retain the properties of the native proteins. The use of such derivatives allows the distribution experiments to be performed at concentrations within the range 0.1 μM–10 pM. This feature is of interest in the study of biological materials that are available in limited quantities and/or show a low solubility in the two-phase system. Further, since specific and sensitive arrays are available for most biologically important macromolecules, it may be possible to use these assays to determine minute concentrations of such macromolecules in the two-phase system without purification from extracts or biological fluids.

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References

Albertsson, P. A. (1970) *Advan. Protein Chem.* **24**, 309–341

Albertsson, P. A. (1971) *Partition of Cell Particles and Macromolecules*, John Wiley and Sons, New York

Berson, S. A. & Yalow, R. S. (1966) *Science* **152**, 205–207

Bromer, W. W., Boucher, M. E. & Patterson, J. M. (1973) *Biochem. Biophys. Res. Commun.* **53**, 134–139

Desbuquois, B. & Aurbach, G. D. (1971) *J. Clin. Endocrinol.* **33**, 732–738

Desbuquois, B. & Aurbach, G. D. (1972) *Biochem. J.* **126**, 717–726

Edelhoch, H. & Lippoldt, R. E. (1962) *J. Biol. Chem.* **237**, 2788–2794

Edelhoch, H. & Lippoldt, R. E. (1969) *J. Biol. Chem.* **244**, 3876–3883

Edelhoch, H. & Schlaff, S. (1963) *J. Biol. Chem.* **238**, 244–250

Fried, M. & Chun, P. W. (1971) *Methods Enzymol.* **12**, 238–248

Herbert, V., Lau, K. S., Gottlieb, C. W. & Bleicher, S. J. (1965) *J. Clin. Endocrinol.* **25**, 1375–1384

Hunter, W.M. & Greenwood, F. C. (1962) *Nature (London)* **194**, 495–496

Juckes, I. R. M. (1971) *Biochim. Biophys. Acta* **229**, 535–546

Perlman, R. L. & Edelhoch, H. (1967) *J. Biol. Chem.* **242**, 2416–2422

Randle, P.J. (1964) in *The Hormones* (Pincus, G., Thimann, K. V. & Astwood, E. B., eds.), vol. 4, pp. 481–530, Academic Press, New York

Rodbard, D., Rayford, P. L., Cooper, J. A. & Ross, G. T. (1968) *J. Clin. Endocrinol.* **28**, 1412–1418

Simpson, R. T. & Vallee, B. L. (1966) *Biochemistry* **5**, 1760–1767

Yalow, R. S. & Berson, S. A. (1964) *Methods Biochem. Anal.* **12**, 69–96