Covalent cross-linking of the bovine somatotropin dimer

Effects on growth-promoting, receptor-binding and immunological activities and preliminary characterization of the self-association

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Bovine somatotropin, at pH 8.5 in 0.02 M-Bicine [*NN*-bis-(2-hydroxyethyl)glycine]/0.09 M-NaCl, showed by frontal analysis the characteristics of a rapid monomer–dimer equilibrium whose dissociation constant was estimated to be 6.6×10^{-6} M. Reaction of the hormone with dimethyl suberimidate lead to covalent cross-linking of the dimeric species. Under the conditions chosen (0.4 mg of bifunctional imidate and 1 mg of protein/ml at room temperature for 1 h) the cross-linked dimers accounted for 26% of the total protein, and these were isolated by molecular sieving in 0.29 M-NH₃/0.12 M-NaCl. Covalent stabilization greatly diminished the growth-promoting activity and the ability to interact with somatogenic sites in both rat liver *in vivo* and rabbit liver microsomal fractions. Evidence indicating a non-critical role for amino groups involved in the covalent cross-linking was provided by a nearly equivalent derivative obtained after reaction with 3,3'-dithiobispropionimidate, which had substantial hormonal activity upon cleavage of the disulphide links. Conversely, immunological reactivity as demonstrated by radioimmunoassay was not affected by cross-linking. Details of the least-squares procedure employed to evaluate the self-association equilibrium constant has been deposited as Supplement SUP 50115 (7 pages) with the British Library Lending Division, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K., from whom copies may be obtained on the terms indicated in *Biochem. J.* (1981) 193, 5.

Circular-dichroism studies showed that the fragment bovine somatotropin-(96–133)-peptide and its complementary peptides [(1–95) and (134–191), connected via a disulphide linkage] undergo conformational transitions (Chen-jung & Sonenberg, 1977) as they are isolated. A similar observation was made for the fragments human somatotropin-(1–134)- and -(141–191)-peptide, the former being associated with some somatogenic activity (Li & Graf, 1974). Recovery of full activity followed the recombination of these later fragments into an

equimolar complex having an overall conformation resembling that of the parent hormone (Li & Bewley, 1976). It is apparent from these experiments that biological activity depends upon the three-dimensional organization of the polypeptide chain, and it may be assumed that an active area is located on the surface of the molecule with a conformation determined by the native folding. By using the particular characteristic of the bovine hormone to form dimers, the aim of the present study was to examine the relationship between the biological activity and the area involved in the interaction. The present paper describes the production of a stable dimer of bovine somatotropin by covalent cross-linking and the measurement of its growth-promoting activity, binding capacity to specific receptors and immunological properties.

Materials and methods

Bovine and human somatotropins were prepared as described by Dellacha & Sonenberg (1964) and Mills *et al.* (1969) respectively. Horse heart cyto-

Abbreviations used: DMS-bovine somatotropin, bovine somatotropin modified by dimethyl suberimidate without forming covalent links with other(s) somatotropin molecule(s); DTBP-bovine somatotropin, bovine somatotropin that failed to originate covalently stabilized oligomers isolated from the reaction mixture of the hormone and dimethyl 3,3'-dithiobispropionimidate; SDS, sodium dodecyl sulphate; R_e , radius of the equivalent hydrodynamic sphere (Stokes radius); Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; Bicine, *NN*-bis-(2-hydroxyethyl)glycine; Caps, 3-(cyclohexylamino)propanesulphonic acid.

chrome *c* was purchased from Nutritional Biochemicals Co., Cleveland, OH, U.S.A.; thyroglobulin, soya-bean trypsin inhibitor, hen's-egg albumin, Sephadex G-100 and G-100 Superfine grade (10/40 μ m dry bead diameter) and dimethyl-3,3'-dithiobispropionimide dihydrochloride were obtained through Sigma Chemical Co., St. Louis, MO, U.S.A., and bovine serum albumin (Pentex fraction 5) was from Miles Laboratories, Elkhart, IN, U.S.A. Dimethyl suberimide dihydrochloride was supplied by Pierce, Rockford, IL, U.S.A. Na¹²⁵I (17 Ci·mol⁻¹) was provided by New England Nuclear Corp., Chicago, IL, U.S.A. Other chemicals were of analytical-reagent quality.

Reaction of bovine somatotropin with bifunctional imidates and isolation of the cross-linked dimers

The protein was dissolved in water at a concentration of 2 mg/ml, adjusted to pH 11 with 0.1 M-NaOH and diluted with an equal volume of 0.2 M-Bicine, pH 8.5. Dimethyl suberimide or dimethyl 3,3'-dithiobispropionimide were dissolved in buffer just before use and added to the protein solution to give a final concentration of 0.4 mg/ml; then the mixture was left for 1 h at room temperature. Reaction was terminated by the addition of a 10-fold molar excess of glycine with respect to the cross-linking agent. The reaction products were dialysed against water at 2–4°C and freeze-dried. Dissociation of non-covalently linked dimers was assured by dissolving the modified hormone in ice-cold 0.29 M-NH₃/0.12 M-NaCl in the case of dimethyl suberimide derivatives or in 0.1 M-glycine/HCl, pH 3.6, for the dimethyl 3,3'-dithiobispropionimide products, then the mixtures were fractionated by gel filtration on Sephadex G-100 at 2–4°C using the same buffers. The selected fractions were recovered after dialysis against cold water and freeze-drying.

Reduction of dimethyl 3,3'-dithiobispropionimide derivatives

Disulphide links of these were cleaved under the conditions described by Gráf *et al.* (1975) with a 25-fold molar excess of dithiothreitol (with respect to hormone), followed by alkylation with a 1.2-fold molar excess of iodoacetamide over dithiothreitol.

Evaluation of the growth-promoting, receptor-binding and immunological activities

Somatotropic activity was measured by the weight-gain assay of hypophysectomized rats, as described by Marx *et al.* (1942) and as utilized by Cascone *et al.* (1980). The specific uptake of the hormone or its derivatives *in vivo* by the rat liver was determined by the method of Turyn & Dellacha (1978). Radioiodination of bovine somatotropin and

its covalent dimer was performed by the method of Roth (1975) as described by Mattera *et al.* (1981). Specific radioactivities of 60–80 μ Ci/ μ g and 8–24 μ Ci/ μ g were obtained for ¹²⁵I-labelled bovine somatotropin and ¹²⁵I-labelled bovine somatotropin cross-linked dimer respectively. Receptor-binding properties were tested in a crude microsomal suspension [sedimenting between 15000 g (r_{av} , 7.5 cm) and 100000 g (r_{av} , 5.9 cm)] from the liver of adult virgin female New Zealand rabbits prepared and assayed as described by Posner *et al.* (1974). Immunological activity was evaluated by displacement radioimmunoassay (Peña *et al.*, 1980). Estimates of relative potency were obtained from statistical analysis as applied to parallel line assays (Finney, 1964) unless otherwise indicated.

Analytical procedures

Analysis by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was carried out in 5 mm-internal-diameter glass columns as described by Weber & Osborn (1969), with a 6% (w/v) final polyacrylamide concentration in the polymerizing mixture. Protein samples were reduced and alkylated with iodoacetamide (Crestfield *et al.*, 1963).

Amino acid analyses of samples hydrolysed in constant-boiling HCl/phenol (1 mg/ml) *in vacuo*, at 110°C for 16 h, were performed on a Technicon TSM 2 apparatus. The total number of lysine residues modified by the imido ester in the protein derivatives was determined from their lysine content. Since amidine bonds are partially hydrolysed in 6 M-HCl at 110°C (Wang *et al.*, 1976), estimates of the amino acid content obtained after 12, 16, 20, 24 and 30 h of hydrolysis were extrapolated to zero time. The content of N^εN^{ε'}-suberimidinobislysine was measured for portions of the hydrolysate applied to the short column of the amino acid analyser (Hartman & Wold, 1966) after acid hydrolysis for 12 h.

Thin-layer gel-filtration experiments were performed with the following buffers: Hepes (pH 7.5), Bicine (pH 8.5–9.3) and Caps (pH 10.0–11.5), all 0.02 M, plus 0.1 M-NaCl, and 0.1 M-glycine/HCl, pH 3.6 (Dellacha *et al.*, 1968), with or without 0.1 M-NaCl. Chromatography was carried out in a Pharmacia TLG apparatus at room temperature on a 0.6 mm layer of Sephadex G-100 (Superfine grade). After application of 10 μ l samples (protein samples at 2 mg/ml, alanine 0.2–1 mg/ml) development took place with a slope of 15°. The chromatographed substances were transferred to a sheet of Whatman 3 MM paper applied on the gel bed surface for 1 min (paper replica). Once dry, replicas were successively sprayed with ninhydrin 0.1% (w/v) in acetone and heated at 100°C in order to localize the alanine spots, and stained with 0.01% (w/v) Nigrosin in methanol/water/acetic acid

(5:4:1, by vol.). The partition coefficients (σ) of the proteins were derived from the migration distances (d) measured on the replicas and the relationship (Ackers, 1975):

$$\sigma = \frac{(1/d) - (1/d_0)}{(1/d_i) - (1/d_0)}$$

d_i and d_0 being the migration distances of alanine and thyroglobulin. Correlation between Stokes radii (R_e) and the partition coefficients was effected by interpolation in a curve (Porath, 1963):

$$\sigma^{\dagger} = c - c'R_e$$

constructed with values obtained at pH 7.5 for the standard proteins. In turn, R_e values for these were inferred from their reported diffusion coefficients ($D_{20,w}$) by the Einstein relationship (Gosting, 1956):

$$R_e = kT/6\pi\eta D_{20,w}$$

where k is the Boltzman constant and η the solvent viscosity [estimated in 1 cP (1 mPa·S)]. Stokes radii (indicated in parentheses) were calculated from the $D_{20,w}$ values: cytochrome c [1.65 nm (16.5 Å)], $13 \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$ (Atlas & Farber, 1956); soya-bean trypsin inhibitor [2.26 nm (22.6 Å)], $9.4 \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$ (Laurent & Killander, 1964); ovalbumin [2.75 nm (27.5 Å)], $7.8 \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$ (Lamm & Polson, cited in Sober, 1970); and bovine serum albumin [3.49 nm (34.9 Å)], $6.15 \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$ (Creeth, 1952). The linear-regression estimates for the parameters c and c' were 1.145 (dimensionless) and 0.1834 nm^{-1} respectively.

Column gel filtration was performed in a silicone-treated glass chromatographic column (Wright; 1.6 cm $\times$ 60 cm; thermostatically maintained at 25°C) packed up to a bed height of 50 cm with Sephadex G-100 (Superfine grade) suspended in 0.02 M-Bicine/0.09 M-NaCl/0.02% NaN_3 , pH 8.5. Samples of native bovine somatotropin were prepared by dissolving the freeze-dried protein (2–10 mg/ml) in water, then the solutions were adjusted to pH 11 by addition of 0.1 M-NaOH and diluted with buffer to the desired concentration, the eventual insoluble material being removed by centrifugation (15000 g, r_{av} , 10.5 cm, for 15 min). The volume was large enough to ensure a plateau in the elution diagram (Ackers & Thompson, 1965), usually from 16 to 20 ml. A 40 μl portion of an alanine solution (20 $\mu\text{mol}/\text{ml}$) was also applied, serving as an internal reference of the chromatographic volumes. Fractions (approx. 1.6 ml) were collected and the volume of effluent was established from the cumulative weight of the fractions. The protein concentration was evaluated from absorbance measurements at 280 nm, using an $A_{1\text{cm}}^{0.1\%}$ of 0.67 to effect the conversion. The distribution of alanine was monitored by fluorescence measurements

on 100 μl portions after reaction with *o*-phthalaldehyde (Mendez & Gavilanes, 1976).

The weight-average distribution coefficient ($\bar{\sigma}_w$) can be evaluated, according to Ackers & Thompson (1965), from the equation:

$$\bar{\sigma}_w = (\bar{V} - V_0)/V_i$$

The internal volume, V_i , was determined in calibration runs from the elution volume of alanine (V_{Ala}) and thyroglobulin (V_{Thy}) as

$$V_i = V_{\text{Ala}} - V_{\text{Thy}}$$

The exclusion volume, V_0 , for each individual frontal-analysis experiment was indirectly estimated from the corresponding elution volume of alanine according to the equation:

$$V_0 = V_{\text{Ala}} - V_i$$

The position of the equivalent sharp boundary of the leading edge (centroid volume, $\bar{V}$), for each of the bovine somatotropin samples, was graphically appraised from the elution diagrams (Ackers, 1975).

The value of the dissociation constant, K , was estimated from the data set $\bar{\sigma}_w$ versus m_0 by a least-squares method based on the following equations (Ackers & Thompson, 1965):

$$\alpha = \frac{\bar{\sigma}_w - \sigma_d}{\sigma_m - \sigma_d}$$

and

$$K = \frac{2\alpha^2 m_0}{(1 - \alpha)}$$

where α is the weight fraction of the monomeric species, m_0 is the protein concentration expressed as mol of monomer per litre, and σ_m and σ_d are the monomer and dimer distribution coefficients. The method (detailed in Supplement SUP 50115) requires solution of the following equation:

$$0 = \frac{\Sigma \bar{\sigma}_w \frac{\partial \alpha_i}{\partial K} - \sigma_d \Sigma \frac{\partial \alpha_i}{\partial K}}{\Sigma \alpha_i \frac{\partial \alpha_i}{\partial K}} - \frac{\Sigma \bar{\sigma}_w \alpha_i - \sigma_d \Sigma \alpha_i}{\Sigma \alpha_i^2}$$

where

$$\alpha_i = \frac{-K + \sqrt{K^2 + 8Km_{0i}}}{4m_{0i}}$$

and

$$\frac{\partial \alpha_i}{\partial K} = \frac{K + 4m_{0i} - \sqrt{K^2 + 8Km_{0i}}}{4m_{0i} \sqrt{K^2 + 8Km_{0i}}}$$

for the root K , allowing the estimation of the parameter without the previous knowledge of σ_m .

Fig. 1. Isolation of bovine somatotropin cross-linked dimer

The chromatographic column (2.5 cm $\times$ 90 cm), packed with Sephadex G-100, was eluted with 0.23 M-NH₃/0.12 M-NaCl at a flow rate of 4.5 ml/h per cm². The fraction volume was 6.5 ml and the temperature 2–4°C. Horizontal bars indicate the pooled fractions. The inset shows SDS/polyacrylamide-gel electrophoresis of materials recovered from pools I and II (staining was with Amido Schwarz).

Table 1. *Body-weight-increase bioassay of covalently cross-linked bovine somatotropin, modified hormone and derivatives*
Long-Evans strain rats of 110–130 g, hypophysectomized 5 weeks in advance, were injected intraperitoneally twice a day during 5 successive days and the weight gain was recorded at the end of this period (Cascone *et al.*, 1980). Growth-promoting activity estimates are expressed as relative potency referred to the native hormone. The 90% confidence limits are given in parentheses. Weight-increase values are means $\pm$ s.d. obtained for the number of animals indicated in parentheses.

	Total dose (μ g)	Body-weight increase (g)	Growth-promoting activity
Native bovine somatotropin	15	2.7 $\pm$ 1.2 (6)	1.0
	120	6.7 $\pm$ 1.2 (6)	
Bovine somatotropin dimer cross-linked with dimethyl suberimide	200	2.9 $\pm$ 1.7 (6)	0.06 (0.02–0.12)
	1600	5.5 $\pm$ 2.2 (6)	
Untreated control	—	–0.4 $\pm$ 1.7 (12)	
Native bovine somatotropin	137	9.8 $\pm$ 2.4 (5)	1.0
	1030	14.6 $\pm$ 2.9 (5)	
DMS-bovine somatotropin	162	11.0 $\pm$ 2.7 (6)	1.4 (0.6–4.0)
	1100	15.8 $\pm$ 2.7 (6)	
Native bovine somatotropin	80	6.8 $\pm$ 1.7 (7)	1.0
	600	11.1 $\pm$ 3.1 (7)	
Bovine somatotropin dimer cross-linked with dimethyl 3,3'-dithiobispropionimide reduced and carboxamidomethylated	80	6.4 $\pm$ 1.5 (6)	1.1 (0.6–2.4)
	600	12.1 $\pm$ 3.2 (7)	
DTBP-bovine somatotropin, reduced and carboxamidomethylated	80	7.0 $\pm$ 1.3 (6)	1.0 (0.4–2.1)
	550	10.6 $\pm$ 2.2 (5)	

Results

Reaction of bovine somatotropin with dimethyl suberimide

Under the standard cross-linking conditions (pH 8.5 and concentrations of 1 mg/ml for the hormone and 0.4 mg/ml for the bifunctional imido-ester), 26% of the protein gave rise to covalent dimers, as estimated after fractionation by molecular sieving (Fig. 1). Analysis of the dimers recovered from pool I (Fig. 1) indicated the formation of 0.4 mol of $N^\epsilon N'^\epsilon$ -suberimidinobislysine/mol of protomer and thereby the presence of a single covalent bridge linking two lysine residues per cross-linked dimer. The total number of modified lysine residues was estimated as 3.3, a similar value being found for the material contained in pool II (Fig. 1), hereafter referred to as 'DMS-bovine somatotropin'.

Growth-promoting activity, binding properties and immunological activity of cross-linked bovine somatotropin dimers and derivatives

The covalent stabilization of the somatotropin dimers with dimethyl suberimide substantially reduced the growth-promoting activity of the hormone (Table 1). DMS-bovine somatotropin, which had a similar number of lysine residues affected by the reaction, remained fully active. More specific evidence indicating a non-critical role for the groups involved in the covalent stabilization of the

dimer was provided by a nearly equivalent derivative, obtained after cross-linking with dimethyl 3,3'-dithiobispropionimidate. This material had demonstrable hormonal activity upon cleavage of the dithiobispropionimidine disulphide links by reduction and blockage with iodoacetamide despite the fact that the amidine groups are not affected by the treatment. Selective modification of the C-terminal disulphide bond in bovine somatotropin is

Fig. 2. Radioreceptor assay of bovine somatotropin (●), cross-linked dimer (□) and DMS-bovine somatotropin (○)

125 I-labelled bovine somatotropin (a, 525 fmol; b, 540 fmol) was incubated for 15 h at 25°C with 1.1 mg of microsomal protein in the presence of the amounts of hormonal protein indicated on the abscissa. B/B_0 is radioactivity bound expressed as a fraction of the radioactivity bound in the absence of unlabelled hormone (B_0), both radioactivity measurements having been previously corrected for non-specific binding. This was evaluated from incubations containing an excess (1 μ g) of unlabelled hormone. For (a) B_0 was 11% of the total radioactivity offered; in (b), B_0 = 14%. Determinations were carried out in triplicate. The points correspond to mean values and the vertical bars to the s.d. Efficiency in displacing the radioligand, relative to that of the native hormone, was 0.26 (0.21–0.31) for the covalent dimer and 1.43 (1.06–2.01) for DMS-bovine somatotropin (95% confidence limits are given in parentheses).

Table 2. Rat liver uptake *in vivo* of native bovine somatotropin and its covalently stabilized dimer

The assays were carried out by the method of Turyn & Dellacha (1978) by injecting to rats (Long-Evans strain, 90–100 g) either 125 I-labelled somatotropin (24 ng plus, unless otherwise indicated, 100 ng of carrier somatotropin) or 125 I-labelled somatotropin dimer (110 ng), alone or together with unlabelled hormonal protein. Results are expressed as the ratio between the radioactivity per unit of mass found in liver and serum; they are presented as means $\pm$ s.d. Non-specific uptake of the radioligand was evaluated after the administration of an excess (80 or 50 μ g) of unlabelled bovine somatotropin.

Hormonal protein	Liver/serum
125 I-labelled bovine somatotropin	2.6 ± 0.4 (4)
125 I-labelled bovine somatotropin plus 80 μ g of bovine somatotropin	0.9 ± 0.3 (4)
125 I-labelled bovine somatotropin plus 50 μ g of cross-linked dimer	1.9 ± 0.4 (4)
125 I-labelled bovine somatotropin plus 5 μ g of bovine somatotropin	1.3 ± 0.3 (4)
125 I-labelled bovine somatotropin cross-linked dimer	1.0 ± 0.0 (3)
125 I-labelled bovine somatotropin cross-linked dimer plus 50 μ g of bovine somatotropin	1.3 ± 0.2 (3)

reported to occur under similar experimental conditions, but this modification does not seriously perturb its biological potency (Gráf *et al.*, 1975). In the rat-liver-uptake assay a radioiodinated preparation of stabilized dimer was unable to become specifically bound (Table 2). This dimer also failed to substantially displace ^{125}I -labelled bovine somatotropin, even when used at doses 10-fold higher than those of native hormone that effectively compete with the tracer. A qualitatively similar behaviour was observed with liver microsomal preparations, when displacements of radiolabelled somatotropin by the dimer took place over a range of concentrations 4-fold higher than for the native hormone (Fig. 2). The immunological reactivity toward antibodies raised against the native protein was not perturbed by covalent cross-linking, as demonstrated by the near equivalence between the hormone and the covalent dimer in radioimmunoassays (Fig. 3).

pH-dependence, stoichiometry and equilibrium constant of bovine somatotropin self-association

The pH-dependent changes on Stokes radius (R_s) observed by thin-layer gel filtration for bovine serum albumin and ovalbumin, included for comparison in Fig. 4, closely parallel those obtained by column gel filtration reported by Martenson (1978). Both proteins evidenced an increase in their estimated R_s values above pH 10 (cf. Martenson, 1978), and in a fundamentally similar fashion also behaved human somatotropin, known to exist in solution as free monomers (Li, 1968), and cytochrome *c*, though the latter to a somewhat lower extent. The covalent dimer did not differ from the other proteins, increasing by 0.38 nm (3.8 Å) its apparent Stokes radius [from 2.76 nm (27.6 Å) to 3.14 nm (31.4 Å)] in the transit from pH 9.3 to 11.5 as compared with increments of 0.59 nm (5.9 Å), 0.58 nm (5.8 Å) and 0.42 nm (4.2 Å) obtained for serum albumin, ovalbumin and human somatotropin respectively. Con-

Fig. 3. Radioimmunoassay of bovine somatotropin (●), its covalently cross-linked dimer (□) and DMS-bovine somatotropin (○)

Each curve depicts competition between ^{125}I -labelled bovine somatotropin (0.5 ng) and the unlabelled protein for rabbit antibodies raised against bovine somatotropin (final serum dilution 1:6000). Maximal ($B_0 + B_1$) and non-specific (B_1) binding were 46 and 17% of the total radioactivity added. Individual values of B were computed, B_1 being subtracted from the radioactivity bound. Relative antigenic efficiencies (R) were estimated from the median effective doses (ED_{50}) (bovine somatotropin, $\text{ED}_{50} = 0.66$ ng; cross-linked dimer, $R = 1.17$, $\text{ED}_{50} = 0.59$ ng; DMS-bovine somatotropin, $R = 0.95$, $\text{ED}_{50} = 0.68$ ng) obtained from plots (inset) of $\text{logit}(B/B_0)$ versus $\text{log}(\text{antigen added})$ (Rodbard & Frazier, 1975).

Fig. 4. Variation with pH of the apparent Stokes radius for bovine somatotropin (●), bovine somatotropin cross-linked dimer (□) and the marker proteins (○): bovine serum albumin (1), ovalbumin (2), human somatotropin (3) and cytochrome c (4)

R_e values were estimated by thin-layer gel filtration at room temperature and approximately constant ionic strength (0.1 M-NaCl plus 0.02 M-buffer). Protein samples were applied at a concentration of 2 mg/ml.

versely, the variation of the native hormone's apparent R_e was clearly unlike the behaviour shown by the other proteins, as could be expected for a self-associating system. Dissociation would occur between pH about 10 and 11.5.

At pH 3.6 and I 0.01, the native hormone is completely dissociated (Dellacha *et al.*, 1968), a value of 2.09 nm (20.9 Å) being obtained for the Stokes radius of this monomeric species; however, it aggregated when the I was increased to 0.11. This result raises a significant differential behaviour between the hormone and the covalent dimer, which readily migrated as a particle of R_e 2.73 nm (27.3 Å) under similar conditions. The addition of native hormone (1 mg/ml) to samples of the cross-linked dimer (1 mg/ml) did not modify the migration of the latter.

A unique asymmetric absorbance peak was observed in the elution diagram when the hormone was subjected to zonal chromatography at pH 8.5. Accordingly, a single concentration plateau, delimited by sharp leading and diffuse trailing boundaries, was obtained after the application of sufficiently large sample volumes. A representative elution profile is shown in Fig. 5. This behaviour, along with a decrease of the centroid volume ($\bar{V}$) caused by an increase of the protein concentration (as can be inferred from the variation of the weight-average distribution coefficient, $\bar{\sigma}_w$, shown in

Fig. 5. Elution profile of a large-zone experiment on Sephadex G-100 for a solution (130 µg/ml) of native bovine somatotropin

The column (1.6 cm × 50 cm), thermostatically maintained at 25°C, was eluted with 0.02 M-Bicine plus 0.09 M-NaCl, pH 8.5, at a flow rate of 4 ml/h per cm². The inset shows the first derivative of the trailing boundary.

Table 1 of Supplement SUP 50115), are characteristics of rapidly polymerizing systems (Ackers & Thompson, 1965). That at pH 8.5 and $I 0.1$ the equilibrium takes place between monomeric and dimeric species was indicated by the monomodal trailing edge shown by the elution diagrams of plateau experiments (Fig. 5). The monomodal character of the boundary is more clearly evidenced by the absence of minimum in the $\partial A/\partial V$ versus volume plot presented in the inset to Fig. 5. The dissociation constant was estimated as $K = 6.6$ $(4.6-8.6) \times 10^{-6} M$, the values in parentheses indicating the 95% confidence limits.

Discussion

On the basis of the molecular-weight values obtained for bovine somatotropin in nearly neutral and either in acidic or strongly alkaline solutions, Dellacha *et al.* (1968) and Edelhoch *et al.* (1966) have shown that the purified protein associates. Under slightly alkaline conditions the hormone forms dimers (Bewley & Li, 1972), which, as it is shown here, are susceptible to becoming covalently stabilized by reaction with dimethyl suberimidate. The existence of dimers under physiological conditions is, however, unlikely, considering the value of the estimated equilibrium constant and the low circulating concentrations at which somatotropins are usually found in mammalian species (García & Geschwind, 1968; Machlin *et al.*, 1968). In fact one can speculate that the monomer is the biologically relevant form, as indicated by the impaired ability of the dimer covalently stabilized to promote increase in body weight. On this later fact rests the main interest for the dimeric particle, since abrogation of the biological potency after cross-linking could be interpreted as being due to eclipsing by the intermolecular contact of an area implicated in the hormonal expression. In support of this hypothesis it was found that cross-linking of the hormone resulted in a substantial decrease in its ability to interact with specific sites, which in both systems tested, rat liver *in vivo* (Turyn & Dellacha, 1978) and rabbit liver microsomal fractions (Posner *et al.*, 1974), are of a somatogenic nature and hence are supposedly related to the growth-promoting activity. Modification of specific amino groups as a cause of inactivation was discarded as an explanation, since scission of the intermolecular arm established after reaction with the dithio-cleavable reagent, dimethyl 3,3'-dithiobispropionimide, resulted in the recovery of activity. The stabilization of inactive conformers as subunits of the dimeric particles is a third alternative that cannot be presently excluded on a firm basis; nevertheless, specific antibodies raised against the native hormone, known to be sensitive to disturbances of the antigen conformation (Zakin *et al.*, 1975), failed to detect differences

between the native protein and the cross-linked dimer by radioimmunoassay. Hence the contact area must be located in such a position as to leave the antigenic site(s) exposed and undisturbed.

Dellacha *et al.* (1968) described the formation of high-molecular-weight complexes by the native hormone in acidic solutions by an increase in the ionic strength. The covalent stabilization of the dimeric species modified this behaviour; the purified cross-linked dimer did not aggregate at $I 0.11$, pH 3.6, as the native protein did. This fact suggests that the areas which are forming part of the intermolecular contact are located in nearly equivalent positions at the surfaces of the respective protomers. Aggregation could be the consequence of an increase of the extension and modification or loss of the specificity of the interacting areas. The existence of a second, separated, contact area does not seem likely, considering the lack of interaction between native somatotropin and dimers, and dimers with themselves, under the conditions causing aggregation of the native hormone.

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