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Growth Hormone Covalently Bound to Sepharose or Glass. Analysis of Ligand Release Rates and Characterization of Soluble Radiolabeled Products†

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ABSTRACT: Purified bovine growth hormone labeled enzymatically with iodine-125 was covalently coupled to cyanogen bromide activated Sepharose 4B gel and to diazotized zirconia-clad glass beads. Under the conditions employed, an average of 0.8 and 7.3 mg of hormone were bound per ml of Sepharose and glass, respectively. When the conjugates were incubated in Krebs-Ringer bicarbonate buffer (pH 7.4), three separate radioactive species were detected in the incubation supernatant by chromatography on Sephadex G-75. The elution volumes of two of the species were identical with those of ^{125}I-labeled growth hormone and Na^{125}I controls, while the third component eluted as a molecule of intermediate size. The rate of release of each species from the solid matrix was linear with time over 4 days and increased with temperature from 4 to 37°. Although significantly less growth hormone was released from glass (0.14%/day) than from Sepharose (0.40%/day) at 37°, active hormone in amounts sufficient to be detectable in a biological assay was nevertheless liberated from the former after as little as 4 hr of incubation. By contrast, the rate of release of $^{125}I^-$ and the intermediate-size compound from glass was significantly greater than from Sepharose, suggesting that protein bound to glass supports is more susceptible to degradation from exposure to ionizing radiation.

The coupling of proteins and other ligands to insoluble supports has provided an important tool for the investigation of molecular interactions (Cuatrecasas, 1970). Insolubilized hormones have been particularly useful in the areas of antibody purification (Wofsy and Burr, 1969; Fellows et al., 1973; Sairam et al., 1974) and hormone receptor isolation (Sica et al., 1973; Shiu and Friesen, 1974). In addition, protein hormone conjugates have been employed in experiments designed to show that receptors for these hormones are located on the surface of the target cell (Cuatrecasas, 1969; Turkington, 1970; Selinger and Civen, 1971). Several recent reports, however, suggest that the interpretation of biological studies carried out with such conjugates is complicated by the slow release of hormones and hormonally active fragments not only from agarose derivatives (Davidson et al., 1973; Fritz, 1971) but also from glass (Yong, 1973) and polyacrylamide supports (Davidson and Van Herle, 1973). When this occurs, it becomes difficult to determine whether part or all of the biological activity of the hormone conjugate may be due to free rather than immobilized hormone.

Previously, we observed that bovine growth hormone covalently coupled to Sepharose released a component with growth hormone activity into the medium after incubation with rat epididymal fat pads (Schwartz et al., 1973). The following studies were undertaken in an attempt to define experimental conditions under which protein hormones would remain covalently bound to a solid matrix for sufficient time to permit investigation of biological activity with the conjugate. The consistent observation of hormone release from both glass and agarose supports compels a reevaluation of investigations attributing biological activity to hormone conjugates unless the amount of hormone released under the conditions employed is clearly demonstrated to be less than the threshold for the biological response.

Materials and Methods

Bovine growth hormone (NIH-BGH-B17) was kindly provided by the Hormone Distribution Program, NIAMDD. Lactoperoxidase (lot No. 300011), grade B, was obtained from Calbiochem; trypsin-TPCK[1] (lot No. 2GB), 190 U/mg, was from Worthington Biochemical Cor-

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[1] Abbreviations used are: HPP, 3-(4-hydroxyphenyl)propionate; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone.

poration; aminopropyltriethoxysilane and *p*-nitrobenzoyl chloride were from Aldrich Chemical Company; *N*-succinimidyl 3-(4-hydroxyphenyl)propionate was from Regis Chemical Company; bovine serum albumin, crystallized, was from Sigma Chemical Company; and guanidine hydrochloride was from Heico. Sepharose 4B and Sephadex G-100, G-75, and G-50 were purchased from Pharmacia Fine Chemicals; and glass beads, MZO-3900 (550 Å), were from Corning Glass Works. Sodium iodide-125, carrier-free (>14 mCi/μg of I^-), was obtained from Amersham/Searle. All other reagents were of the highest purity available from commercial sources.

Purification and Iodination of Growth Hormone. Bovine growth hormone was additionally purified by gel filtration at 4° on a column, 2.5 × 90 cm, of Sephadex G-150 equilibrated and eluted with 0.05 *M* Tris-HCl (pH 9.0) (Fellows and Rogol, 1969). The monomer was iodinated at room temperature by a modification of the method of Miyachi et al. (1972). In a typical iodination, 5 μg of the hormone dissolved in 5 μl of 0.05 *M* $NaHCO_3$ (pH 9.0) and 77 ng of lactoperoxidase dissolved in 10 μl of 0.05 *M* sodium acetate (pH 5.0) were added to 1 mCi of carrier-free $Na^{125}I$ buffered with 25 μl of 0.4 *M* sodium acetate (pH 5.0). The reaction was started by the addition of 200 ng of freshly prepared H_2O_2 in 10 μl of deionized water and additional 10-μl aliquots were added at 7 and 14 min. After 20 min the solution was adjusted to pH 9 with 50 μl of 0.05 *N* NaOH and applied to a column, 0.9 × 90 cm, of Sephadex G-100 equilibrated and eluted at 4° with 0.05 *M* $NaHCO_3$ (pH 9.0). The specific activities of the monoiodinated growth hormone preparations used in this study were calculated to be between 66.2 and 75.7 Ci/g.

Coupling of Growth Hormone to Sepharose and Glass. Growth hormone was coupled to Sepharose as previously described (Fellows et al., 1973). The synthesis of the growth hormone-glass conjugate was performed by a modification of the method of Weetal (1969), with the substitution of zirconia-clad glass beads, which have greater stability than silica beads between pH 5 and 10. In a typical preparation, 0.5 g of glass beads was refluxed at 105–115° for 1 hr with 5 ml of a 3% solution of aminopropyltriethoxysilane in toluene, washed with 15 ml of toluene, and dried at 150° for 2 hr. They were then refluxed for 1 hr at 65–75° with 5 ml of chloroform containing 50 mg of *p*-nitrobenzoyl chloride and 25 mg of triethylamine. The beads were washed with 15 ml of chloroform, dried at 80° for 30 min, refluxed at 105–115° for 1 hr with 5 ml of an aqueous solution of 1% sodium dithionite, and washed with 15 ml of deionized water. Five milliliters of 2 *N* HCl was added to the beads, the solution was cooled to 0° in an ice bath, and 125 mg of $NaNO_2$ was added. The solution was maintained at 0° under reduced pressure (<5 mm) for 30 min, following which the beads were washed with 15 ml of ice-cold 1% sulfamic acid followed by 15 ml of ice-cold water and were reacted immediately with 30 mg of growth hormone containing 3×10^7 cpm of ^{125}I-labeled growth hormone in 10 ml of 0.05 *M* $NaHCO_3$ (pH 9.0). Coupling was allowed to proceed for 1 hr at 0° with gentle shaking. Finally the beads were sequentially washed with 100 ml of each of 0.05 *M* $NaHCO_3$ (pH 9.0), water, 0.05 *M* sodium acetate (pH 5.0), water, 10 ml of guanidine hydrochloride, and 200 ml of water. A portion of each preparation was lyophilized, weighed, hydrolyzed with 6 *N* HCl at 110° for 24 hr under reduced pressure, and subjected to amino acid analysis on a Spinco Model 120B analyzer. Hormone conjugates were stored at 4° in 0.05 *M* $NaHCO_3$ (pH 9.0), containing 0.2% sodium azide.

Synthesis of HPP-Growth Hormone and [^{125}I]HPP-Growth Hormone. In a typical synthesis, a tenfold molar excess of *N*-succinimidyl 3-(4-hydroxyphenyl)propionate was added to 15 mg of growth hormone in 15 ml of 0.05 *M* $NaHCO_3$ (pH 9.0) and the solution was magnetically stirred for 45 min at 0°. After dialysis, the number of tyrosine residues in the derivatized hormone was determined spectrophotometrically by the method of Edelhoch (1967). The 3-(4-hydroxyphenyl)propionate (HPP) derivative of growth hormone was iodinated in the same manner described above for the unmodified hormone. Alternately, iodination was carried out by the method of Bolton and Hunter (1973) without modification other than purification of the iodinated hormone on a column, 0.9 × 90 cm, of Sephadex G-100 equilibrated and eluted at 4° with 0.13 *M* NH_4HCO_3 (pH 8.0). The specific activity of [^{125}I]HPP-growth hormone prepared by either method was typically calculated to be between 68.4 and 74.6 Ci/g.

In order to compare the two methods of synthesizing and labeling HPP-growth hormone, each was subjected to digestion with trypsin (2% by weight) for 2 hr at 37° in 0.13 *M* NH_4HCO_3 (pH 8.0), containing 0.05 *M* methimazole and 3.5 m*M* sodium dodecyl sulfate. Fingerprints of the soluble peptides were then made by the method of Seavey et al. (1971). After thorough drying, chromatograms were exposed to X-ray film (Kodak RP Royal X-Omat) for 2–4 days, according to the amount of radioactivity applied, and the film was manually processed according to the manufacturer's instructions.

Incubation. Before use in incubation studies, aliquots of growth hormone-Sepharose (3–8 ml) or growth hormone-glass (0.5–1.5 ml) were washed with 200 ml of deionized water and 100 ml of Krebs-Ringer bicarbonate buffer (pH 7.4). The amount of Sepharose or glass conjugate containing 1 mg of growth hormone and 1×10^6 cpm of ^{125}I-labeled growth hormone was suspended in 1.0 ml of the bicarbonate buffer containing 0.1% bovine serum albumin and 0.2% sodium azide and incubated in a water bath with constant shaking at either 4, 23, or 37°; 0.5-ml aliquots of the supernatant were removed at various times during the incubations and subjected to gel filtration on a column, 0.9 × 90 cm, of Sephadex G-75 equilibrated and eluted at 4° with bicarbonate buffer at a flow rate of 2–3 ml/hr. In pilot experiments, the column was equilibrated and eluted with bicarbonate buffer containing 0.1% bovine serum albumin in order to minimize protein losses due to nonspecific absorption. This was discontinued when control studies revealed that it did not enhance recoveries if the column was preconditioned with 0.5 ml of 3% bovine serum albumin. Eluate fractions of 1 ml were counted in a Nuclear Chicago-γ Counter (Model 4233) having a 57% counting efficiency for ^{125}I.

The lipolytic activity of the growth hormone conjugates in isolated fat cells was determined by Dr. James Malgieri and Dr. John Fain (Department of Health Sciences, Brown University) according to published methods (Fain et al., 1965).

Results

Coupling efficiencies averaged 41% for the growth hormone-Sepharose (0.8 mg of hormone/ml of packed Sepharose) and 31% for the growth hormone-glass (7.3 mg of hormone/ml of glass). The percentage of radioactivity bound

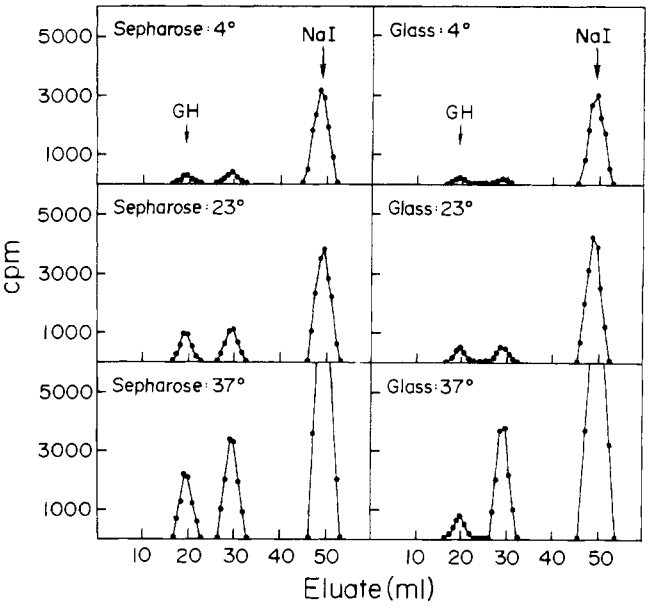

FIGURE 1: Comparison of the amount of radioactivity released from growth hormone-Sepharose (left) to that released from growth hormone-glass (right) after incubating for 4 days at 4, 23, and 37°. Incubations were carried out in bicarbonate buffer (pH 7.4) containing 0.1% bovine serum albumin and 0.2% sodium azide and the supernatants were chromatographed on a column, 0.9 × 90 cm, of Sephadex G-75 equilibrated and eluted at 4° with bicarbonate buffer (pH 7.4) at a flow rate of 2-3 ml/hr.

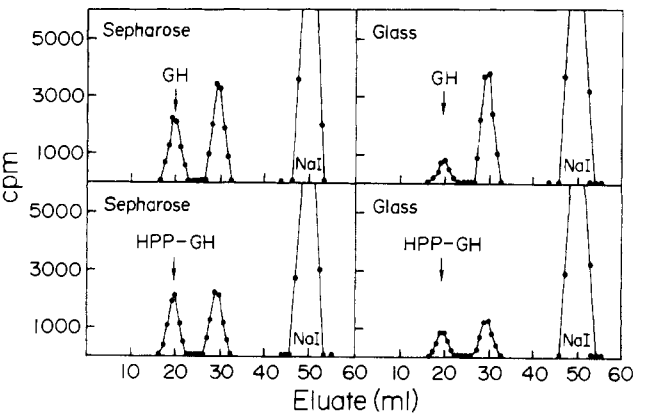

FIGURE 2: Comparison of the amount of intermediate peak released from Sepharose (left) and glass (right) to which the unmodified growth hormone (upper) or HPP-growth hormone (lower) had been coupled. Conjugates were incubated for 4 days at 37° in bicarbonate buffer (pH 7.4) containing 0.1% bovine serum albumin and 0.2% sodium azide, after which the supernatant was chromatographed on a column, 0.9 × 90 cm, of Sephadex G-75 equilibrated and eluted at 4° with bicarbonate buffer (pH 7.4) at a flow rate of 2-3 ml/hr.

to Sepharose (44%) and to glass (33%) agreed closely with the percentage of protein bound to either matrix, indicating the absence of preferential coupling of either labeled or unlabeled hormone. Using a tenfold molar excess of *N*-succinimidyl 3-(4-hydroxyphenyl)propionate over growth hormone, it was possible to incorporate an average of 1.3 mol of HPP/mol of protein, as determined by the method of Edelhoch (1967).

Chromatograms of incubation supernatants typically revealed three radioactive peaks (Figure 1). The first peak to emerge coincided with the position of growth hormone on preceding calibration runs, and the last peak coeluted with the sodium iodide-125 standards. The identification of $^{125}I^-$ as the only component in the salt peak was further confirmed by thin layer chromatography in pyridine-1-butanol-glacial acetic acid-water (50:75:15:60). An unanticipated peak (K_{av} = 0.32) was consistently observed in an intermediate position and was suspected to be a fragment of growth hormone, since trypsin digestion of this fraction for 2 hr at 37° displaces all of the radioactivity into a broad, salt peak. A possible source of this fragment might be the enzymatic cleavage of growth hormone by contaminating proteases (Padayatty and Van Kley, 1967; Ellis et al., 1968). However, incubation of NIH-BGH-B17 under the conditions given above, followed by chromatography on a column, 0.9 × 90 cm, of Sephadex G-100 in 6 *M* guanidine hydrochloride with or without 0.05 *M* dithiothreitol, failed to generate this species.

This fragment might also be generated during enzymatic iodination by oxidative cleavage at tryptophan (Alexander, 1974). In order to examine this possibility, 5 mg of growth hormone was iodinated with nonradioactive potassium iodide by the protocol used for radiolabeling, modified to provide proportionate increases in the volumes and amounts of reactants and to substitute ammonium acetate for sodium acetate buffers. After lyophilization, the protein was redissolved in 0.6 ml of 50% acetic acid containing 0.05 *M* dithiothreitol. Both iodinated and native growth hormone eluted as single peaks at the exclusion volume. The tryptophan content, determined by the method of Edelhoch (1962), was found to be 1.12 residues/molecule of growth hormone before iodination and 1.14 residues/molecule after iodination, indicating that the intermediate fragment was not produced by cleavage at this residue.

To test the hypothesis that this fragment might be generated as a result of radiation damage, the 3-(4-hydroxyphenyl)propionate derivative of growth hormone was enzymatically iodinated and then coupled to both glass and Sepharose. This method was preferred over that of Bolton and Hunter (1973) in which *N*-succinimidyl 3-(4-hydroxyphenyl)propionate is iodinated directly before coupling to growth hormone, because it requires lesser amounts of $Na^{125}I$. Radioautography of tryptic peptide maps showed that with [^{125}I]HPP-growth hormone prepared by either method, most of the label was located in a single spot near the origin. The peptide map of the [^{125}I]HPP-growth hormone prepared by our method exhibited a second, minor spot which contained 6.1% of the total radioactivity recovered and which had the same electrophoretic mobility as the major spot, but a greater chromatographic mobility. This may represent a longer or shorter fragment of the major peptide, or a peptide from a second region containing either an iodinated tyrosine residue or another iodinated HPP group.

Since in this derivative the ^{125}I is located farther from the peptide backbone, radiation damage should be minimized and consequent release of the fragment should be diminished. This decrease is readily seen in the chromatograms of the 4-day incubations (Figure 2). As shown in Table I, the rate of release of the intermediate peak from the HPP-growth hormone was significantly less than from the unmodified growth hormone with $p < 0.005$ for both glass and Sepharose by the *t* test for the comparison of two slopes (Zar, 1974), while the growth hormone and $^{125}I^-$ release rates were not significantly different.

When the release of radioactivity was measured as a function of temperature (Figure 1), it was seen that the leakage of all three species increases with increasing temperature and that the increment for each species is linear to

Table I: Comparison of the Intermediate Peak Release Rate form Insolubilized Growth Hormone and HPP-Growth Hormone.[a]

Matrix	Species	Ligand: Growth Hormone (%/day ± SE)	Ligand: HPP-Growth Hormone (%/day ± SE)	p
Sepharose	Growth Hormone	0.40 ± 0.04	0.35 ± 0.01	NS[b]
	Intermediate	0.61 ± 0.04	0.40 ± 0.01	<0.005
	$Na^{125}I$	2.74 ± 0.06	2.80 ± 0.05	NS
Glass	Growth Hormone	0.14 ± 0.02	0.15 ± 0.01	NS
	Intermediate	1.61 ± 0.10	0.23 ± 0.02	<0.005
	$Na^{125}I$	5.13 ± 0.04	4.42 ± 0.05	NS

[a] The conjugates were incubated for various time intervals at 37° in bicarbonate buffer (pH 7.4) containing 0.1% bovine serum albumin and 0.2% sodium azide. The supernatants were then fractionated on Sephadex G-75 and the radioactivity for each species at each time interval expressed as a percentage of the total radioactivity originally bound at the start of the incubation. These data were fitted to a straight line by the method of least squares and the slopes with their standard error of estimate given above. [b] Not significant ($p > 0.05$).

Table II: Comparison of the Effects of Temperature and Time on the Release of Radiolabeled Species from GH-Sepharose and GH-Glass.[a]

Species	Incubation Time (hr)	% Release of Total Bound Radioactivity: GH-Sepharose 4°	GH-Sepharose 23°	GH-Sepharose 37°	GH-Glass 4°	GH-Glass 23°	GH-Glass 37°
Growth Hormone	1			0.01			0.00
	2			0.04			0.00
	4			0.05			0.00
	8			0.12			0.00
	12	0.02	0.08	0.19	0.00	0.08	0.04
	48	0.09	0.34	0.81	0.03	0.30	0.31
	96	0.18	0.69	1.57	0.14	0.62	0.54
Intermediate	1			0.03			0.00
	2			0.05			0.05
	4			0.09			0.17
	8			0.25			0.48
	12	0.02	0.08	0.42	0.00	0.08	0.78
	48	0.10	0.35	1.23	0.03	0.30	2.91
	96	0.19	0.71	2.45	0.09	0.64	6.44
$Na^{125}I$	1			0.16			0.31
	2			0.28			0.49
	4			0.44			0.83
	8			0.96			1.73
	12	0.31	0.42	1.52	0.34	0.51	2.64
	48	1.41	1.87	5.47	1.48	1.84	10.62
	96	2.76	3.33	11.01	2.55	3.66	20.46

[a] The conjugates were incubated in bicarbonate buffer (pH 7.4) containing 0.1% bovine serum albumin and 0.2% sodium azide. The supernatants were then fractionated on Sephadex G-75 and the radioactivity for each species expressed as a percentage of the total radioactivity originally bound at the start of the incubation.

Table III: Comparison of the Effect of Temperature on the Release Rates of Radiolabeled Species from GH-Sepharose and GH-Glass.[a]

Species	Temp (°C)	Conjugate: GH-Sepharose (%/day ± SE)	Conjugate: GH-Glass (%/day ± SE)	p
Growth Hormone	4	0.05 ± 0.002	0.04 ± 0.001	NS[b]
	23	0.17 ± 0.02	0.16 ± 0.02	NS
	37	0.40 ± 0.04	0.14 ± 0.02	<0.005
Intermediate	4	0.05 ± 0.002	0.03 ± 0.004	NS
	23	0.18 ± 0.02	0.16 ± 0.02	NS
	37	0.61 ± 0.04	1.61 ± 0.10	<0.005
$Na^{125}I$	4	0.70 ± 0.02	0.63 ± 0.09	NS
	23	0.83 ± 0.10	0.90 ± 0.01	NS
	37	2.74 ± 0.06	5.13 ± 0.04	<0.005

[a] The data from Table II were fitted to a straight line by the method of least squares and the slopes with their standard error of estimate given above. [b] Not significant ($p > 0.05$).

4 days (Table II). Although there is no statistically significant difference between the rate of release of growth hormone from either matrix at 4 or 23°, the release from Sepharose is much greater ($p < 0.005$) than from glass at 37° (Table III). Furthermore, the release rates of the intermediate peak and the $^{125}I^-$ from both supports are similar at 4 and 23°, but the rates are considerably higher ($p < 0.005$) from glass at 37°.

Since it has been suggested that soluble hormone could be generated by hydrolysis of glycosidic linkages in the agarose matrix rather than by cleavage of amide or ester bonds, the anthrone reaction (Kabat and Mayer, 1961) was carried out on the supernatant of the 4-day incubation at 37°. By this method, less than 5 μg of agarose could be detected in standard solutions. Although none of the incubation supernatants were significantly different from the blank, it can be calculated that as many as 45 sugar residues could still be bound to each molecule of released growth hormone and escape detection in this assay. However, there is certainly no gross breakdown of Sepharose after 4 days at 37°, since less than 0.025% of the agarose could be solubilized during this time and escape detection.

We also considered that, despite the extensive washing protocol, some of the hormone is only adsorbed to the matrix and that this is the source of the free hormone seen in the incubation medium. However, in control experiments, ^{125}I-labeled growth hormone and either nonactivated Sepharose or silanized glass beads were mixed, allowed to stand at 4° for 24 hr or at 0° for 1 hr, respectively, and subjected to the washing procedure described. After washing, 1929 cpm remained adsorbed to each milliliter of Sepharose per 1×10^6 cpm added. One milliliter of growth hormone-Sepharose exposed to the same amount of radioactivity during the coupling reaction bound 881,563 cpm and released 4042 cpm into the incubation supernatant in 24 hr. Similarly, 1023 cpm remained adsorbed to every 100 mg of glass beads per 1×10^6 cpm added, while 100 mg of the glass beads coupled to 1×10^6 cpm of ^{125}I-labeled growth hormone bound 662,951 cpm and released 2131 cpm in 24 hr. Therefore, although a small amount of adsorption occurs, it is insufficient to account for the soluble hormone.

When the supernatant from a 4-hr incubation of growth hormone-glass at 37° in the absence of adipocytes was reincubated with isolated fat cells, there was a significant increase in glycerol release which was enhanced by dexamethasone, a characteristic of growth hormone-induced lipolysis in adipocytes (Malgieri, J., and Fain, J. N., personal communication). Accurate quantitation of the released growth hormone was complicated by technical difficulties in handling the glass supports. Column fractions containing the intermediate peak were not tested in the lipolytic assay.

Discussion

The valid use of iodinated hormones depends upon the demonstration that the hormone is not damaged by the iodination procedure. Initially, most investigators were concerned about the number of iodide atoms incorporated per hormone molecule (Freychet et al., 1971; Gandolfi et al., 1971; Miyachi et al., 1973); however, recent observations of peptide chain cleavage during enzymatic iodinations (Alexander, 1974) suggest that other forms of damage may be occurring.

Experimental data presented in this paper indicate that bovine growth hormone is not degraded in the course of enzymatic iodination. The difference between these data and those of Alexander (1974) probably is a function of the size of the peptides studied. In very short peptides, tryptophanyl residues may be exposed to the reaction medium, while in larger proteins, including growth hormone, tryptophanyl residues are presumably in a protected environment in the molecule and less accessible to various reactants.

In addition, the valid use of hormone conjugates depends upon the demonstration of their stability under the various bioassay conditions. In this study, the release of growth hormone from either glass or Sepharose at 4° was found to be nearly imperceptible. Even at 37°, the leakage rate is quite small, amounting to 0.14% (1.4 μg/mg of coupled growth hormone incubated) per day from glass and 0.40% (4.0 μg/mg of coupled hormone incubated) per day from Sepharose. Although this leakage is insignificant with respect to chemical applications of these conjugates (e.g., affinity chromatography), it nevertheless exceeds the limits of detectability in sensitive biological assays where, for example, as little as 10 ng of growth hormone/ml can be measured in the lipolytic assay in the presence of dexamethasone (Fain et al., 1965). In addition, the stronger binding between glass and growth hormone is offset by various mechanical problems presented by the glass itself; in particular, its high density makes ligand-to-cell contact difficult even with vigorous shaking, and it also complicates accurate aliquoting of the material.

Although the glass matrix was found to release less growth hormone than did Sepharose, it liberates much more free $^{125}I^-$ and intermediate peak. This could be accounted for if two separate processes are operating independently: (a) instability of the coupling bond, and (b) radiation damage. This study suggests that the former process appears to be more important in the leakage of intact, biologically active growth hormone; and the latter is a more significant factor with respect to the leakage of free $^{125}I^-$ and the intermediate peak.

The major products of coupling to cyanogen bromide activated Sepharose are isourea derivatives, N-substituted imidocarbonates, and N-substituted carbamates (Axén and Ernback, 1971). Although the last derivative is stable under most conditions, the first two resemble Schiff bases in their ease of hydrolysis. The N-substituted imidocarbonates are particularly susceptible to hydrolysis because of the strain placed on the galactose ring of the agarose by the imidocarbonate ring. Both of these bonds are most stable between pH 4 and 6 and the rate of hydrolysis increases rapidly as the pH rises. This is consistent with the findings of Yong (1973) that the leakage of catecholamines from Sepharose increased as the pH was raised above pH 6. Recently, Tesser et al. (1974) have identified the liberated ligand as the intact molecule or, at higher pH's, its carbamoyl derivative; and they give strong evidence that the leakage results from an attack on the N-substituted imidocarbonate by hydroxyl ions. Since the diazo bond is much more stable than either the isourea derivative or the N-substituted imidocarbonates, this probably contributes to the reduced leakage rate from glass. Although we were not able to rule out possible cleavage of the polysaccharide linkages, the relative stability of glycosidic and peptide bonds compared to the coupling bonds make this an unlikely mechanism for hormone release.

Tesser et al. (1972) have noted that ligand release is more of a problem with smaller molecules, such as cyclic 3′,5′-adenosine monophosphate and steroids, than with larger ones, such as proteins, and suggest that this difference occurs because the former are bound at one site, while the latter are bound at several positions. For biological studies, hormones frequently are coupled to Sepharose at suboptimal pH values (Turkington, 1970; Selinger and Civen, 1971) or at submaximal cyanogen bromide concentrations (Fellows et al., 1973), on the assumption that the hormone would be more active if it were bound to the ma-

trix at fewer sites. Unfortunately, these procedures also decrease the number of the bonds between the matrix and hormone, thereby increasing the leakage rate and complicating experimental interpretation.

These findings add to the growing literature (Fritz, 1971; Tesser et al., 1972; Davidson et al., 1973; Yong, 1973; Katzen and Vlahakes, 1973; Butcher et al., 1973) urging cautious interpretation of experiments with insolubilized hormones, particularly when these materials are being used to study hormone–cell interactions. It is clear that insolubilized hormones constitute powerful investigative tools, but only when their stability has been thoroughly characterized. In addition, it is important to recognize that the release of total radioactivity into the incubation medium is not necessarily quantitatively equivalent to the release of the intact, biologically active hormone or an active fragment.

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