Human Chorionic Gonadotropin, Reaction with Tetranitromethane†

Valerie G. Hum, J. E. Knipfel,‡ and K. F. Mori*

ABSTRACT: Treatment of human chorionic gonadotropin (HCG) with tetranitromethane resulted in polymerization of the hormone molecule and nitration of two of the seven tyrosyl residues in the monomer. Polymers were the main reaction products, and the overall yield of nitrated monomers was about 25% of the starting materials. The modified monomers were characterized to show that the nitration occurred only in the α subunit of HCG at positions 88 and 89 and that it was accompanied by more than 90% reduction in biological activity. The α subunit of [Tyr(NO_2)]$_2$-HCG recombined with intact HCG-β subunit as judged by gel electrophoresis, but the recombined product showed only 10% of the biological activity regenerated by recombination of native subunits. When isolated HCG α and β subunits were subjected to the same reagent under similar conditions, three tyrosyls at 65, 88, and 89 were nitrated in the α subunit, whereas in the β subunit Tyr-58 and Tyr-81 were nitrated. The [Tyr(NO_2)]$_3$-HCG-α apparently did not recombine with intact HCB-β, whereas recombination took place between the [Tyr(NO_2)]$_2$-HCG-β and intact HCG-α to regenerate biological activity to nearly the same extent as a recombination product of native subunits. These results suggest that Tyr-88 and Tyr-89 in the α subunit of HCG are important though not essential for biological activity; that Tyr-65 in the same subunit is involved in holding the two subunits together in the native conformation; and that Tyr-58 and Tyr-81 in the β subunit are of little importance in the biological activity of the hormone.

Of the seven tyrosyl residues of HCG,[1] four have been found reversibly titratable under *normal* conditions (Mori and Hollands, 1971). A solvent perturbation study of this hormone also has shown that about four tyrosyls are accessible to perturbants (Mori, 1972). Further studies were carried out to characterize these seemingly easily accessible tyrosyls by modifying with a known tyrosine-specific reagent, $C(NO_2)_4$ (Sokolovsky *et al.*, 1966). A preliminary account of the reaction of HCG with $C(NO_2)_4$ was given by Brossmer *et al.* (1971).

Experimental Section

Materials. HCG was purified from a commercial preparation (3000 IU/mg, Organon, Inc.) as described previously (Mori, 1970) and had a biological potency of 12,200 IU/mg when determined by the seminal vesicle weight method (Van Hell *et al.*, 1964). HCG-α and -β were prepared by the method of Morgan and Canfield (1971) and each was purified by gel filtration on Sephadex G-100. HCG or its subunits were reduced with a 30-molar excess of dithiothreitol in 0.2 M Tris buffer (pH 8.5) containing 8 M urea at 37° for 4 hr under N_2 gas and then alkylated with iodoacetic acid. The final products were subjected to amino acid analysis to confirm that all disulfides were converted to *S*-carboxymethylcysteine. Dithiothreitol (Calbiochem), $C(NO_2)_4$ (Sigma), 3-nitrotyrosine, and 3,5-dinitrotyrosine (K & K Labs), and trypsin treated with L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone (Worthington) were used without further purification. Urea solutions were freshly prepared by dissolving a commercial product (Ultra Pure grade, Mann). All other chemicals were of reagent or analytical grade.

Nitration with $C(NO_2)_4$. $C(NO_2)_4$, 10% (v/v) in 95% ethanol, was added to 250–530 nmol of protein solutions in 0.05 M Tris buffer (pH 8.1) at molar ratios ($C(NO_2)_4$:protein) of 10 to 480 and the mixtures were incubated as described by Sokolovsky *et al.* (1966) for 2 hr at room temperature and then immediately placed on Sephadex G-25 columns in 1% NH_4HCO_3. The molecular weights of HCG and its subunits were estimated as 38,000 (Mori, 1970) and 19,000, respectively. Since a number of preliminary experiments indicated that treatment of HCG with $C(NO_2)_4$ polymerized the hormone molecule, the reaction products were subjected to gel filtration on Sephadex G-100 (fine) in 1% NH_4HCO_3 to separate monomers from polymeric forms. All of the products were recovered by lyophilization. The extent of tyrosyl nitration was determined spectrophotometrically at 428 nm (Sokolovsky *et al.*, 1966) and/or by amino acid analysis.

Protein concentrations were determined using $E^{1\%}_{1\,cm} = 5.47$ at 276 nm (Mori and Hollands, 1971) for native HCG and SCM-HCG either with the Folin phenol reagent (Lowry *et al.*, 1951) using bovine serum albumin as a standard; by dry weight or by amino acid analysis for HCG subunits and modified products.

Homogeneity of samples was examined by polyacrylamide gel electrophoresis in the absence or presence of sodium dodecyl sulfate (Weber and Osborn, 1969), and their molecular size was determined by gel filtration in 1% NH_4HCO_3 as described previously (Mori, 1970). *Amino acid and sialic acid analyses* were carried out in the same manner as reported earlier (Mori, 1970) except that HCl hydrolysis prior to amino acid analysis was continued for 22 hr. Some difficulty was encountered in quantitating the number of nitrotyrosine by amino acid analysis due to coemergence of glucosamine and 3-nitrotyrosine as noted by Cheng and Pierce (1972) and this

† From Drug Research Laboratories, Health Protection Branch, Department of National Health & Welfare, Ottawa, Ontario K1A OL2. *Received September 27, 1973.*

‡ Present address: Food Research Laboratories, Health Protection Branch, Department of National Health & Welfare.

[1] Abbreviations used are: HCG, human chorionic gonadotropin; $C(NO_2)_4$, tetranitromethane; HCG-α, α subunit of HCG; HCG-β, β subunit of HCG; SCM-HCG, completely reduced and carboxymethylated HCG; LH, luteinizing hormone.

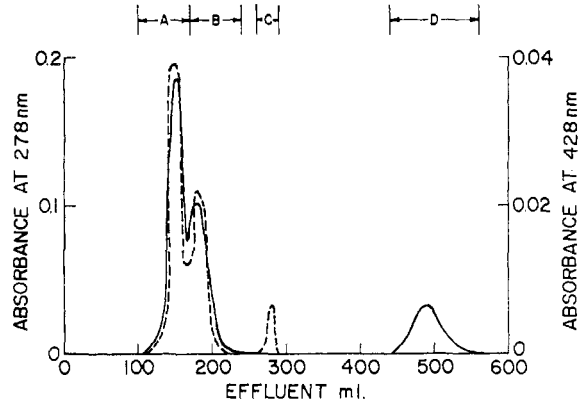

FIGURE 1: Gel filtration of HCG treated with a 10 molar excess of $C(NO_2)_4$ on Sephadex G-100 (2.5 × 90 cm) in 1% NH_4HCO_3. After 14.6 mg of nitrated HCG was applied, the column was eluted with 1% NH_4HCO_3 at a rate of 10 ml/hr. Solid line: absorbance at 278 nm; broken line: absorbance at 428 nm.

was resolved with the same procedure as described by these authors.

Subunit Separation and Recombination. $C(NO_2)_4$-treated HCG products were separated into two subunits by the same method as described for native subunits (Morgan and Canfield, 1971). No significant difference was noted in the subunit separation pattern between native and treated HCG. Recombination of these subunits was achieved by incubating two subunits mixed in equal proportions by weight in 0.01 M phosphate buffer (pH 7.0) at 37° for 17 hr and then examining gel electrophoretic patterns and regeneration of biological activity of the mixtures.

Preparation and Analysis of Tryptic Peptides. Subunits separated from nitrated HCG or isolated and nitrated subunits were reduced and S-carboxymethylated in the same way as in the preparation of SCM-HCG. The SCM subunits were dissolved in 1% NH_4HCO_3 buffer (pH 8.0), containing 8 M urea, to which trypsin was added at an enzyme/substrate ratio (w/w) of 1/50. Reduced and S-carboxymethylated α subunits were treated, before tryptic digestion, with maleic anhydride in 1% $NaHCO_3$ (Butler *et al.*, 1969) to block the lysine residues against the action of trypsin. Enzymic digestion was allowed to continue for 2–4 hr at 37°, the hydrolysates were fractionated on Bio-Gel P-10 columns in 1% NH_4HCO_3, and each fraction was lyophilized. The purity of each fraction was checked by the number and identity of N-terminal amino acid(s) by the combined dansyl-Edman degradation technique of Gray (1967). Further separation of the tryptic peptides was effected by descending paper chromatography with 1-butanol–pyridine–acetic acid–water (90:60:18:72, v/v) for 17 hr, followed by high-voltage electrophoresis at pH 3.6 in pyridine–acetate buffer for 90 min at a gradient of 40 V/cm. Peptides containing nitrotyrosine were detected as yellow spots when paper was exposed to NH_3 vapor. The yellow peptides were eluted with 30% aqueous pyridine, and the solutions were lyophilized for further analytical work.

Results

Nitration of HCG. Several attempts were made to control the nitration rate of tyrosyls in HCG by changing the molar ratio of $C(NO_2)_4$ to HCG and/or the reaction time, but this failed to produce any consistent results aud the overall recovery of tyrosyl residues after $C(NO_2)_4$ treatment (the sum of free and nitrotyrosyls) was low, ranging from 51 to 85% of the theoretical value. Further investigation revealed that polymers were the major reaction product. Nevertheless, in a few cases nitration of tyrosyls in the monomer was confirmed, the overall yield of modified monomers being about 25% of the starting materials. For example, when a reaction mixture of HCG and a 10-molar excess of $C(NO_2)_4$ was passed through a Sephadex G-100 column, fraction B (Figure 1) had a molecular weight estimate of 33,400 and contained 1.7 mol of 3-nitrotyrosyl residues/mol, whereas fraction A was a polymer with a molecular weight of 64,000. Fractions C and D represented nonprotein components and were presumably salts. In the nitrated HCG monomer the loss of tyrosyl residues was fully accounted for by the formation of 3-nitrotyrosine and no other amino acid was affected. Neither did $C(NO_2)_4$ treatment affect the sialic acid content of the hormone. Nitro-HCG (monomer) retained less than 10% of the original biological activity (Table I).

TABLE I: Biological Activity of Nitrated HCG and Combined Products of Various HCG Subunits.[a]

Sample	Biol Act.[b]
Intact HCG	11,720 (10,850–13,600)
Nitro HCG (monomer)	960 (710–1,050)
Intact HCG-α + intact HCG-β	7,940 (6300–9020)
α from nitro-HCG + β from nitro-HCG	655 (384–020)
α from nitro-HCG + intact HCG-β	730 (370–930)
[Tyr(NO_2)]$_3$-HCG-α + intact HCG-β	nr[c]
Intact HCG-α + [Tyr(NO_2)]$_2$-HCG-β	8,270 (5890–11,900)
[Tyr(NO_2)]$_3$-HCG-α + [Tyr(NO_2)]$_2$-HCG-β	nr[c]

[a] In recombination experiments only monomers of modified subunits were used and the combined products were examined by gel electrophoresis prior to bioassay. [b] Mean, followed by (range), in IU/mg. [c] No response at a maximum dose of 20 μg of nitro α plus 20 μg of intact or nitro β per animal.

When nitro-HCG was separated into two subunits and each subunit submitted to amino acid analysis, the amino acid compositions obtained for the two subunits agreed with those for native subunits except that in the α subunit isolated from nitro-HCG there were 1.9 mol of free and 1.5 mol of 3-nitrotyrosyl residues per mol. A tryptic digest of this α subunit, after being reduced, S-carboxymethylated, and maleylated, yielded three fractions with exclusion chromatography on a Bio-Gel column (Figure 2). Fraction I showed no absorbance at 278 nm, whereas fractions II and III had peaks at this wavelength. Only fraction II displayed significant absorption at 428 nm. Fractions I and III were rechromatographed on Bio-Gel to yield homogeneous fractions, whereas fraction II did not. Further separation of this fraction by paper chromatography and electrophoresis produced two subfractions, II-a that was light absorbing at 428-nm and II-b that was nonabsorbing. N-Terminal amino acid residues and compositions of amino acids other than tyrosine of subfractions II-a and II-b agree well with those which should be obtained theoretically on the two peptides corresponding to residues 43–67 and 68–92 (T(M)-2 and T(M)-3, Figure 3) in HCG-α, respec-

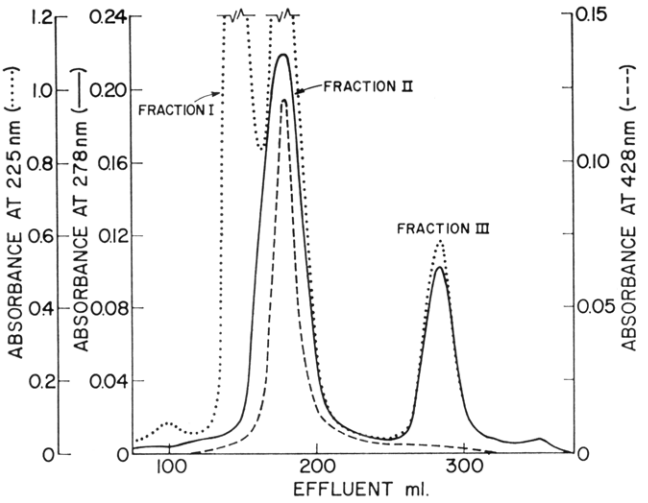

FIGURE 2: Gel filtration of a tryptic digest of reduced, S-carboxymethylated, and maleylated α subunit separated from nitro-HCG. The 2-hr hydrolysate (5 mg) was applied to a Bio-Gel P-10 (200–400 mesh) column (1.6 × 180 cm) and eluted with 1% NH_4HCO_3 at room temperature at a flow rate of 6 ml/hr.

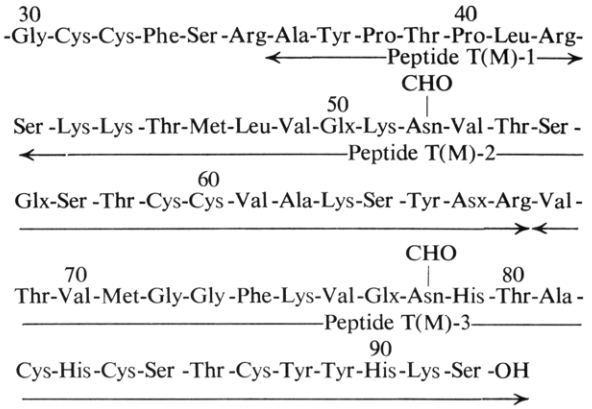

FIGURE 3: Nitrated tyrosyl residues circled in the partial linear amino acid sequence of HCG-α taken from Bahl *et al.* (1972).

tively. No 3-nitrotyrosine was present in fraction II-a, but there were 1.5 residues/mol in II-b. Only 0.1 free tyrosine was detected in the latter. It was concluded, therefore, that the two nitrotyrosines must correspond to Tyr-88 and Tyr-89.

When $[Tyr(NO_2)]_2$HCG-α was allowed to react with β subunit derived from either nitro or intact HCG, recombination of the two was apparent as judged by gel electrophoresis (Figure 4). Yet, regeneration of biological activity observed for this recombined product was about 10% of that shown for a recombined product of native subunits (Table I).

Nitration of Isolated HCG Subunits. When isolated HCG-α or -β was treated with $C(NO_2)_4$ in the same manner as intact HCG, polymer formation again yielded the major product, and nitrated α and β subunits (monomers) were formed only in about 10 and 30% yields, respectively, regardless of whether $C(NO_2)_4$ was in a 10 or 60 molar excess. In nitro HCG-α 0.9 mol of free tyrosyl and 2.7 mol of 3-nitrotyrosyls per mol were found after nitration with a 60 molar excess of $C(NO_2)_4$. In HCG-β, on the other hand, 1.6 mol of tyrosyls was found as 3-nitrotyrosine and 0.8 remained as free. In both nitrosubunits no amino acid other than the tyrosyl residue was significantly affected.

When a tryptic digest of a reduced, S-carboxymethylated, and maleylated material of this $[Tyr(NO_2)]_3$-HCG-α was subjected to gel filtration under similar conditions to those used for the α subunit of nitro-HCG, its elution pattern was essentially the same, qualitatively, as that shown in Figure 2. A second fraction was again found heterogeneous after rechromatography on Bio-Gel and accordingly was further fractionated by paper chromatography and electrophoresis to yield two 3-nitrotyrosine-containing subfractions, II-a and II-b. Fraction II-a contained no free but 0.8 mol of 3-nitrotyrosines, whereas fraction II-b no free but 1.6 mol of 3-nitrotyrosines. Analyses for N-terminal amino acid residues and the amino acid composition of the two subfractions showed that fractions II-a and II-b correspond to peptides T(M)-2 and T(M)-3 (Figure 3), respectively.

When a tryptic digest of reduced and S-carboxymethylated $[Tyr(NO_2)]_2$-HCG-β was applied on Bio-Gel, two peaks with absorbance at 428 nm were obtained (fractions I and II; Figure 5). Each fraction was further fractionated to produce a few peptides, one of which from fraction I (fraction I-a) contained 0.8 tyrosyl but no 3-nitrotyrosyl residue per molecule and is judged to correspond to peptide T-1 (Figure 6). Two peptides, one from fraction I (fraction I-b) and another from fraction II (fraction II-a), were shown to contain nitrotyrosine by yellow color on paper chromatograms and by amino acid analysis (0.7 and 0.6 mol per mol). Agreement in the amino acid composition between fraction I-b and peptide T-2 was excellent but rather poor between fraction II-a and peptide T-6. However, when a newly proposed sequence of the region of HCG-β corresponding to peptide T-6 (Morgan *et al.*, 1973) is considered, the amino acid composition of II-a becomes nearly identical with that of peptide T-6. The amino acid analyses on which the identification of the individual peptides was based are summarized in Table II.

Attempts to recombine $[Tyr(NO_2)]_3$-HCG-α with intact HCG-β failed to regenerate any biological activity (Table I). Nor did the mixture of the two subunits show any sign of recombination on gel electrophoresis (Figure 4). On the other hand $[Tyr(NO_2)]_2$-HCG-β, after incubation with intact HCG-α, regenerated biological activity to the same extent as intact HCG-β with intact HCG-α (Table I). Recombination did not take place between $[Tyr(NO_2)]_3$-HCG-α and $[Tyr(NO_2)]_2$-HCG-β as neither regeneration of biological activity nor appearance of recombined products on gel electrophoresis was observed. It should be pointed out here that nitration apparently did not affect the gel electrophoretic patterns of HCG or its isolated subunits significantly.

Polymerized Products. As has already been described herein, the major $C(NO_2)_4$ reaction products of HCG or its isolated

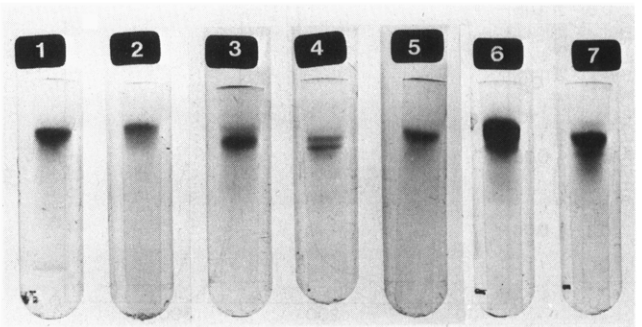

FIGURE 4: Polyacrylamide gel electrophoresis patterns of various combinations of native and nitrated (monomer) HCG subunits. Electrophoresis was conducted in glycine buffer, pH 9.2: 1, native HCG; 2, nitro HCG; 3, α subunit from nitro-HCG; 4, native HCG-β; 5, (3 + 4); 6, $[Tyr(NO_2)]_3$-HCG-α; 7, (4 + 6).

TABLE II: Amino Acid Composition of Tryptic Peptides of Nitro HCG-α and HCG-β.[a]

Amino Acid	Nitro-HCG-α		Nitro-HCG-β		
	Fraction II-a	Fraction II-b	Fraction I-a	Fraction I-b	Fraction II-a
N terminus	Serine	Valine	Glutamic acid	Valine	Glycine
Lysine	3.9 (4)	2.0 (2)			
Histidine		2.9 (3)			
Arginine	0.8 (1)		0.9 (1)	1.0 (1)	0.8 (1)
Aspartic acid	2.1 (2)	1.0 (1)	1.1 (1)	0.9 (1)	1.1 (1)
Threonine	2.8 (3)	2.6 (3)	3.7 (4)		
Serine	3.7 (4)	1.8 (2)			1.7 (1)
Glutamic acid	2.2 (2)	1.1 (1)	1.2 (1)	2.2 (2)	0.7 (1)
Proline			2.1 (2)	2.5 (2)	1.1 (1)
Glycine		2.3 (2)	2.3 (2)	0.9 (1)	1.2 (1)
Alanine	1.0 (1)	1.3 (1)	1.1 (1)	1.0 (1)	3.1 (2)
SCM-cysteine	1.9 (2)	2.7 (3)	3.6 (4)	0.8 (1)	2.4 (1)
Valine	2.8 (3)	2.8 (3)	1.9 (2)	2.8 (3)	3.7 (4)
Methionine	0.6 (1)	0.8 (1)	0.6 (1)		
Isoleucine			2.1 (2)		
Leucine	0.9 (1)			3.9 (4)	1.8 (1)
Tyrosine	0.0 } (1)	0.0 } (2)	0.8 } (1)	0.0 } (1)	0.1 } (1)
3-Nitrotyrosine	0.8 }	1.6 }	0.0 }	0.7 }	0.6 }
Phenylalanine		0.9 (1)			
Total residue	25 (25)	25 (25)	22 (22)	18 (17)	19 (14)

[a] Given as residues per molecule. Corrections for destruction during acid hydrolysis were not made. The numbers in parentheses are the theoretical values based on the sequence given by Bahl *et al.* (1972).

subunits were higher molecular weight compounds. The use of larger molar excess of $C(NO_2)_4$ tended to yield more polymers, though a 10 molar excess occasionally produced as much polymers as a 120 molar excess. The polymers produced from HCG had molecular weights of about 60,000–120,000 but had essentially the same amino acid composition as native HCG. However, a few (0.4–2.7/monomer molecule) 3-nitrotyrosyl residues were present and the total tyrosine recovery (the sum of free and 3-nitro tyrosines) was low ranging from 2.8 to 5.0 as compared with 7.0 for native HCG. These products apparently did not show any dissociation into subunits on gel electrophoresis in the presence of sodium dodecyl sulfate. Their biological activity varied from 28 to 56%, the average being 34.2%, of that of native HCG.

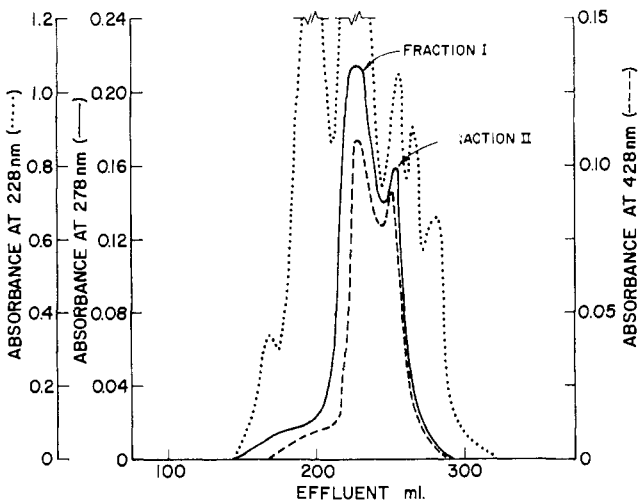

FIGURE 5: Gel filtration of a tryptic digest of reduced, and S-carboxymethylated dinitrotyrosyl-HCG-β. The 2.5-hr digest, 5.2 mg, was applied to a 1.6 × 185 cm Bio-Gel P-10 (200–400 mesh) column. The conditions used were the same as given in Figure 2.

Discussion

In agreement with the finding of Brossmer *et al.* (1971), in the native form of HCG two tyrosyls were nitrated with $C(NO_2)_4$, though nitration was a minor reaction event. The two nitrated tyrosyls were located in the α subunit, identified as Tyr-88 and -89 (see Figure 3), but none of the tyrosyls in the β subunit were nitrated. Based on the results of the tyrosyl nitration, the two tyrosyls in the α subunit are apparently the most easily accessible to the reagent in the native hormone. The fact that the biological activity of the hormone after nitration of Tyr-88 and Tyr-89 in the α subunit was greatly reduced but was not completely abolished may suggest that

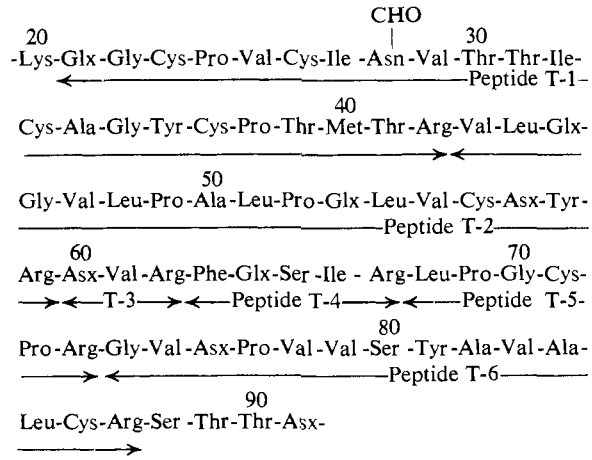

FIGURE 6: Nitrated tyrosyl residues (circled) in the partial linear amino acid sequence of HCG-β taken from Bahl *et al.* (1972).

these two tyrosyls are important but not essential for the biological activity. It is not unreasonable to assume that Tyr-88 and Thr-89 of HCG-α are involved in the binding of the hormone molecule to its receptors at the target organ and/or are important in maintaining the native structure of the hormone in such a manner as to protect it against various *in vivo* destructive processes. Attempts were made to determine the validity of these possibilities by examining the binding ability of modified HCG monomer and various combinations of native and nitrated subunits, using the radioligand–receptor assay of Catt *et al.* (1971). Although a considerable degree of receptor binding was shown for some products at minimum effective dose levels, the *in vitro* assay responses of these products were nonparallel to that of native HCG, thereby leaving the matter unsettled.

Polymers which formed the major reaction product, are covalently cross-linked because of their nondissociation on gel electrophoresis in the presence of sodium dodecyl sulfate, thereby adding one more example to a growing list of the occurrence of cross-linking of proteins with $C(NO_2)_4$ (Boessel and Carpenter, 1970; Vincent *et al.*, 1970; Williams and Lowe, 1971; Shifrin and Solis, 1972; Cheng and Pierce, 1972). The low recovery of tyrosyls in the polymers suggests that some of the tyrosyls, but unlikely Tyr-88 and Tyr-89 in the α subunit, are involved in this cross-linking. Thus the nitration reaction of tyrosyls in HCG with $C(NO_2)_4$ cannot be regarded as a sensitive measure for determining the reactivity of the tyrosyls toward the reagent and accordingly, a consideration of the location of these tyrosyls in the three-dimensional arrangement of the hormone structure in terms of "exposed" against "buried" would be meaningless. Also it should be borne in mind that the possibility of some side reactions of $C(NO_2)_4$ with HCG or its subunits other than those mentioned above cannot be ruled out.

Some of the present findings appear to merit some consideration with regard to the role of the tyrosyl residues in the subunit structure of HCG (see Figures 3 and 6). Since as evidenced by regeneration of biological activity and by gel electrophoretic patterns, $[Tyr(NO_2)]_2$-HCG-α in which Tyr-88 and Tyr-89 were nitrated, was shown to recombine with unmodified HCG-β, these two tyrosyls are apparently not involved in the subunit combination, *i.e.*, the formation of noncovalent bond between α and β subunits in the native conformation of the hormone molecule. However, $[Tyr(NO_2)]_2$-HCG-α, in which one more tyrosyl at position 65 was nitrated, failed to combine with intact HCG-β, inferring that Tyr-65 in the α subunits is involved in the combination of the two subunits. On the other hand, in the β subunit two tyrosyls at positions of 58 and 81 which were nitrated only after the subunit was separated from the α subunit, are independent of the subunit combination, since the $[Tyr(NO_2)]_2$-HCG-β regenerated the hormonal activity almost completely upon incubating with intact HCG-α. This fact also suggests that Tyr-58 and Tyr-81 in the β subunit are not important in the biological activity of the hormone.

The cross-linked polymers of HCG obtained after treatment with $C(NO_2)_4$ possessed about one-third of the biological activity of native hormone. Of some interest is the fact that HCG polymers, having molecular weights comparable to those of the present cross-linked polymers but being produced by other means, are also reported to have lower biological activity than native hormone (Brossmer *et al.*, 1971).

Recently the reaction of $C(NO_2)_4$ with LH, which resembles HCG in many respects, has been studied by two groups of investigators (Sairam *et al.*, 1972; Cheng and Pierce, 1972).

TABLE III[a]

	Bovine LH		Ovine LH			HCG
α subunit						
Tyr-21	+	++		++		
Tyr-30			+	++		
Tyr-41		++	+	++	Tyr-37	
					Tyr-65	++
Tyr-92	+	++	+	++	Tyr-88	+ ++
Tyr-93	+	++	+	++	Tyr-89	+ ++
β subunit						
Tyr-37		++	+	++	Tyr-36	
Tyr-59		++		++	Tyr-58	++
					Tyr-81	++

[a] Those tyrosyls which were nitrated with $C(NO_2)_4$ in the native form are marked with +, whereas those in the isolated subunit form are done with ++. Also it should be noted here that Tyr-37, Tyr-88, and Tyr-89 of HCG-α correspond to Tyr-41, Tyr-92, and Tyr-93 of LH-α, whereas Tyr-36 and Tyr-58 of HCG-β to Tyr-37 and Tyr-59 of LH-β.

In agreement with the findings of these workers, the low yield of monomeric nitrated products, the high reactivity of tyrosyl residues in the α subunit toward $C(NO_2)_4$ for nitration in the native form of the hormone, and the higher nitration rate of tyrosyls in isolated subunits as opposed to in native molecule have also been observed in this study, reaffirming the homology between the two glycoprotein hormones. However, the location of the either completely or partially nitrated tyrosyl residues shows some differences as demonstrated in Table III.

References

Bahl, O. P., Carlsen, R. B., Bellisario, R., and Swaminathan, N. (1972), *Biochem. Biophys. Res. Commun. 48*, 416.

Boessel, R. W., and Carpenter, F. H. (1970), *Biochem. Biophys. Res. Commun. 38*, 678.

Brossmer, R., Dörner, M., Hilgenfeldt, U., Leidenberger, F., and Trude, E. (1971), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 15*, 36.

Butler, P. J. G., Harris, J. I., Hartley, B. C., and Leberman, R. (1969), *Biochem. J. 112*, 679.

Catt, K. J., Dufau, M. L., and Tsuruhara, T. (1971), *J. Clin. Endocrinol. 32*, 860.

Cheng, K.-W., and Pierce, J. G. (1972), *J. Biol. Chem. 247*, 7163.

Gray, W. R. (1967), *Methods Enzymol. 11, 139*, 469.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Morgan, F. J., Birken, S., and Canfield, R. E. (1973), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 31*, 101.

Morgan, F. J., and Canfield, R. E. (1971), *Endocrinology 88*, 1045.

Mori, K. F. (1970), *Endocrinology 86*, 97.

Mori, K. F. (1972), *Biochim. Biophys. Acta 257*, 523.

Mori, K. F., and Hollands, T. R. (1971), *J. Biol. Chem. 246*, 7223.

Sairam, M. R., Papkoff, M., and Li, C. H. (1972), *Biochim. Biophys. Acta 278*, 421.

Shifrin, S., and Solis, B. G. (1972), *J. Biol. Chem. 247*, 4121.

Sokolovsky, M., Riordan, J. F., and Vallee, B. L. (1966), *Biochemistry 5*, 3582.

Van Hell, H., Matthijsen, R., and Overbeek, G. A. (1964), *Acta Endocrinol. 47*, 409.
Vincent, J. P., Lazdunski, M., and DeLeage, M. (1970), *Eur. J. Biochem. 12*, 250.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.
Williams, J., and Lowe, J. M. (1971), *Biochem. J.121*, 203.

Thyroid Tubulin: Purification and Properties†

B. Bhattacharyya and J. Wolff*

ABSTRACT: Colchicine-binding protein was purified from beef thyroid glands with a procedure which allows isolation of this protein from a source low in tubulin. The protein dimer has a molecular weight of 114,000. Sodium dodecyl sulfate–acrylamide gel electrophoresis indicates that the subunits have molecular weights of 55,000 ± 2000. Two monomeric subunits were identified by urea–acrylamide gel electrophoresis. Amino acid analysis shows differences from other tubulins in lysine, aspartic, and glutamic acids contents. The protein binds ~0.85 mol of colchicine and contains 0.8 mol of alkali-labile phosphate/mol of dimer. Combination of colchicine with the tubulin dimer induces marked fluorescence at 430 nm. This constitutes a convenient method for studying colchicine binding. On the basis of molecular weight, subunits, sedimentation constant, amino acid composition, and colchicine-binding activity, the protein is very similar to the subunit protein of cilia, flagella, and brain tubules.

Ample evidence has accumulated which indicates that tubulin, or the colchicine-binding protein of many tissues, is the subunit of the microtubule (Olmsted and Borisy, 1973). At the same time it has become apparent that colchicine interferes with the secretion of hormones and other cellular products and granule movement (Lacy *et al.*, 1968; Malawista, 1965; Williams and Wolff, 1970; Wolff and Williams, 1973). In the thyroid, colchicine and other agents that disaggregate microtubules inhibit TSH[1] and dibutyryl cyclic AMP-stimulated colloid droplet formation and ^{131}I release from the thyroid gland (Williams and Wolff, 1970, 1972; Nève *et al.*, 1972). The effect was specific in the sense that the hormone biosynthetic pathway and glucose-metabolizing reactions were undisturbed by colchicine concentrations that totally abolished secretion (Wolff and Williams, 1973). By correlating the antisecretory potency of various drugs with antimitotic potency and their ability to displace [^{3}H]colchicine from the binding sites, it was established that this inhibition of secretion produced by colchicine is most probably due to an effect on microtubules and is connected with binding of this drug to a specific site in the subunit tubulin. The mechanics of the participation of microtubules in the secretory process are difficult to specify. Unlike the microtubules of cilia, basal bodies, mitotic spindles, axons and even the pancreatic β cells, the microtubules of the thyroid gland show no obvious organization into structures that might participate in mechanical events (Wolff and Williams, 1973). The question arose whether or not the "unorganized" tubules from thyroid tissue consist of tubulin that is identical with the well-characterized tubulins of brain or cilia. We therefore purified and characterized this protein from beef thyroid glands.

Materials and Methods

Materials. $(NH_4)_2SO_4$ was Mann Enzyme grade. GTP (Grade II-S) was obtained from Sigma. Tritiated colchicine (ring C, methoxy-^{3}H), a product of New England Nuclear Corp., had a specific activity of 2.5 Ci/mmol. All other chemicals used were of reagent grade. The standard buffer used in most experiments contained 0.01 M sodium phosphate–0.01 M $MgCl_2$ (pH 6.5) and 0.1 mM GTP and is called PMG. When 0.25 M sucrose was present in the same buffer for homogenization it is called SPMG.

Methods. Protein was determined by the method of Lowry *et al.* (1951). Crystalline bovine albumin was used as standard. For colchicine-binding assays the DEAE-cellulose filter disk method of Weisenberg *et al.* (1968) was used as modified by Williams and Wolff (1972).

Disc gel electrophoresis was carried out in three different systems. (1) Sodium dodecyl sulfate–polyacrylamide gels were run as described by Weber and Osborn (1969) with phosphate buffer (pH 7.1). The gel contained 5% acrylamide and 0.2% bisacrylamide. The samples were run on a Büchler electrophoresis apparatus at a current of 4 mA/tube at 23° until the tracking dye reached the end of the gel. (2) Urea–polyacrylamide gels were made according to Everhart (1971) with the inclusion of 8 M urea in 5% polyacrylamide gels. Electrophoresis was performed at 400 V in 5 mM Tris–glycine buffer (pH 8.4) at 23°. (3) Discontinuous buffer systems at different gel concentrations (5, 5.5, 6, and 6.5%) were run according to Davis (1964), with 0.05 M Tris–glycine (pH 8.7) in the upper chamber, and 0.06 M Tris–0.05 M HCl (pH 7.4) in the lower and 2.5 mA/tube at 23°. In all gels, polymerization was initiated by the addition of Temed (*N,N,N′,N′*-tetramethylethylenediamine) and ammonium persulfate. The gels were stained with 0.02% Coomassie Brilliant Blue in 50% methanol and 9% acetic acid and destained in 5% methanol and 7.5% acetic acid; they were scanned with a Gilford spectrophotometer equipped with a linear transport attachment.

† From the National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20014. *Received November 29, 1973.*

[1] Abbreviations used are: TSH, thyroid-stimulating hormone; dibutyryl cyclic AMP, N^6,2-*O*-dibutyryladenosine 3′:5′-monophosphate; DEAE, diethylaminoethyl.