Pituitary Growth Hormone from the Turtle and Duck: Purification and Immunochemical Studies[1] (36436)

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We report here our preliminary studies on the purification and characterization of growth hormone (GH) from the pituitaries of two submammalian species, the turtle and the duck, representing the reptilian and avian vertebrate classes, respectively. All reports to date have dealt with the isolation and characterization of GH from mammalian pituitary glands, with the exception of an early study on GH from modern bony fishes (1). Considerable data are now available with regard to the chemical (2–7) and immunochemical properties (8–12) of a number of mammalian GHs. The possibility that pituitary GH from various vertebrate species, including nonmammalian species, may possess common structural cores, has been an intriguing question for many years (1, 13, 14). Recently the results of extensive comparative immunochemical and biological investigations of GH in pituitary extracts from various vertebrate species have been reported in which monkey antiserum to a purified mammalian GH (rat) was employed for the immunochemical studies and a mammalian species (the hypophysectomized rat) was utilized for the biological studies (12). The findings based on these studies with crude pituitary extracts have provided strong suggestive evidence that GH from species representing the various submammalian vertebrate classes share immunochemical determinants in common with mammalian GHs, and that as the phylogenetic distance of a particular species from the mammal widens, there are fewer common determinants shared with mammalian GH by the GH from that particular species.

Purification. In the present study, 1000 pituitaries (9.2 g total wt) of the Mallard duck (a variety of *Anas platyrhynchos*) and 520 pituitaries (7.0 g total wt) of the snapping turtle (*Chelhydra serpentina*) were fractionated by modification of techniques previously developed in this laboratory for the isolation of a variety of species of mammalian GH (15–17). In brief, the pituitaries (fresh-frozen prior to use) were homogenized in a Waring Blendor with cold (4°) water and extracted at pH 9.0 by adjustment of the pH with $Ca(OH)_2$ solution. The extract was fractionated with $(NH_4)SO_4$ and the fraction precipitating at 0.5 saturation was subjected to chromatography on Amberlite IRC-50 as previously described (15–17). The GH-containing fraction was applied to columns of Sephadex G-100 equilibrated with 0.05 *M* NH_4HCO_3, and the fraction which eluted with a V_e/V_o of approximately 2.0 was taken for the characterization studies reported here. A yield of 2.5 mg of duck GH and 6.1 mg of turtle GH was obtained.

Characterization studies. Preliminary bioassay studies have indicated that the purified GH from both the duck and the turtle are capable of significantly stimulating growth in the mammal on the basis of the tibial epiphyseal plate assay in hypophysectomized rats (18). This finding is in accord with previous results (12) which have shown that the crude pituitary extracts (PE) from these two particular species as well as those from a number of other submammalian species representing most of the major verte-

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TABLE I. Amino Acid Composition[a] of Turtle, Duck, and Human Growth Hormone.

Amino acid	Turtle	Duck	Human
Lysine	7.2	8.2	4.7
Histidine	2.1	2.6	1.6
Arginine	5.1	6.7	5.3
Aspartic	10.0	11.2	10.5
Threonine	5.6	5.0	5.3
Serine	8.7	7.0	9.5
Glutamic	13.7	16.3	13.7
Proline	5.7	7.2	4.2
Glycine	9.6	6.2	4.2
Alanine	7.4	6.7	3.7
½ Cystine	2.6	1.2	2.1
Valine	4.7	1.1	3.7
Methionine	ND	1.1	1.6
Isoleucine	3.8	3.5	4.2
Leucine	8.1	10.5	13.2
Tyrosine	1.6	2.1	4.2
Phenylalanine	3.1	3.7	6.9
Tryptophan	ND	ND	0.5

[a] Results are calculated as residues/100 residues analyzed; HGH data calculated from structure composition (2); ND, not determined.

brate classes are capable of effecting significant growth promotion in this mammalian test system.

The purified duck and turtle GH was examined by polyacrylamide gel disc electrophoresis (19). Both preparations showed one major band located in the area where GH is expected to migrate (20). Analysis for the presence of amino terminal amino acids was performed by the dansyl procedure (21–23). The results showed the presence of phenylalanine and leucine in both the duck and turtle GH preparations. It may be recalled here that most mammalian GHs show phenylalanine while bovine and ovine GH show alanine as well. Samples for amino acid content were hydrolyzed in constant boiling HCl at 105° for 20 hr in sealed, evacuated tubes and analyzed in a Beckman amino acid analyzer by the method of Spackman, Stein, and Moore (24). The results are tabulated in Table I and compared with human GH. All three GHs show close similarities with respect to the content of histidine, arginine, aspartic, threonine, and isoleucine. The turtle and duck have similar contents of lysine, alanine, leucine, tyrosine, and phenylalanine, in addition; while human and turtle GH are also similar in their content of serine, glutamic, 1/2 cystine, and valine.

Immunochemical characterization. In the immunochemical studies both precipitin reactions in agar gel and radioimmunoassay procedures were utilized. For the comparative immunochemical investigation cited above (12), monkey antiserum to purified rat GH had been employed in the demonstration of cross-reactions with GH in crude extracts of pituitaries from the various vertebrate species. The same antiserum was used in the present investigation. Figure 1A illustrates the results obtained with the double diffusion technique of Ouchterlony (25), in which the antiserum placed in the center well was tested against various preparations in the peripheral wells, namely, highly purified rat GH, rat pituitary extract (PE), purified duck GH, duck PE, normal rat serum, and highly purified rat prolactin. As shown, the rat GH (bottom well) gave a strong single precipitin line with the antiserum as did also a substance within the rat PE, presumably the native rat GH in the extract, as indicated by the reaction of identity (complete fusion) of this substance with the line due to the purified rat GH. No reactions were noted with rat serum proteins nor with rat prolactin. Purified duck GH gave a good precipitin line, showing a reaction of partial identity with rat GH, as indicated by the spur formed by rat GH at the point of juncture of the lines due to these two hormones. The same type of reaction had been observed in the earlier studies with the crude extract of duck pituitaries which had been utilized in place of the purified duck GH. The results of the present study shown in Fig. 1A suggest that the reaction line of duck PE is indeed due to GH contained in the extract, indicated by the complete fusion of the line with that due to purified duck GH. Essentially identical results were obtained in the current studies when purified turtle GH and turtle PE were substituted for the duck GH and PE in tests which were set up in a similar manner. In Fig. 1B is illustrated the results obtained with a guinea pig antiserum we have recently

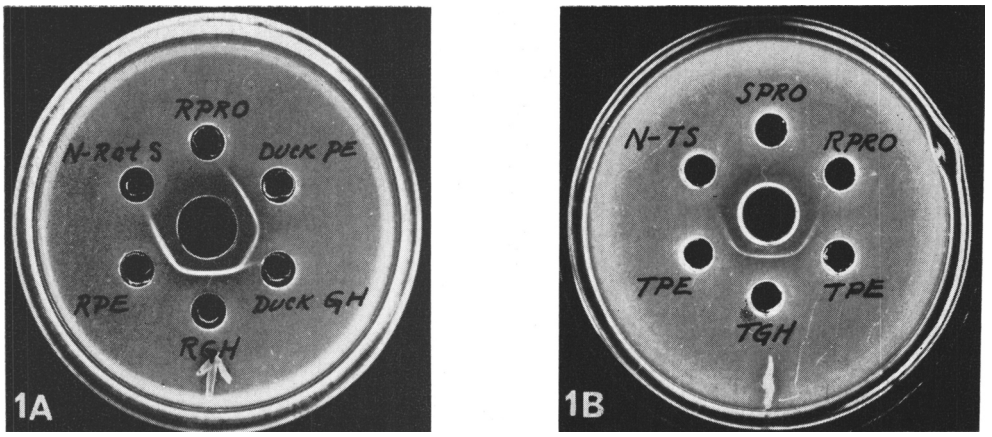

FIG. 1A. Ouchterlony plate showing the reaction of a monkey antiserum to rat growth hormone (center well, 0.15 ml) with purified rat growth hormone (RGH, 7 μg), purified duck GH (30 μg), a crude phosphate–saline extract of duck pituitaries (duck PE) or rat pituitaries (RPE). The PEs represent 0.25 mg and 0.5 mg wet weight of pituitary tissue for the rat and duck, respectively. Note the lack of reaction with rat prolactin (50 μg) or normal rat serum (1:10 dilution) (N-rat S). All peripheral wells contain 50 μl each. Photographed at 48 hr. (B) Ouchterlony plate showing the reaction of a guinea pig antiserum to turtle growth hormone (center well, 0.15 ml) with purified turtle growth hormone (TGH, 30 μg) and a crude phosphate–saline extract of turtle pituitaries (TPE). Each PE well contains the equivalent of 0.25 mg wet weight of pituitary tissue. Note the lack of reaction with normal turtle serum (1:10 dilution) (N-TS) and 50 μg each of sheep prolactin (SPRO) and rat prolactin (RPRO). All peripheral wells contain 50 μl each. Photographed at 28 hr.

prepared against turtle GH itself. It may be observed from the results shown on this plate that the antiserum gave a good precipitin line with purified turtle GH, which in turn gave a reaction of identity with a substance in turtle PE, presumably the native GH itself. The results further showed no reaction of the antiserum with turtle serum proteins nor with sheep or rat prolactin. When the same guinea pig antiserum was used in the microimmunoelectrophoresis procedure of Scheidegger (26), a single precipitin arc was formed with a substance in a crude extract of turtle (*C. serpentina*) pituitaries, presumably the native turtle GH, showing the same electrophoretic mobility as the purified turtle GH.

The immunochemical reactivities of the purified GHs from the duck and turtle sources were also examined by means of the double antibody radioimmunoassay procedure as previously described (12); the monkey antiserum to rat GH was employed at a final dilution of 1:250,000. Highly purified rat GH (3.0 USP units/mg) was used for the preparation of standards as well as for iodination with ^{131}I according to the method of Greenwood, Hunter, and Glover (27). The second antibody employed for the immunoprecipitation of the labeled rat GH consisted of rabbit antihuman γ-globulin serum. The results of radioimmunoassays shown in Fig. 2A and B were obtained several months apart, employing antisera from different bleedings taken from the same monkey. As shown, the addition of increasing amounts of unlabeled rat GH standard competitively inhibited the antiserum binding of labeled rat GH. Increasing amounts of duck PE (Fig. 2A) likewise competitively inhibited the binding of labeled rat GH, but less efficiently than did the rat GH standards, as indicated by the lesser slope for the duck PE. The purified duck GH gave a slope that was almost identical to that due to the duck PE. Turtle PE (Fig. 2B) gave a slope that was less steep than that due to purified turtle GH. This has now been observed in several assays employing 3 different lots of turtle PEs. The significance of this difference in

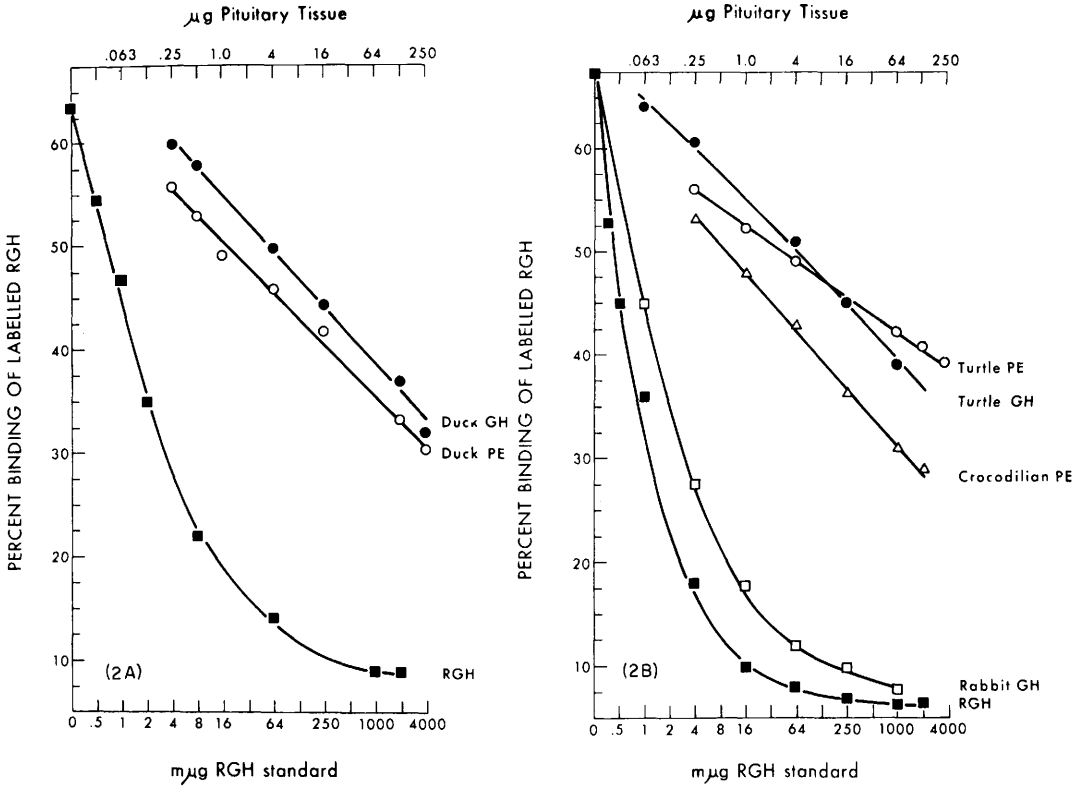

FIG. 2. Cross-reactions of duck and turtle GHs or pituitary extracts demonstrated by radioimmunoassay with monkey antiserum to rat GH (RGH). (A) Cross-reactions of duck GH and duck pituitary extract (PE). (B) Cross-reactions of turtle GH and turtle PE. Note that the slope of the curve due to the crocodilian PE closely resembles that due to turtle GH, while the curve due to GH from a mammal (rabbit) closely resembles the curve for RGH.

slopes is under current investigation. A PE from another reptilian species (a crocodilian, *Caiman sclerops*), gave a slope that was very similar to that of purified turtle GH, while a purified mammalian GH (rabbit GH, Ellis) gave a curve whose characteristics were very different from those of the reptilian species in Fig. 2B or the avian species in Fig. 2A, but rather closely resembled the curve of the rat GH standard.

The results of the studies reported here on growth hormone from nonmammalian vertebrate species show that such investigations are not only feasible with the limited amount of tissue available, but that meaningful characterization data can be required. The availability of purified nonmammalian GHs will allow more comprehensive comparative studies to be undertaken and give us further insight into the nature of this important hormone molecule and how it has been modified during the process of evolution.

Summary. Turtle and duck pituitaries were fractionated and preparations of purified growth hormone (GH) were obtained. Phenylalanine and leucine are present as N-terminal residues in both species of GH and the amino acid composition of both is similar. Studies by agar-gel diffusion and radioimmunoassay employing antirat GH serum and antiturtle GH serum showed that the purified GHs were identical to the immunoreactive material in pituitary extracts.

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