

## GROWTH HORMONE- AND PROLACTIN-LIKE PROTEINS OF THE BLUE SHARK (*PRIONACE GLAUCA*)

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### ABSTRACT

The two major disc-electrophoretic components, designated as "slow" and "fast," which are seen in fresh homogenates of the pars distalis of the blue shark (*Prionace glauca*) pituitary gland were isolated. Some alteration of the proteins occurred during the purification as evidenced by appearance of a slightly faster migrating band in each. The slow component and its altered form had a molecular weight of 22,000 daltons; the fast, and its modified form, 20,000 daltons. The two proteins were very similar chemically and immunologically, but definite differences were noted. Both gave a precipitin line with antiserum to mouse growth hormone, although quantitatively different, but neither reacted to antiserum to mouse prolactin. The peptide maps of the two proteins were identical except for three peptides. Both contained two moles of tryptophan and six half-cystine residues. These results indicate that the pars distalis of the blue shark contains two very similar proteins that resemble both growth hormone and prolactin of mammals.

There have been few reports that have dealt with the isolation of purified preparations of growth hormone and prolactin from pituitary glands of fish. This most probably stems from the fact that there is no convenient bioassay for these piscine hormones. Wilhelmi (1955) purified growth hormone from the hake; Donaldson, Yamazaki, and Clarke (1968) reported on prolactin of salmon; and Emmart and Bates (1968) described purification of prolactin of the pollack. As a means of circumventing bioassays, we thought that disc electrophoresis might prove successful. The method has been used by us to follow the purification of growth hormone and prolactin of the mouse (Cheever, Seavey, and Lewis, 1969), human (Lewis, Singh, and Seavey, 1971), and turtle (Singh, Seavey, and Lewis, 1972).<sup>4</sup> The approach appeared even more rea-

sonable for the isolation of the shark hormones when we found that the electrophoretic pattern of the pars distalis extract contained only two major bands. This has been found to be the case for many species, and growth hormone usually has been identified as the slower migrating of the two bands (Nagy, Kurz, and Baranyai, 1969; Nicoll and Nichols, 1971; Nicoll and Licht, 1971). An exception to this has been found in the human (Friesen, Guyda, and Hardy, 1970; Lewis et al., 1971) where growth hormone is more acidic than prolactin and, therefore, migrates more rapidly during electrophoresis at an alkaline pH.

Making the assumption, then, that the two major bands seen in the electrophoretic pattern of the shark pituitary extract were growth hormone and prolactin, we devised a procedure for their isolation and used electrophoresis as a means of following the purification. The isolation procedure and some properties of the purified proteins are described in this communication.

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<sup>4</sup> Singh, R. N. P., B. K. Seavey, and U. J. Lewis. 1972. Purification and some properties of growth hormone and prolactin of the turtle. (Manuscr.)

## MATERIALS AND METHODS

### DISC ELECTROPHORESIS

Our use of the method has been described (Lewis, Litteria, and Cheever, 1969). For routine analytical analyses the gel was made of 7.5% acrylamide and 0.25% N-N'-methylenebisacrylamide. For quantitation of the amount of protein in a stained band, ethylene diacrylate was substituted for the cross linking agent.

### MOLECULAR WEIGHT

Molecular weights were estimated by using disc electrophoresis (Cheever and Lewis, 1969).

### PEPTIDE MAPPING AND AMINO ACID ANALYSES

Our procedure for peptide mapping has been described (Seavey and Lewis, 1971). Total amino acid analyses were done on performic acid oxidized samples by means of automated analysis. Tryptophan was determined by the method of Beaven and Holiday (1952).

### IMMUNODIFFUSION

Mouse growth hormone prepared by the method of Cheever et al. (1969) was used for immunization of monkeys; antiserum to mouse prolactin was produced in rabbits by using hormone prepared as described in the same publication. The antiserum to mouse growth hormone was not contaminated with antibodies to mouse serum proteins or mouse prolactin; the antiserum to mouse prolactin, likewise, did not give a precipitin line with serum proteins nor did it cross react with mouse growth hormone. Immunodiffusion was carried out by the method of Ouchterlony.

### COLLECTION AND STORAGE OF GLANDS

The pars distalis was removed within 30 min after the animals were killed and immediately homogenized in water (5 ml/g wet tissue) at 5°C. The homogenate was stored at -20°C until it could be fractionated. This procedure was

used to minimize alteration of the proteins, as judged by electrophoretic analysis. More rapid changes occurred when the glands were stored intact.

### ISOLATION PROCEDURE

All processes were carried out at 5°C. The pars distalis (7.6 g wet weight) from 87 blue sharks were homogenized in 38 ml water. The pH of the homogenate was adjusted to 10 with dilute NaOH and made  $10^{-3}$  M in diisopropylphosphofluoridate. After 4 hr of stirring the homogenate was centrifuged at  $20,000 \times g$  for 40 min. The supernatant fluid was decanted, its pH lowered to 8 with dilute HCl and set aside. The insoluble residue was suspended in 0.05 M  $\text{Na}_2\text{CO}_3$ - $\text{NaHCO}_3$  buffer, pH 10 (2.5 ml/g original tissue) and stirred for 16 hr. After centrifugation, the supernatant fluid was pooled with the first extract and the pH of the mixture adjusted to 8. The insoluble tissue was discarded when electrophoresis showed that all the desired components had been extracted.

The crude extract was concentrated to about 15 ml on a Diaflo UM-10 membrane<sup>5</sup> (Amicon, Lexington, Mass.) and placed on a column of Sephadex G-150,  $5 \times 90$  cm and developed with 0.01 M  $\text{NH}_4\text{HCO}_3$ . Every fifth tube was analyzed by disc electrophoresis (100  $\mu$ liter aliquots) and the tubes that contained the two proteins that were assumed to be growth hormone and prolactin were combined and concentrated to about 10 ml on a UM-10 Diaflo membrane. Figure 1 shows the elution pattern of the Sephadex column; the stippled area contained the desired components, S and F.

The concentrated solution from the Sephadex chromatography was applied to a column of DEAE-cellulose (Whatman 32) with dimensions of  $0.9 \times 10$  cm and which had been equilibrated with 0.01 M  $\text{NH}_4\text{HCO}_3$ . The flow rate was 38 ml/hr. Once the sample had been applied, the column was washed with 25 ml of 0.01 M  $\text{NH}_4\text{HCO}_3$ . No appreciable amount of protein

<sup>5</sup> Reference to trade names in this publication does not imply endorsement of commercial products by the National Marine Fisheries Service.

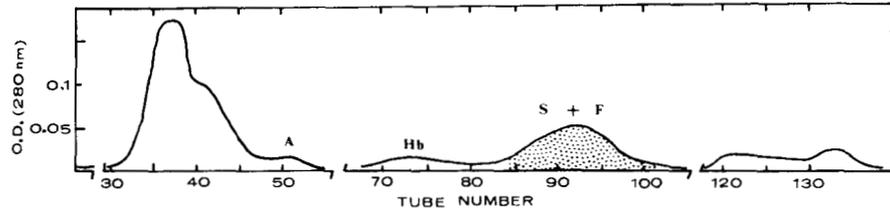


FIGURE 1.—Elution pattern obtained by chromatography of an alkaline extract of the pars distalis on Sephadex G-150. Each tube had a volume of 14 ml. The slow and fast electrophoretic components (S and F) were found in the stippled area. A = albumin; Hb = hemoglobin.

was eluted by the process. A gradient was next begun and was made by mixing equal portions (100 ml each) of 0.01 M and 0.1 M  $\text{NH}_4\text{HCO}_3$ . This eluted a major peak. A second gradient of equal volumes (150 ml each) of 0.1 M and 0.2 M  $\text{NH}_4\text{HCO}_3$  was begun immediately. This gradient eluted a second peak. The results obtained with the DEAE-cellulose chromatography are shown in Figure 2.

The two major peaks eluted during chromatography on DEAE-cellulose were lyophilized. The yield of component F was 43 mg; that of component S, 22 mg.

## RESULTS

### ELECTROPHORETIC ANALYSES

#### Pituitary Extracts

The water homogenate of the pars distalis showed two principal electrophoretic components when analyzed within 30 min after the sharks were taken from the sea. An alkaline extract of the glands, made about a week later, gave an almost identical pattern except that a definite band could be seen just ahead of each of the major components S and F. This is shown in Figure 3. The S and F components were assumed to be growth hormone and prolactin, respectively, and were the components for which we devised an isolation procedure. The bands designated as A, B, and C were minor components seen in both the water homogenate and the alkaline extract. A fast component also migrated near the buffer front.

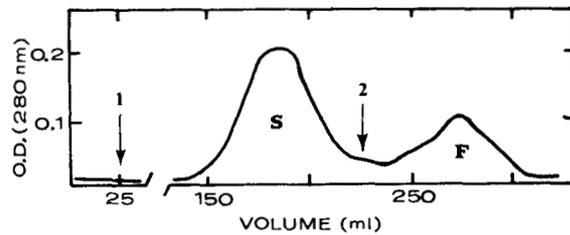


FIGURE 2.—Elution pattern obtained by chromatography of the S + F fraction of Figure 1 on DEAE-cellulose. The first gradient was begun at 1 and a second at 2. The S and F components were resolved as indicated in the drawing.

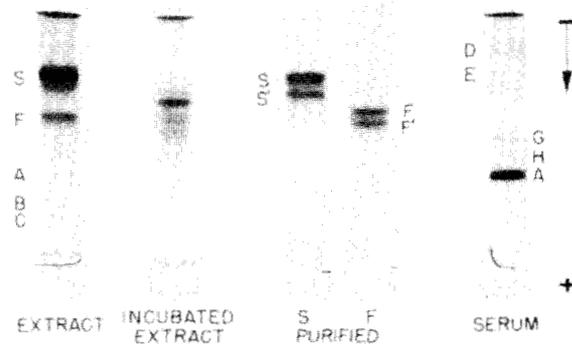


FIGURE 3.—Electrophoretic patterns (pH 9.5) of various shark materials. The pattern for the incubated sample was obtained after heating the alkaline extract at  $37^\circ\text{C}$  for 2 hr. S and F are the slow and fast components; S' and F' are the bands that formed during isolation of the proteins. Components A through E are discussed in the text. A thread marks the original buffer front (bromphenol blue) in two of the gels.

The conversion of the S and F bands to more rapidly migrating components became more pronounced as the fractionation of the extract progressed. As seen in Figure 3, by the time the proteins were isolated, each was a definite doublet. The new components are designated as S' and F'. The same type of alteration of the S and F proteins occurred even during storage of intact glands at  $-20^{\circ}\text{C}$ . After one month, the S' and F' were of about equal intensity to that of the original S and F bands. The alteration was barely noticeable however in a water homogenate of glands stored for the same length of time at  $-20^{\circ}\text{C}$ . We determined also how incubation at  $37^{\circ}\text{C}$  affected the conversion of components S and F. After 2 hr at that temperature, S and F had virtually disappeared and a new band intermediate in mobility between S and F was present. This is shown in Figure 3. All bands disappeared if the extract was left at  $37^{\circ}\text{C}$  for 6 hr.

#### Serum

To determine whether either S or F might be serum components we analyzed a sample of shark serum (Figure 3). There was a faint band (E) in the region of S but none for F in gels of 7.5% acrylamide. At 10% and 12% acrylamide band E was more retarded than S, however. Component A of the pituitary extract lined up with the major component of serum in four concentrations of acrylamide.

#### PURIFICATION

The elution pattern obtained from chromatography of the alkaline extract on Sephadex G-150 is shown in Figure 1. As described in the experimental section above, the S and F components were located by disc electrophoresis and both were found in the same elution peak (stippled area). The peak of high molecular weight material at the beginning of the elution pattern gave an abnormally high optical density reading because of opalescence. The peak designated as A had the same electrophoretic pro-

perties of band A of Figure 3 and, as indicated above, was presumed to be albumin. The peak eluted just before S and F was labeled Hb (hemoglobin) because of its red color. Electrophoretically the material gave two very diffuse bands.

Figure 2 shows the elution pattern obtained by chromatography of Sephadex peak S + F on DEAE-cellulose. No protein was eluted with 0.01 M  $\text{NH}_4\text{HCO}_3$ . The first major peak, found to be F by electrophoretic analysis, was eluted by gradient 1 (0.01 M to 0.1 M  $\text{NH}_4\text{HCO}_3$ ). A second gradient (0.1 M to 0.2 M  $\text{NH}_4\text{HCO}_3$ ) eluted a second peak which was found to be component F by electrophoresis. The shoulder on the leading edge of peak F was probably a result of a small, rapid increase in ionic strength when the second gradient began.

The material of peaks S and F were lyophilized. The yield of S was 43 mg and 23 mg for F. Figure 3 shows the electrophoretic pattern of each of these samples. That each was a doublet was a result of alteration that took place during isolation. The conversion became progressively more pronounced during the purification.

#### MOLECULAR WEIGHTS

The molecular weights of components S and F as seen in electrophoretic patterns of the alkaline extract were  $S = 22,000$  and  $F = 20,000$ . Identical values were obtained when the purified S and F fractions were analyzed. Likewise S' and F' had molecular weights indistinguishable from the corresponding S or F form although the molecular weight determination is accurate only to  $\pm 2,000$ . The  $\log R_m$  vs. gel concentration curves for these components are shown in Figure 4. Of significance was the fact that there was a greater charge difference between S and S' than between F and F'. This is additional evidence that S and F are different substances. The slopes of the curves of Figure 4 were converted to molecular weight values by reference to a standard curve constructed from proteins of known molecular weight (Cheever and Lewis, 1969).

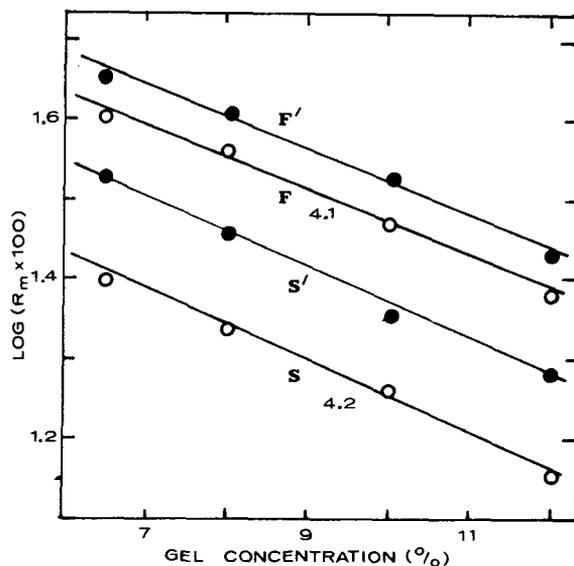


FIGURE 4.—A Ferguson plot of the relative mobility ( $R_m$ ) of the S and F components and their altered forms ( $S'$  and  $F'$ ) versus the concentration of acrylamide used for the gel-electrophoresis.

The molecular weights of the other electrophoretic components seen in Figure 3 were A = 68,000; B = 65,000; C = 60,000; D = 47,000; E = 71,000; G = 64,000; H = 72,000.

#### IMMUNODIFFUSION

The purified preparations of S and F, which also contained  $S'$  and  $F'$ , respectively, produced a precipitin line when tested against antiserum to mouse growth hormone (Figure 5). When tested at the same concentration, S gave a more pronounced line than did F. Neither preparation gave a precipitin line with antiserum to mouse prolactin.

#### AMINO ACID COMPOSITION

Table 1 gives the amino acid analyses of components S and F. The composition of the two proteins was quite similar but there were definite differences and component S had a greater total number of amino acids. Both contained six half-cystines and two tryptophans.

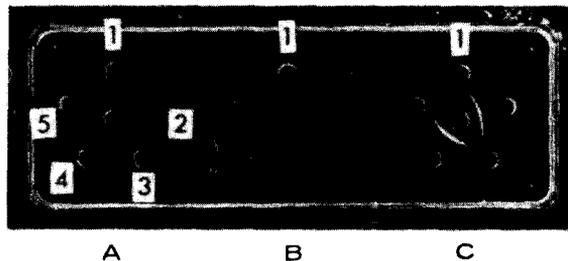


FIGURE 5.—Immunodiffusion plate for the S and F proteins. Antiserum to mouse growth hormone (10  $\mu$ liter) was in the center well of A and B; antiserum to mouse prolactin was in the center well of C. The outer wells of A contained: 1, 10  $\mu$ g component S; 2 and 5, 5  $\mu$ g mouse growth hormone; 3, 5  $\mu$ g component S; 5, 2  $\mu$ g component F. The outer wells of C contained: 1, 10  $\mu$ g component S; 2, 5  $\mu$ g mouse prolactin; 3, 10  $\mu$ g component F; 4, 5  $\mu$ g mouse prolactin. The picture was taken after 24 hr at room temperature.

TABLE 1.—Amino acid composition of performic acid oxidized components S and F.

Amino acid	Component S residues <sup>1</sup>	Component F residues <sup>1</sup>
Cysteic acid	6.0	6.0
Aspartic acid	22.6	21.7
Methionine sulfone	4.8	4.0
Threonine	10.3	8.0
Serine	19.7	13.1
Glutamic acid	31.2	31.5
Proline	10.6	7.4
Glycine	10.2	8.4
Alanine	15.7	10.3
Valine	9.0	5.6
Isoleucine	6.3	6.9
Leucine	25.8	20.6
Tyrosine	6.9	3.8
Phenylalanine	10.1	7.2
Histidine	6.5	4.7
Lysine	17.5	15.0
Arginine	14.0	7.5
Tryptophan <sup>2</sup>	2.1	2.1

<sup>1</sup> Based on 22,000 daltons for S and 20,000 for F.  
<sup>2</sup> Spectrophotometric (Beaven and Holiday, 1952).

#### PEPTIDE MAPS

Figure 6 is a drawing of the peptide map obtained with component S. The F component gave an almost identical map but was different in that peptides labeled S were not seen and another peptide labeled F was noted.

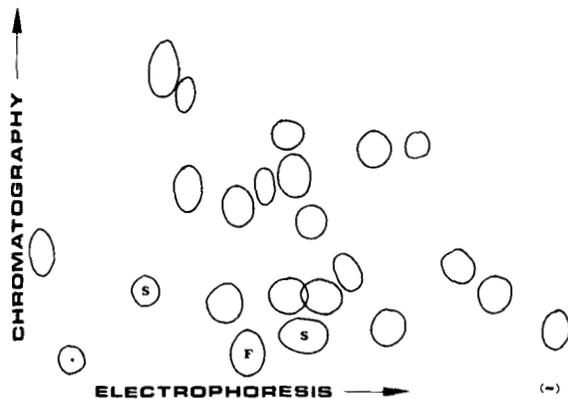


FIGURE 6.—Peptide map of a tryptic digest of component S. Chromatography was done with n-butanol-acetic-acid-water (4:1:5); electrophoresis was at pH 3.6 in a pyridine-acetic acid-water buffer (1:10:289). The peptide map of component F was identical except that peptides designated S were not seen and the peptide F was present.

## DISCUSSION

From the immunodiffusion data we know that the S and F proteins are related to mammalian growth hormone. Their molecular weights also were near the values found for mammalian growth hormone and prolactin. One other observation which will be reported in detail later (Seavey et al., 1972)<sup>o</sup> was that the amino acid compositions of the tryptic peptides of the peptide maps closely resembled those of bovine growth hormone (Seavey et al., 1971). But because there were six half-cystine residues in both S and F and because each contained two residues of tryptophan, these two shark proteins are more like mammalian prolactins than the growth hormones which have only four half-cystines and one residue of tryptophan. Therefore, the proteins are not only very similar to each other, but are immunologically related to growth hormone and have structural resemblances to both growth hormone and prolactin. These results support the view that growth hormone and prolactin may have developed from a common ancestral

<sup>o</sup> Seavey, B. K., R. N. P. Sigh, U. J. Lewis, and R. Lasker. 1972. Tentative structure for a growth hormone-like, prolactin-like protein of the shark.

protein. In the blue shark they appear as separate but yet quite similar substances with similarities to both growth hormone and prolactin. It will be interesting to see if there are two such proteins in the pituitary gland of cyclostomes.

If not already clear we would like to stress the point that no biological activities have been determined for the S and F components. Our assignment of growth hormone-like and prolactin-like properties are based on immunological and structural data alone. How these two components are related to the biological activities that have been reported for the shark pituitary gland is not known. Extensive biological studies are now needed to characterize these proteins. Also, more study will be given to the altered forms of the substances, and in particular the modification that was produced by incubation at 37°C. This type of study will be given to the altered forms of the hormones because of the rapid alterations that occurred even in the intact pituitary of the shark. We have observed that the prolactin activity of human growth hormone is increased when a hexapeptide is removed from the hormone by an enzyme present in pituitary extracts (Lewis et al., 1971). Biological activity of the shark hormones may also be affected by such changes. The nature of the alteration that occurs in the shark proteins is not known, but from our experiences with mammalian hormones we suspect that it is a result of deamidation, enzymic action, or a combination of both. The ease with which the shark's S and F components are converted to more acidic forms, however, strongly suggests deamidation, and the unusually large number of glutamic acid residues may actually be glutamines which rapidly lose ammonia.

## ACKNOWLEDGMENTS

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### ADDENDUM

The S component of the shark pituitary gland was tested for its ability to produce growth in hypophysectomized *Fundulus heteroclitus*. The results are shown in Figure 7. The preparation was found to be as potent as a previously assayed sample of hake growth hormone (Wilhelmi, 1955).

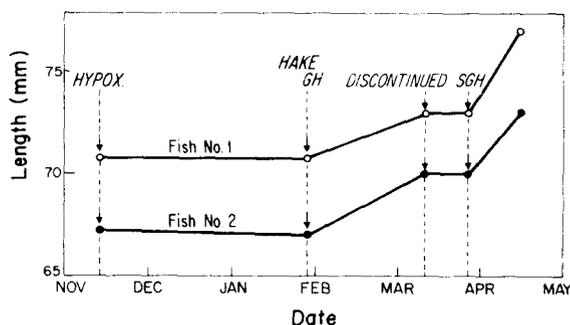


FIGURE 7.—Growth of two hypophysectomized male *Fundulus heteroclitus* after administration of growth hormone. Eleven weeks after hypophysectomy (HYPOX), injections of hake growth hormone (HAKE GH) (Wilhelmi, 1955) were begun and continued for five weeks. The injections were discontinued for two weeks and then resumed with shark growth hormone (SGH) for a period of four weeks. The S component of the shark pituitary gland was used. Both the hake and shark growth hormones were injected three times weekly at a dosage of 20  $\mu$ g of hormone per gram (wet weight) of fish.

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