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PURIFICATION OF GONADOTROPIN FROM RAINBOW TROUT (*SALMO GAIRDNERII* RICHARDSON) PITUITARY GLANDS

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SUMMARY

A method of purifying trout gonadotropic hormone is discussed. The technique consists of the following stages : alcoholic extraction, two successive gel filtrations on Sephadex G-50 and G-100, chromatography on DEAE-cellulose and ultrogel ACA54. The biological activity of fractions obtained is measured using as assay the maturation *in vitro* of trout oocytes.

The preparation obtained has a molecular weight of about 27500, determined by behavior on Sephadex-G 100 and analytical ultracentrifugation for a KS_{20w} of 2.45. Polyacrylamide gel electrophoresis shows two strips of electrophoretic mobility, 0.50 and 0.58. Analysis of the amino acid composition of the preparation indicates a number of 160 AA residues per mole.

These different values are compared to the corresponding ones for c-GtH, s-GtH and mammalian gonadotropins.

INTRODUCTION

The purification of fish gonadotropin has encountered a major problem up to now, namely, a lack of sufficiently sensitive and specific biological assays for a given species (FONTAINE *et al.*, 1972 ; BRETON *et al.*, 1973). However, a Carp pituitary gonadotropin (c-GtH) has been prepared for the first time in a highly purified state by BURZAWA-GERARD (1971) using the frog spermiation assay, criteria of little specificity. The same assay was also used by GONDCHAROV (1972) to prepare sturgeon GtH, while DONALDSON *et al.* (1972) employed a variation (YAMAZAKI and DONALDSON, 1968) of the goldfish gonad hydratation assay (CLEMENS *et al.*, 1964 *a* et *b*) to obtain gonadotropic hormone from salmon pituitary. In all cases, gametogenesis promoting activity in fish was only controlled *a posteriori* (BILLARD *et al.*, 1970 ; DONALDSON *et al.*, 1972 ; SUNDARARAJ *et al.*, 1972).

In the present work, the purification of a rainbow Trout gonadotropin was undertaken using as bioassay the *in vitro* intrafollicular maturation of Trout oocytes

(to avoid species specificity), which can be considered as representative of a typical gonadotropic activity (JALABERT *et al.*, 1974) since, as far as now, only one gonadotropin seems to promote both follicular growth (vitellogenesis) maturation and ovulation in fishes (SUNDARARAJ *et al.*, 1972 ; BURZAWA-GERARD and FONTAINE, 1972 ; BURZAWA-GERARD, 1973).

MATERIAL AND METHODS

Pituitaries were taken from adult (3 years minimum) rainbow trout during spawning.

The following preparation techniques were used :

— alcohol extraction according to SCHMIDT *et al.* (1965) ;

— gel filtration on Sephadex G-50 and G-100 ;

— ion-exchange chromatography on DEAE-cellulose Whatman DE 23 with ionic force gradient ;

— chromatography on agarose acrylamide gel ACA54 (I. B. F. ultrogel commercialized by LKB France) ;

— concentration of protein solutions between each purification stage was done on Amicon UM 05 membrane in a Diaflo chamber under nitrogen pressure, followed by equilibrium dialysis against the proper buffers for subsequent chromatography ;

— protein concentration of chromatographic effluents was determined by Folin reaction using the technique of LOWRY *et al.* (1951).

The preparations obtained were analyzed using the following physico-chemical techniques :

— determination of electrophoretic mobilities by polyacrylamide gel electrophoresis in 8 p. 100 gel, using ORNSTEIN's technique (1964), and Isco gel scanner reading ;

— study of comportment on Sephadex G 100 (exclusion volume-molecular weight), according to LAURANT and KILLANDER's technique (1964) ;

— analysis of ultraviolet absorption spectra on a Carry spectrophotometer ;

— measurement of sedimentation coefficient $KS_{20}w$ and molecular weight (equilibirium sedimentation) by analytical ultracentrifugation (Beckman Spinco analytic ultracentrifuge, Model E, equipped with an ultraviolet scanning device) ;

— analysis of amino acid composition after 24-hours acid hydrolysis with HCl 6N at 110°C. Free amino acid assay was done using the technique of MOORE *et al.* (1958) with a Beckman 120B analyzer for preparation 1, and MENEZO's technique (1973) with an Optica aminolyzer for preparation 2.

RESULTS

1. — *Alcohol extraction*

Immediately after collection, pituitaries are frozen into liquid nitrogen, lyophilized and reduced to acetonic powder, according to the technique described for mammalian glycoprotein hormones by COURTE (1970).

The acetonic powder is then extracted for 24 hours at 4°C with a solution 40 p. 100 ethanol and 2 p. 100 NaCl, (100 ml of solution for 2.5 g of acetonic powder). After 20 minute centrifugation at 5 500 g, the precipitate is extracted twice again in the same volume of alcoholic solution. The supernatants are pooled and adjusted to pH 5.2 with glacial acetic acid, and the alcohol content brought to 85 p. 100 by a slowly addition of absolute alcohol. After precipitation for 24 hours at 4°C, the suspension obtained is centrifuged 20 minutes at 36 000 *g*. The dry centrifuged residue constitutes the pituitary protein extract, EPH.

In two successive year preparations the ponderal and activity yields are similar (tabl 1).

TABLE I

Ponderal and biological activity yields at different stages of t-GtH purification

Prepa-ration	Yields	Purification stage						
		Acetone powder	EpH	G-50	G-100	DEAE C	Ultrogel ACA54	Overall yield
1	mg active material	2 400	290	117	34.18	6.72		
	p. 100 ponderal yield		12.08	40.3	29.2	19.7		0.28
	mean activity/mg		1	2.14	5.64	2.83		
	p. 100 activity yield	0.125	96.6	86.3	67.9	19.6		6.33
2	mg active material	3 500	376	113	49.2	18.12	5.04	
	p. 100 ponderal yield		10.74	30.0	43.5	36.9	27.8	0.14
	mean activity/mg	0.125	1	3.01	5.75	6.07	8.44	
	p. 100 activity yield		86.04	90.40	82.9	39.8	39	24.90

2. — *Gel filtration on Sephadex*

EPH is fractionated by Sephadex G-50 gel filtration on a 60 × 2.5 cm column with *Tris*-HCl 0.05M buffer, pH 7.8 for the first preparation ; the same buffer of 0.01M molarity at pH 8.0, containing 0.5mM dithiothreitol, is used for the second preparation.

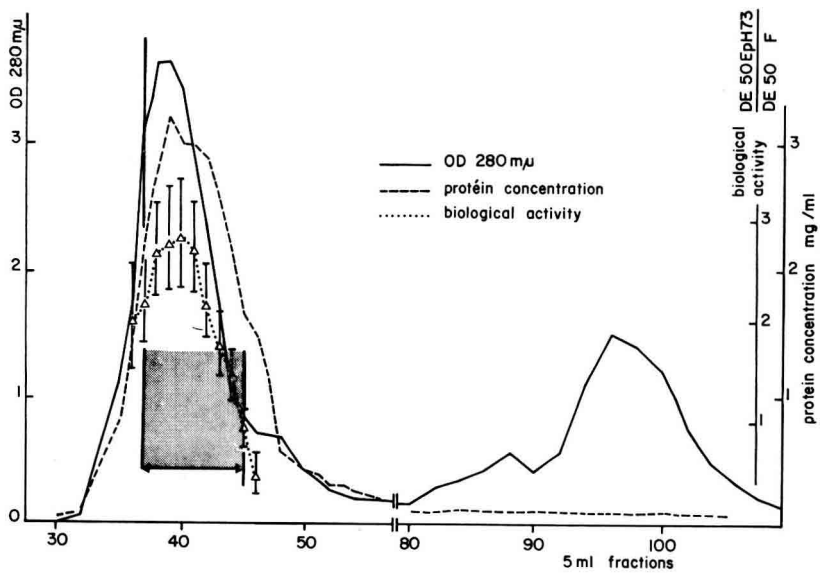


FIG. 1. — *Gel filtration on Sephadex G-50*
(buffer *Tris* HCl 0.05M, pH 7.8, 2.5 × 60 cm column)
of alcoholic extract of rainbow trout pituitary glands.
Fractions 37 to 45 were pooled for chromatography on Sephadex G-100
Vertical bars indicate the confident limits at the 95 p. 100 probability level

Figure 1 shows the elution diagram obtained in the first purification. Biological activity is found between fractions 37 and 45, in the drop of the protein peak coming out in the exclusion volume of the column. Employing the second buffer system does not change the yield or biological activity.

After concentration, the active fractions are re-chromatographed on Sephadex G-100 in the same two buffer systems on a 100 × 2.5 cm column. Activity peaks are found between fractions 30 and 37 (fig. 2) for the first preparation (Kd : 0.29), and between 29 and 39 for the second (Kd : 0.29). In this case, yields are higher than those obtained at pH 7.8 in absence of dithiothreitol, and there is no notable variation of mean activity per mg of protein.

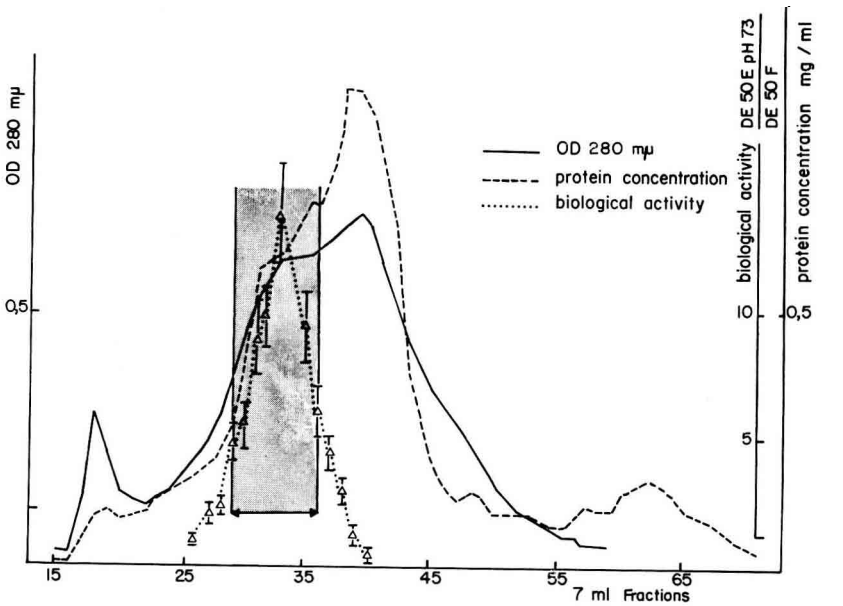


FIG. 2. — *Gel filtration on Sephadex G-100*
(buffer *Tris* HCl 0.01M, pH 8.0, dithiothreitol 0.5 mM, 2.5 × 100 cm column)
of gonadotropically active fractions after chromatography on Sephadex G-50. Fractions 30 to 37 were pooled for chromatography on DEAE-cellulose

3. — *Chromatography on DEAE-cellulose DE 23*

Table 2 shows results obtained in different buffer systems of fixation and elution. Best results are obtained by using a *Tris*-HCl 0.01M fixation system, pH 8.4, containing 0.5 mM dithiothreitol, elution is carried out using a linear gradient of the buffer and NaCl 0.5M (500 cc — 500 cc) (fig. 3). Protein yield is similar at this pH in absence of dithiothreitol and a shorter gradient (400-400 starting at 0.05M). However, protein activity drops as compared to that obtained at the preceding stage (table 2). At pH 7.8, besides loss of biological activity (0.7 U/ml), yields become very poor due to incomplete fixation (54 p. 100) of active material on the ion exchanger and to considerable dilution of activity on the whole chromatogram (fig. 4). The purification of the t-GtH_1 is stopped at this stage.

TABLE 2

Comparison of different chromatographic systems on DEAE-cellulose for purification of trout gonadotropin (t-GtH)

Chromatographic system	Deposit activity	Uptake of activity	Recovered activity	Mean activity/mg	Total protein yield
Tris-HCl, 0.05M, pH 7.8 (400) NaCl, 0.05M (400)	96.3	54.9 (51 %)	5.79 (6 %)	0.70	23.66 %
Tris-HCl, 0.05M, pH 8.4 (400) NaCl, 0.05M (400)	96.3	91.4 (95 %)	19 (19.6 %)	2.83	69.7 %
Tris-HCl, 0.01M, pH 8.4 dithiothréitol 0.5M (500) NaCl 0.5M (500)	281	266.9 (95 %)	109 (38.7 %)	6.07	66.5 %

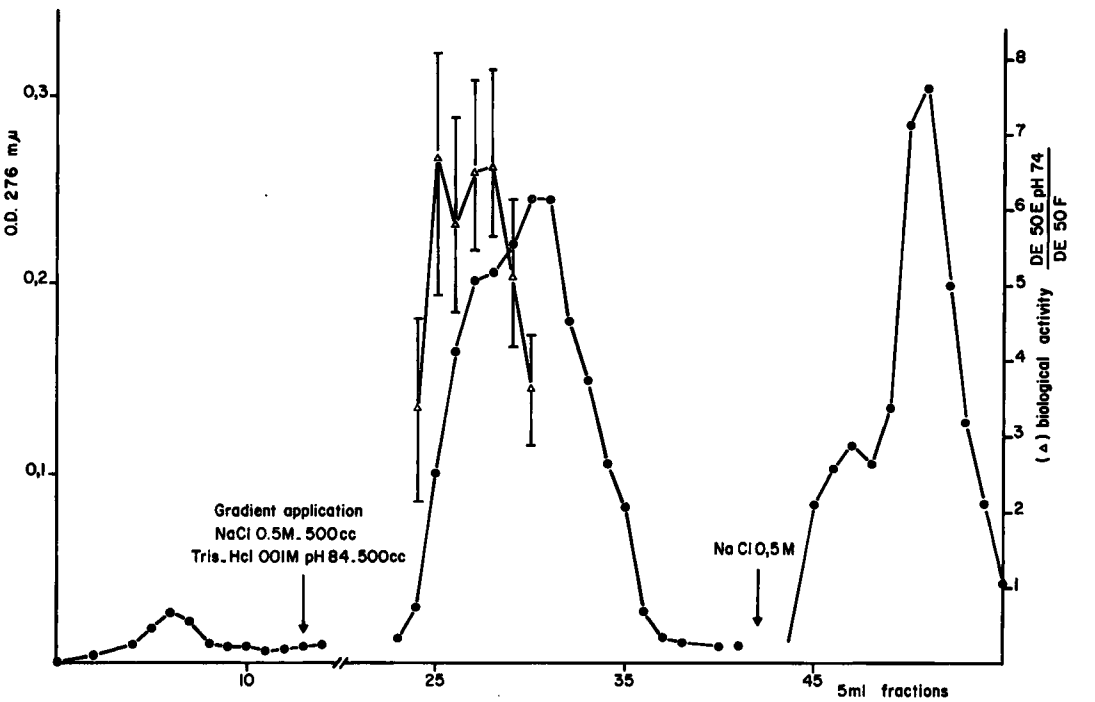


FIG. 3. — *Chromatography on DEAE-cellulose Whatman DE 23* (equilibriated in buffer *Tris* HCl 0.01M, pH 8.4, dithiothreitol 0.5 mM) *of eluted gonadotropic activity after filtration on Sephadex G-100. Elution is done with linear ionic force gradient* (500 cc of equilibriating buffer, 500 cc NaCl 0.5M), *1.5 × 25 cm column*

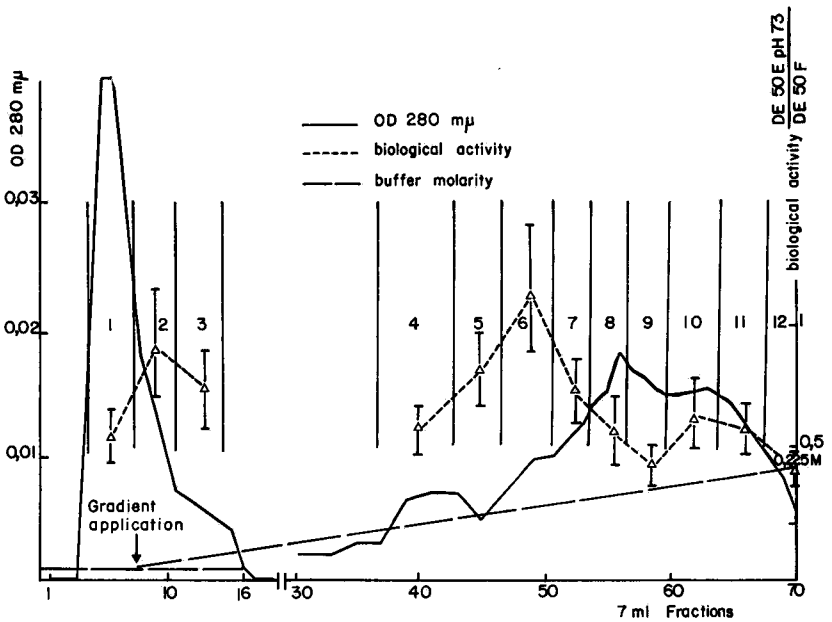


FIG. 4. — *Chromatography on DEAE-cellulose Whatman DE 23* (equilibriated in buffer *Tris* HCl 0.05M, pH 7.8) *of eluted gonadotropic activity after filtration on Sephadex G-100. Elution is done with linear ionic force gradient* (400 cc of buffer and equilibriator, 400 cc NaCl 0.5M), *1.5 × 25 cm column*

4. — *Chromatography on ultrogel ACA54 gel*

Only t-GtH$_2$ was submitted to this chromatography in ammonium bicarbonate buffer 0.01M, pH 8.0. Activity is found in the first elution peak (Kd : 0.285). No activity is found in the second peak, which includes a protein complex of about 15 to 17 000 in molecular weight (fig. 5).

Table 3 shows the plan used for purification of trout (t-GtH) gonadotropin : 3 500 mg pituitary acetonic powder give 5.04 mg active material presenting an activity gain of 67.5, as compared to the acetonic powder.

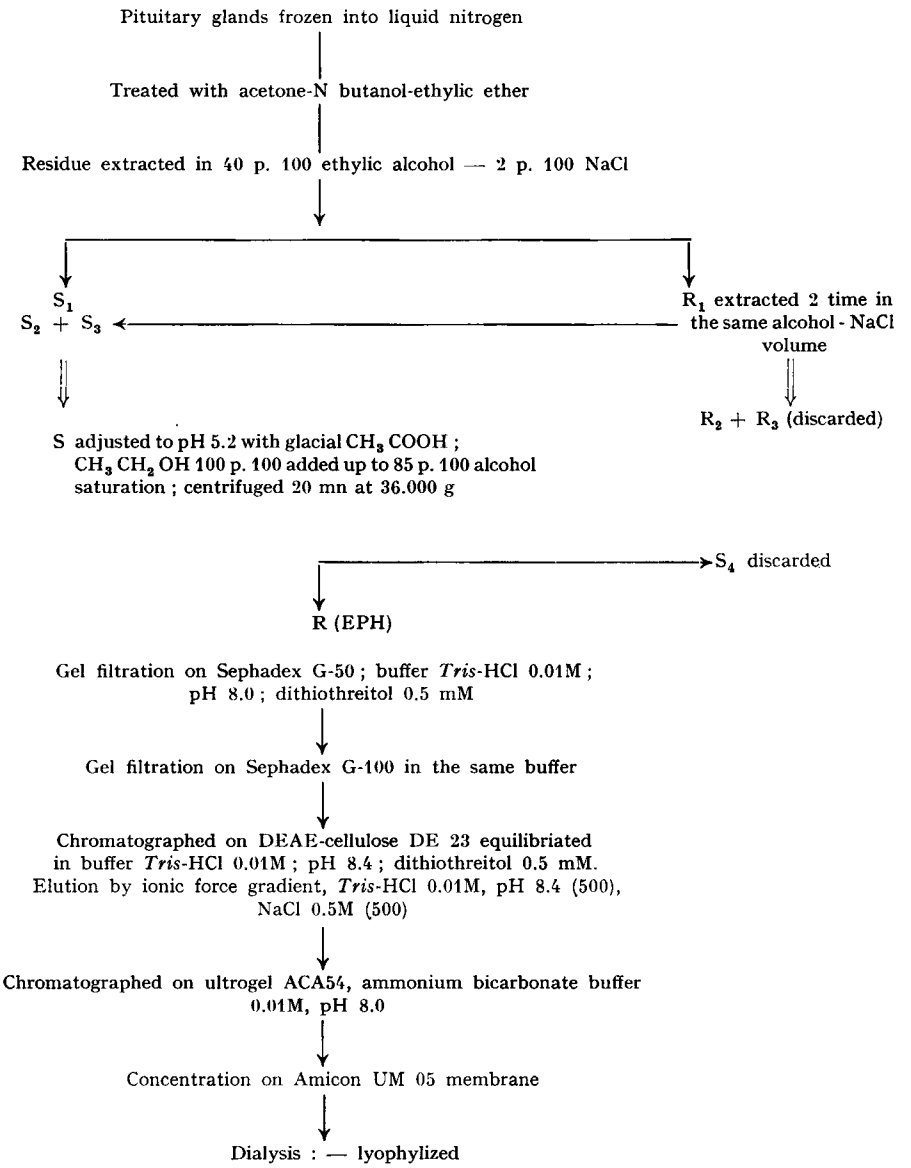
5. — *Physico-chemical characteristics*

Overall results are shown in table 4. The Kd and molecular weight, determined on Sephadex G-100 are identical for the two t-GtH preparations and comparable to those of the c-GtH prepared by BURZAWA-GERARD (1973). On the other hand, sedimentation coefficients and molecular weight, determined by equilibrium ultracentrifugation, are different for the two trout preparations, but comparable for the t-GtH$_2$ and c-GtH. The equilibrium sedimentation peak is apparently homogenous.

In polyacrylamide gel electrophoresis at 8 p. 100 pH 9.4 (at concentration of 1 mg/cc), the protein obtained seems to be heterogenous with two electrophoretic Rf strips 0.50-58.

TABLE 3

Outline of purification procedure for trout gonadotropin (t-GtH)



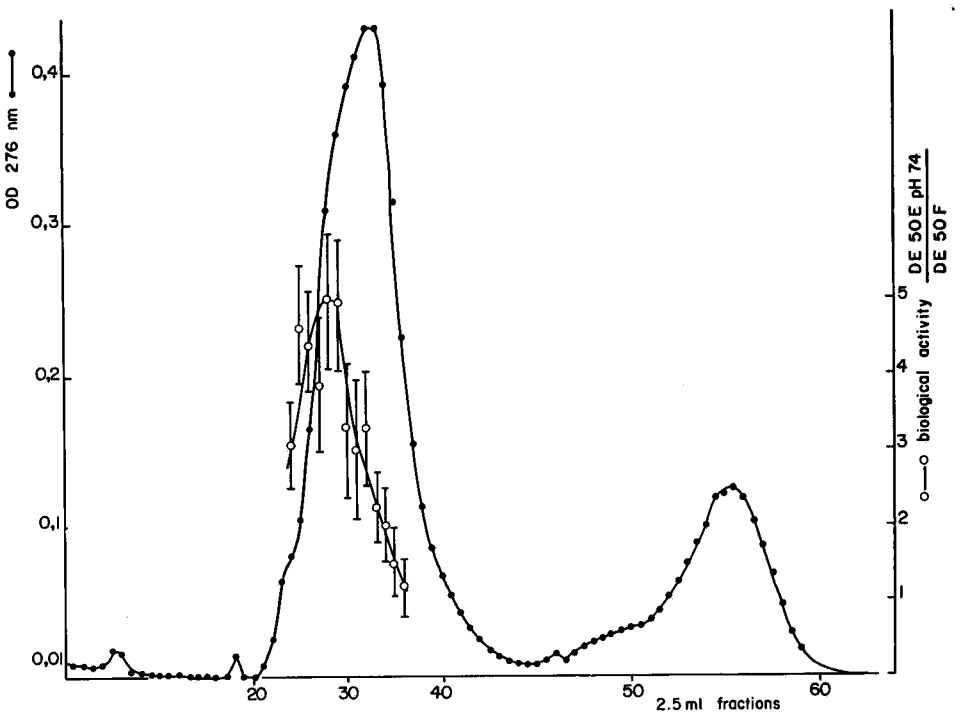


FIG. 5. — *Chromatography on ultrogel ACA54*
(ammonium bicarbonate buffer 0.01M, pH 8.0, dithiothreitol 0.5 mM, 1.5 × 40 cm column)
of active fractions after chromatography on DEAE-cellulose

TABLE 4

Comparison of physico-chemical characteristics of t-GtH$_1$, t-GtH$_2$ and c-GtH

Preparation	Kd	PM_{G100}	Ks_{20W}	PM_{20W}	Mobility electrophoretic
t-GtH$_1$	0.29	27 500	1.40	15 500	0.31-0.41-0.45
t-GtH$_2$	0.29	27 500	2.45	28 500	0.50-0.58
c-GtH BURZAWA (1973)	0.280	27 000	2.67	28 500	0.50-0.54

Kd : exclusion coefficient on Sephadex G-100
PM_{G100} : Apparent molecular weight determined on Sephadex G-100
KS_{20W} : Sedimentation coefficient with analytical ultracentrifugation
PM_{20W} : Apparent molecular weight obtained with equilibrium ultra-centrifugation.

Amino acid compositions of t-GtH_1 and t-GtH_2 are given in table 5 and compared to values obtained by BURZAWA-GERARD (1973) on c-GtH.

Compositions, expressed in residues/100 amino acid residues, are comparable from one preparation to another. Comparison of the numbers of residues/mole for t-GtH_2 and c-GtH (160-168) shows large differences, mainly in valine and tyrosine, and many similarities : arginine, glutamic acid, glycine, alanine, hemicystine, phenylalanine. Between the two preparations of t-GtH the number residues per mole increases from 125 for t-GtH_1 to 160 for t-GtH_2.

The ultraviolet absorption spectra presents a maximum at 276 mμ, characteristic of tyrosinized protein.

TABLE 5

Amino acid composition of trout and carp gonadotropic hormones

Hormone	t-GtH_1		t-GtH_2		c-GtH BURZAWA GERARD (1973)	
AA	residues/ mole	residues/ 100 residues	residues/ mole	residues/ 100 residues	residues/ mole	residues/ 100 residues
Lysine	4.68	3.74	9.80	5.69	11.3	6.7
Histidine	3.11	2.48	6.3	3.65	5.4	3.2
Arginine	4.46	3.56	6.9	4.00	7.0	4.1
Aspartic acid	14.32	11.45	17.76	10.3	14	8.4
Threonine	11.23	8.98	15.19	8.8	13	7.7
Serine	8.35	6.67	8.88	5.10	11.5	6.8
Glutamic acid	15.74	12.58	15.75	9.10	15.5	9.2
Proline	11.61	9.28	11.6	6.74	14	8.3
Glycine	6.06	4.84	7.0	4.30	6.5	3.9
Alanine	3.57	2.85	5.79	3.36	6.4	3.8
Hemicystine	11.38	9.09	12.03	6.97	11.5	6.9
Valine	8.89	7.08	12.0	7.09	19.4	11.5
Methionine	2.02	1.81	3.70	2.15	2.6	1.5
Isoleucine	4.92	3.98	7.0	4.06	4.8	2.8
Leucine	6.98	5.58	10	5.79	12.5	7.4
Tyrosine	3.82	3.05	4.97	2.88	8.1	4.8
Phenylalanine	3.97	3.17	5.4	3.13	4.7	2.8
N. residues/mole	*125*		*160*		*168*	

DISCUSSION

With the techniques described, a gonadotropic hormone (t-GtH) has been obtained from trout pituitary which is 67 times more active than the crude pituitary acetonic powder. The ponderal yield is 0.14 p. 100. This technique resembles those used by BURZAWA-GERARD (1971) for carp gonadotropic hormone and by DONALDSON *et al.* (1972) for the salmon one (s-GtH).

The physico-chemical characteristics of the three hormones are similar as regards molecular weight determined on Sephadex G-100, sedimentation coefficients and molecular weight determined by equilibrium ultracentrifugation. The constants are compa-

rable to those of o. LH and o.FSH (PAPKOFF, 1971 ; CAHILL and HART, 1969). As do carp and salmon hormones, trout gonadotropin gives two strips of similar mobility in polyacrylamide gel electrophoresis characteristic of a molecular polymorphism (BRASELTON and McSHAN, 1970 ; COURTE, 1970). If the t-GtH$_1$, obtained after chromatography on DEAE-cellulose in absence of dithiothreitol, and t-GtH$_2$ (the most completely purified) have the same molecular weight determined on Sephadex G-100, they behave very differently with ultracentrifugation. Biological activity of the t-GtH$_1$ decreases after chromatography on DEAE-C in comparison to the preceding purification stage. Mammalian hormones, LH and FSH, may be dissociated into two sub-units, α and β (LI and STARMAN, 1964; PAPKOFF and EKBLAD, 1970), having a molecular weight of about 15 000, possessing KS of about 1.5 without biological activity. BURZAWA-GERARD (1971) also observed that after 15 hours of ultracentrifugation in saccharose gradient at 65 000 r.p.m., the sedimentation peak at TS 2.67 of native c-GtH gives a second fraction of TS 1.62 without biological activity, having a molecular weight of 15 800. This author thus suspected spontaneous dissociation of c-GtH, which was confirmed by her later studies (BURZAWA-GERARD, 1975).

The characteristics of t-GtH$_1$ (KS : 1.4; equilibrated molecular weight of sedimentation 15.500 ; drop in biological activity) leads us to suppose that this trout gonadotropic preparation was obtained in a partially dissociated state, dissociation being completed during ultracentrifugation. The t-GtH would present no differences in quaternary structure (dissociation into sub-units) as compared to other glycoprotein hormones, such as TSH, LH, FSH, and c-GtH.

It also seems that dissociation could be spontaneous for both t-GtH and c-GtH. Fish gonadotropic hormones are thus more fragile than mammalian gonadotropins.

Ending purification at the DEAE-cellulose chromatography stage (DONALDSON *et al.*, 1972) seems insufficient to obtain pure hormone. Amino acid composition analysis of the preparation obtained after this stage gives an overall composition of 125 residues per mole, corresponding to 50 p. 100 of the molecular weight. On the other hand, after ultrogel ACA54 chromatography, the number of residues per mole increases to 160, representing 70 p 100 of hormone weight. This values is similar to that obtained by BURZAWA-GERARD (1973) for c-GtH (168 residues/mole). We have equivalent results (BRETON *et al.*, 1975) comparing two c-GtH preparations obtained according to BURZAWA-GERARD's technique after DEAE-cellulose (1) and G-100 post-DEAE-cellulose (2) chromatographies.

A comparison of the numbers of residues of each amino acid for t-GtH and c-GtH reveals notable differences which, besides a different sequence, may explain immunological and biological specificity of fish gonadotropins. These values are also different from those obtained for mammalian LH and FSH. Analysis of carbohydrate and sialic acid contents in the protein obtained could not be done, the amounts procured being too small.

In conclusion, trout pituitary gonadotropin was obtained with a sufficiently good degree of homogeneity to allow the radioimmunological determination of this hormone in the plasma of the Trout (BRETON *et al.*, unpublished data). This hormone has physico-chemical characteristics comparable to corresponding fish c-GtH and s-GtH hormones and to mammalian LH and FSH.

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RÉSUMÉ

PURIFICATION D'UNE HORMONE GONADOTROPE HYPOPHYSAIRE DE LA TRUITE ARC-EN-CIEL (*SALMO GAIRDNERII* RICHARDSON)

Une méthode de purification d'une hormone gonadotrope de Truite est proposée. La technique comprend les étapes suivantes : une extraction alcoolique, deux gel filtrations successives sur Séphadex G-50 et G-100, une chromatographie sur DEAE-cellulose et une chromatographie sur ultrogel ACA54. L'activité biologique des fractions obtenues a été mesurée en utilisant le test de maturation des ovocytes de Truite *in vitro*.

La préparation obtenue présente un poids moléculaire d'environ 27500 déterminé par comportement sur Séphadex G-100 et ultracentrifugation analytique pour un $KS_{20}w$ de 2,45. L'électrophorèse sur gel de polyacrylamide révèle la présence de deux bandes de mobilité électrophorétique 0,50 et 0,58. L'analyse de la composition en acides aminés de la préparation indique un nombre de résidus 160 AA par mole.

Ces différentes valeurs sont comparées aux valeurs correspondantes pour la c-GtH, la s-GtH et les gonadotropines mammaliennes.

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