Summary. An antagonism between prolactin and thyroxine, similar to that found in amphibian tissues at at metamorphosis, has been recently shown to occur at the level of the rat mammary epithelium. This phenomenon may be implicated in the pathogenesis of human breast cancer. This experiment demonstrates that two analogues of thyroid hormone, triiodothyropropionic acid and triiodothyroacetic acid, which are relatively very weak in their calorigenic action, are as potent as thyroxine and triiodothyronine in inhibiting the prolactin-mediated mammary growth in thyroidectomized rats. The possible implication of this finding in the treatment of mammary cancer is discussed.

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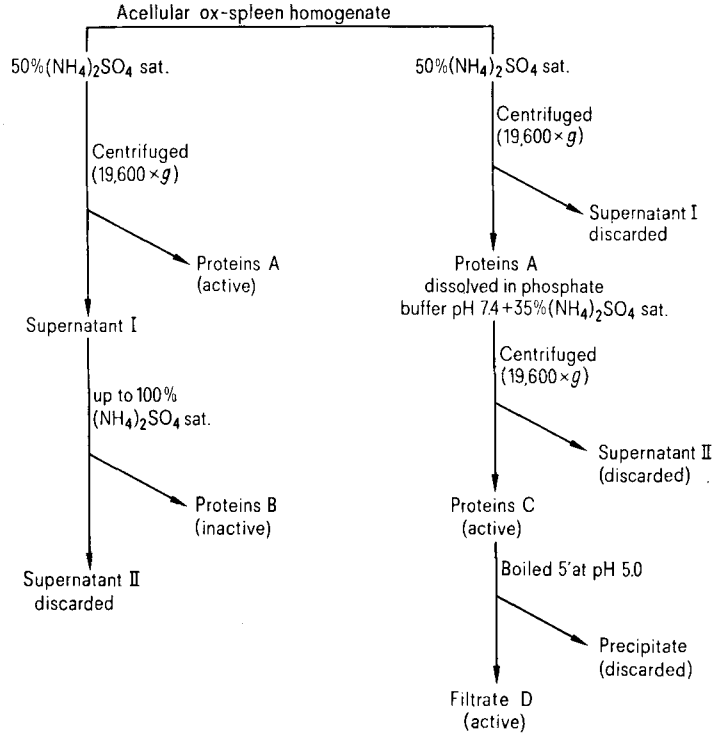
Preliminary Purification and Dosages of the Erythropoietic Factor from Ox-Spleen

The existence of a splenic erythropoietic stimulating factor is well known; in fact, since 1926, KRUMBHAAR[1] emphasized the indirect influence of the spleen on blood formation through a stimulating action on bone marrow. Later GLEY, DELOR and LAUR and RUHENSTROTH-BAUER[2] concluded that the spleen may represent one of the sites producing an erythropoietic-stimulating factor. More recently DE FRANCISCIS[3,4] reported the results of his investigations: Acellular ox spleen homogenate, when injected i.p. in rats, caused a great increase of reticulocytes in peripheric blood. In mice there was no increase of reticulocyte concentration after treatment with homogenates of kidney, muscle or liver of splenectomized animals; there was, on the contrary, a pronounced and significant reticulocyte increase in the animals treated with spleen extract or with liver of normal rats.

Table I.



In this work we proposed to study a purification method for this active factor and to assay its erythropoietic activity with different methods.

Materials and methods. An acellular ox-spleen homogenate was prepared by homogenization of the organ, immediately removed after the animals death, with 2 volumes of distilled water in a blender at 15,000 rpm for 2 min and at 0 °C. The coarse homogenate was then centrifuged for 90 min at 54,000 × *g* and at 0 °C. The acellular supernatant was precipitated by different percentages of ammonium sulphate following the reported outline (Table I).

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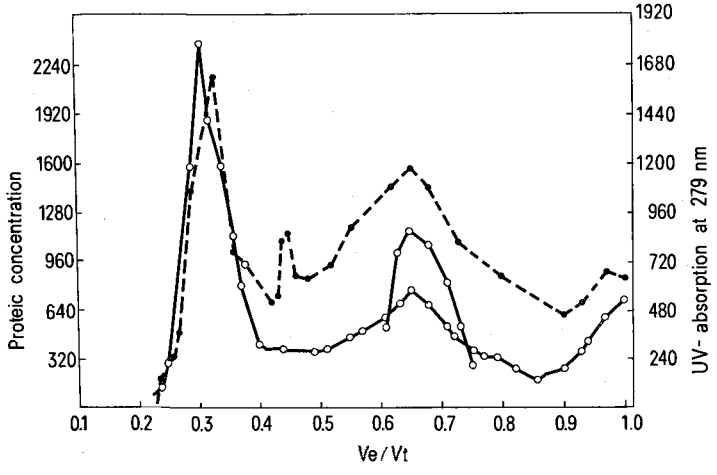

Fig. 1. Elution of protein A on Sephadex G-200. Eluant: 0.1 *M* *Tris*-HCl buffer, pH 8.0 and 1 *M* NaCl; Flow rate: 10 ml/h, Temperature 4 °C. ○—○, UV-absorption; ●—●, proteic concentration.

Table II. Effects of acellular homogenate, proteins A, B, C and filtrate D on % RBC ^{59}Fe incorporation in polycythemic rat

Group No.	Treatment	Injected protein % RBC ^{59}Fe incorporated	
		mg	mean ± SEM
1[a]	1.0 ml × 2 saline		3.98 ± 0.90
2	1.0 ml × 2 saline		0.97 ± 0.11
3	0.5 ml × 2 acell. homogenate	30	1.18 ± 0.12
4	1.0 ml × 2 acell. homogenate	60	1.97 ± 0.22[b]
5	1.0 ml × 2 proteins A	60	2.36 ± 0.25[b]
6	1.0 ml × 2 proteins B	44	1.02 ± 18
7	1.0 ml × 2 proteins C	28	2.83 ± 0.27[b]
8	0.5 ml × 2 filtrate D	2	3.13 ± 0.72[b]

[a] Normal rats. [b] $p < 0.01$ when compared to control values.

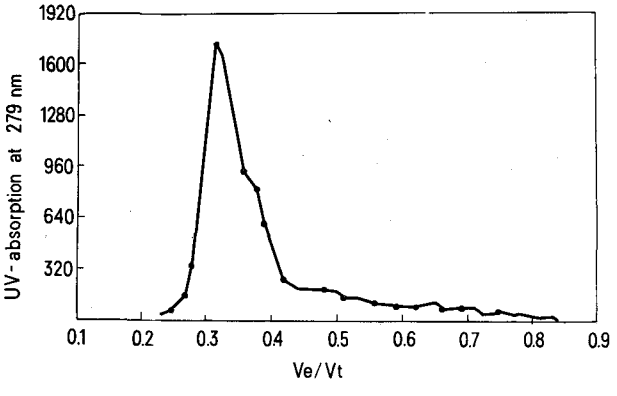

Fig. 2. Elution of proteins C on Sephadex G-200. Eluant: 0.1 *M* *Tris*-HCl buffer, pH 8.0 and 1 *M* NaCl; Flow rate: 10 ml/h, Temperature 4 °C. ○—○, UV-absorption.

Proteins A, B and C were dissolved in phosphate buffer pH 7.4, 0.05 *M* following dialysis at 4 °C against phosphate buffer pH 7.4, 0.05 *M* until the elimination of the ammonium sulphate (Nessler test). Filtrate D, obtained by acidification with HCl 0.2 *N* and ebollition of proteins C, was dialyzed against tap water in a cold room until the elimination of Cl^-; it was then freeze-dried and dissolved in phosphate buffer pH 7.4, 0.05 *M* (proteic concentration 2 mg/ml). The filtrate D exhibited a positive sialic acid and hexoses reaction.

Proteins A and C were analyzed by elution on Sephadex G-200 (40–120 μ) using 0.1 *M* *Tris*-HCl buffer, pH 8.0 and NaCl 1 *M*. After 3 days of swelling in *Tris*-HCl buffer, gel was packed in column of 2.6 × 70 cm at 4 °C and under an operating pressure of ca. 8.0 cm. Homogeneity of the bed was controlled eluing blue-dextran (M.W. 2×10^6; concentration 3.0 mg/ml). Protein dosages were made either by spectrophotometrically at 279 nm or according to LOWRY's method. (Figures 1 and 2).

In Figure 1 the proteins A show substantially 2 peaks whose Ve/Vt were 0.326 (LOWRY) or 0.31 (spectroph.) and 0.65 respectively; the last peak was read at 420 nm owing to the presence of haemoglobin. In Figure 2 the spectrophotometric graph of proteins C shows an unchanged peak with Ve/Vt = 0.32 and the disappearance of the peak with Ve/Vt = 0.65.

Electrophoresis on agarose with veronal pH 8.4 buffer of proteins C revealed the presence of γ-globulins and some other substances moving among α-β globulins.

Erythropoietic activity of acellular homogenate, proteins A, B, C and filtrate D were assayed. Polycythemic rat assay: Sprague Dawley rats, maintained ad libitum on normal diet enriched in vitamins and minerals, were employed. Donors 300 g in weight were bled by cardiac puncture and a volume of blood equal to 2.5% of the body weight was withdrawn. The packed cell volume of pooled samples was adjusted to 70% and approximately 5 ml of this concentrated erythrocyte preparation was transfused into the tail veins of recipient rats weighing 200 g.

Packed cell volume of normal rats ranged from 47 to 49%; those in transfused rats were substantially 50 ± 3% one day after transfusion and 58 ± 4% four days later. An i.v. injection of test materials was given for 2 days and the 3rd day 0.5 μCi ^{59}Fe (ferrouscitrate – Radiochemical Centre England) was injected. 1 day after this injection, radioactivity was assayed in 2 ml of whole blood withdrawn from the heart of recipient animals and percent RBC ^{59}Fe incorporation was calculated[5]. 8 groups of animals were treated; the 1st and the 2nd

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Table III. Effect of acellular homogenate and filtrate D on % RBC ^{59}Fe incorporation in polycythemic mouse

Group No.	Treatment	Injected proteins % RBC ^{59}Fe incorporated	
		mg	mean ± SEM
1[a]	0.5 ml × 2 saline		0.76 ± 0.09
2	0.5 ml × 2 acell. homogenate	40	1.89 ± 0.55[b]
3	0.25 ml × 2 filtrate D	1	2.39 ± 0.31[c]
4	0.05 IU erythropoietin		2.21 ± 0.18
5	0.25 IU erythropoietin		21.90 ± 4.99
6	0.90 IU erythropoietin		30.20 ± 3.25

[a]Control mice. [b]$p < 0.05$. [c]$p < 0.01$.

Table IV. Effects of acellular homogenate and filtrate D obtained from washed spleen on % RBC ^{59}Fe incorporation in polycythemic mouse

Group No.	Treatment	Injected proteins % RBC ^{59}Fe incorporated	
		mg	mean ± SEM
1[a]	0.5 ml × 2 saline		0.76 ± 0.09
2	0.5 ml × 2 acell. homogenate	8	2.02 ± 0.55[b]
3	0.25 ml × 2 filtrate D	1	3.20 ± 1.17[c]
4	0.05 IU erythropoietin		2.21 ± 0.18

[a]Control mice. [b]$p < 0.05$. [c]$p < 0.01$.

group (20 rats each) were used as control, including the 1st normal rats and the 2nd polycythemic rats. The values 3.96 ± 0.90 for normal rats and 0.97 ± 0.11 for polycythemic rats were obtained. The other groups of polycythemic rats (10 rats each) were used to assay the action of acellular homogenate and of the different proteic preparations too. The results obtained were grouped in the reported outline (Table II).

Polycythemic mouse assay: the ex-hipoxic polycythemic mouse assay used in these studies was made according to FISHER's[6]. CF/1 strain female mice 8–10 weeks of age and in a weight range of 22–26 g at the beginning of the assay were employed. The mice were placed in a hypobaric chamber at 0.42 atm. (319 mm Hg) for 2 weeks; the mice remained in the tank 22 h per day and were removed from the tank 2 h each day. Food and water were provided ad libitum while the mice were in the tank. We have treated 6 groups of polycythemic mice (6 mice each). The first was the control group; the other 5 groups were treated with: 1 acellular homogenate; 2 filtrate D; 3 0.05 IU of erythropoietin (erythropoietin Step 1, Arnold R Horwell); 4 0.25 IU; 5 0.9 IU. These substances were injected s.c. on the 4th and 5th day following the tank treatment and 0.5 μCi ^{59}Fe were injected i.v. 24 h later. 48 h later each mouse was weighed and bled via cardiac puncture. Microhematocrits were determined (63 ± 0.93) and 1.0 ml blood counted for the calculation of percent RBC ^{59}Fe incorporation in red cells. The results obtained are grouped in the report (Table III).

Results. As shown in Table II, the acellular homogenate assayed in doses of 30 mg ($p < 0.3$) and proteins B ($p < 0.4$) have no erythropoietic effect.

In the other groups, every injection was followed by an expressive increase of percent RBC ^{59}Fe incorporation. Still it appears that this increase is evident also if the proteic concentration was progressively lowered to 2 mg, as the case of the filtrate D.

The values of Table III show an expressive increase of percent RBC ^{59}Fe incorporation when polycythemic mice were given acellular homogenate and filtrate D. In particular the administration of 1.0 mg of the filtrate D elicited a significant stimulating effect on the rate of erythropoiesis similar to that observed when 0.05 IU erythropoetin was given. We know that this erythropoietic activity is just produced by the spleen. In fact the homogenate, obtained from spleen treated with isotonic buffer pH 7.4 up to whole blood elimination, keeps unchanged its erythropoietic activity. Simultaneously with experiments of Table III, 2 groups of mice treated with homogenate from washed spleen were tested and the corresponding filtrate D. The results show that the administration of the homogenate (proteic concentration = 8.0 mg/ml) or the filtrate D (proteic concentration = 2.0 mg/ml) is followed by an expressive increase of percent RBC ^{59}Fe incorporation (Table IV).

We calculated that whole ox-spleen, set free by aponeurosis and weighing about 600 g in 12-18-month-old animals contains a total concentration of erythropoietic factor equal to 16 IU erythropoietin. This steady activity is normally produced from the spleen and it is not in correlation with any hypoxic stimulus or other condition able to stimulate the production of the erythropoietic factor.

Summary. The administration of ox-spleen homogenate, whether in rats made polycythemic by blood transfusion or in mice polycythemic by hypoxia, elicited a significant stimulatory effect on the rate of erythropoiesis. This activity remained unchanged even if the spleen, before being homogenized, was exhaustively washed with isotonic buffer pH 7.4 up to the total blood elimination.

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