OESTROGEN-INDUCED PROTEIN (IP) OF RAT UTERUS. ISOLATION AND PRELIMINARY CHARACTERIZATION

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1. Introduction

The oestrogen-stimulated synthesis of a rat specific uterine protein (Induced Protein, IP) is the subject of considerable interest at the present time. The synthesis of this protein becomes detectable 30 min after hormone administration [1, 2], and represents certainly one of the earliest biochemical events occurring in the uterus in response to oestrogen. Contrary to the first report, evidence has been presented that oestrogen is inducing the synthesis of IP through an actinomycin D-sensitive step, presumably synthesis of a new RNA [3]. The induction of IP has also been observed after *in vitro* treatment of isolated uteri with 17β-oestradiol [4].

It is of particular interest that inhibitors of protein synthesis when administered just before or even immediately after oestrogen injection abolish the early synthesis of RNA which normally follows the administration of the hormone [5, 6]. Moreover, recent experiments seem to suggest that the antibiotics block the synthesis of an early Key Intermediary Protein (KIP) [7] decisive for the subsequent increase of the synthesis of RNA's and proteins implicated in the growth of the uterus. Although there is up to now no direct evidence that IP is KIP, the rapid turnover (half-life, 15–30 min) of both the proteins and the same sensitivity to the suppressive action of α-amanitin [8] suggest that they are identical. However, the isolation of IP or potential KIP had not been accomplished, and no trial had yet been done to explore directly the above possibility.

In a first attempt to elucidate the physiological role of these proteins, the isolation and preliminary characterization of IP from rat uterus has been performed.

2. Methods

2.1. *Materials and general procedures*

Wistar rats 21–24 days old (immature) were used. [4, 5-^{3}H]Leucine (1 Ci/mmole) and [^{14}C]leucine (331 mCi/mmole) were obtained from Amersham Radiochemical Centre. Eagle's HeLa medium (liquid, Difco) was used without modification. DEAE-cellulose (DE 52, microgranular) was purchased from Whatmann Biochem., Ltd. 17β-oestradiol was a gift of Organon Pharmac. Disc electrophoresis was performed according to the original method of Davis [9]. Sodium dodecyl sulfate gel electrophoresis was carried out by the procedure of Weber and Osborn [10] in 0.7 × 8 cm 5% polyacrylamide gel. Standard proteins of known molecular weight were from Schwarz-Mann. Isoelectric focusing and determination of pI was carried out according to Conway-Jacobs and Lewin [11]. Samples for analysis were dialyzed exhaustively against deionized water and subjected to electrofocusing in 6% polyacrylamide gel using a pH gradient of 3–5 provided by Ampholine (LKB, Stockholm). The pI values were taken directly from the pH reading of a 0.001 M KCl extract of slices of the gel. Amino acid analysis was obtained on a C. Erba (mod 3A27) amino acid analyzer following hydrolysis of the sample in 6 N HCl at 112° for 18 hr under N_2. Radioactivity

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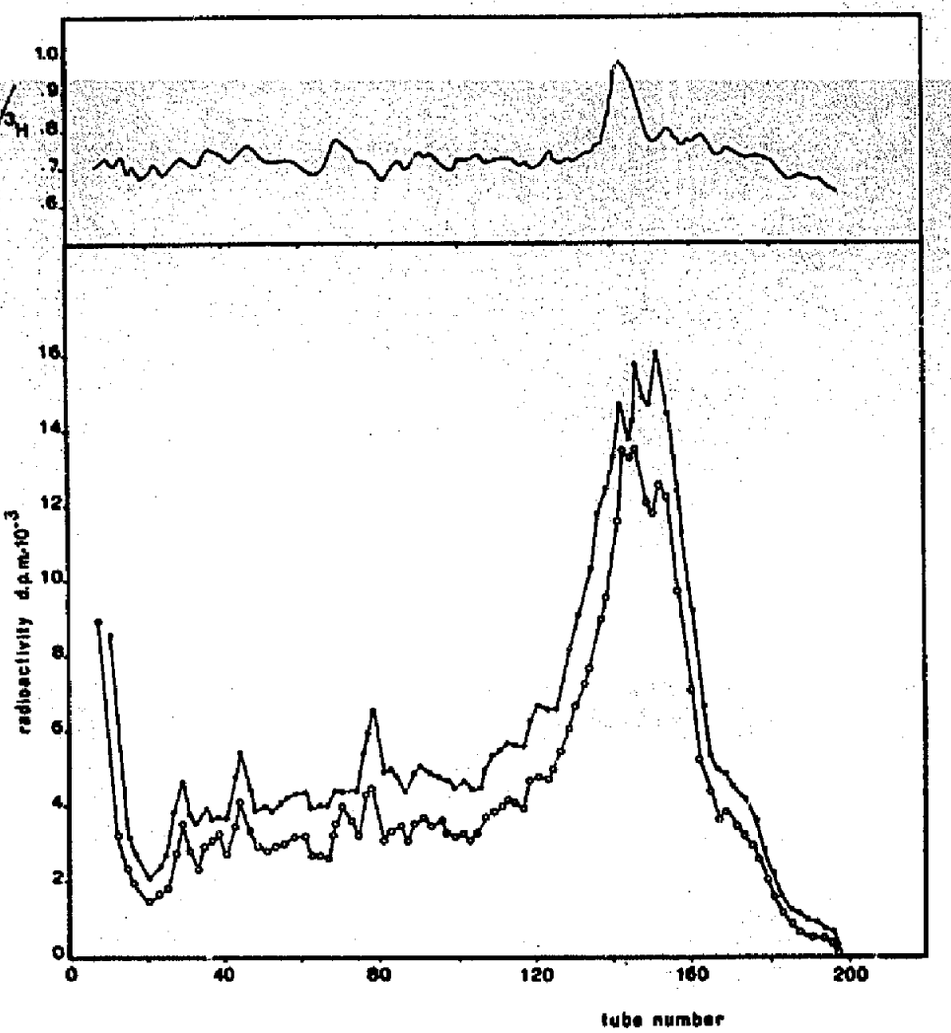

Fig. 1. Chromatography of soluble proteins from experimental and control uteri on DEAE-cellulose. The column (1 × 20 cm) was eluted as described in the text. Fractions (3.0 ml) were collected and aliquots were used for the determination of ^{3}H-radioactivity (●---●---●) and ^{14}C-radioactivity (○---○---○). The upper curve indicates the $^{14}C/^{3}H$ ratio.

counting of stained bands from polyacrylamide gel was performed as described by Mayol and Thayer [2]. Protein was measured by the method of Lowry et al. [12].

2.2. *Treatment of animals and incubation*

Rats were injected intraperitoneally with 5 μg of 17β-oestradiol in aqueous 5% ethanol. Control animals were injected with the solvent. One hour later, animals were killed by decapitation and the uteri quickly removed and stripped of connective tissue. The excised, intact uteri from 25 to 30 rats were placed into flasks containing 5 ml Eagle's medium and incubated at 37° for 30 min under an atmosphere of 95% O_2–5% CO_2. Uteri removed from hormone-treated animals were incubated in the presence of [^{14}C]leucine (10 μCi/ml), while control uteri were incubated in the presence of [^{3}H]leucine (15 μCi/ml). At the end of the incubation period, the uteri of both groups were combined, rinsed thoroughly with cold saline solution and stored frozen at −80° until enough had been accumulated (250 in each group).

2.3. *Isolation of IP*

The combined uteri were homogenized in 0.0015 M EDTA, pH 7.6 (0.05 ml/uterus) and centrifuged at 105,000 *g* for 30 min. The supernatant fraction was dialyzed against 0.005 M Tris-phosphate buffer, pH

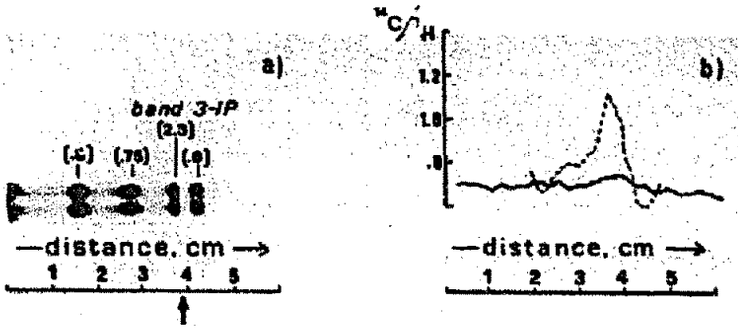

Fig. 2. a) Polyacrylamide gel electrophoresis of fraction containing oestrogen-induced protein from DEAE-cellulose chromatography. Electrophoresis was performed on 0.5 × 6 cm tubes of 7.5% polyacrylamide. The numbers in parenthesis indicate the $^{14}C/^{3}H$ ratio. Corresponding migration of rat serum albumin is indicated by the vertical arrow. b) Effect of actinomycin D on synthesis of oestrogen-induced protein. Two groups of immature rats were used. One group was treated with actinomycin D (6 mg/Kg), followed in 30 min by an injection of 5 μg of 17β-oestradiol. The animals were sacrificed 60 min after hormone treatment. The other group served as control and was injected with solvent. The uteri from each group were incubated for 1 hr in HeLa medium containing [^{3}H]leucine (controls) or [^{14}C]leucine (stimulated). Aliquots of the mixed 105,000 g fraction were applied to 0.5 × 6 cm tubes of 7.5% polyacrylamide. The gels were sliced and counted, and the ratio of ^{14}C to ^{3}H in each slice was determined. The dashed line illustrates, for comparison, the usual 1-hr response to the hormone in the absence of actinomycin.

7.3, and applied to a DEAE-cellulose column in the same buffer. After washing the column, fractionation was performed by means of a parabolic gradient to 1.0 M NaCl (total vol, 720 ml) provided by a nine-chambered gradient device [13]. Fractions were collected at a flow rate of 15 ml/hr and aliquots were taken up in 10 ml Instagel for the determination of [^{3}H]- and [^{14}C]radioactivity. Fractions of high $^{14}C/^{3}H$ ratio (indicating the oestrogen-induced protein) were eluted in tubes 140–148, the peak corresponding to 0.2 M NaCl. The fractions were pooled, desalted and concentrated by membrane filtration to 1.0 ml and applied to the top of a preparative gel electrophoresis apparatus using a 7.5% polyacrylamide gel [9] as separating gel. Electrophoresis was performed at 4° with a constant current of 15 mA for 12 hr. The elution buffer was Tris-glycine, pH 8.7 [14]. Fractions were collected at a flow rate of 20 ml/hr and aliquots were used for radioactivity counting as described above.

3. Results and discussion

Since a marked increase in the rate of IP synthesis has been shown to occur at 45–60 min after hormone administration [15], we injected 17β-oestradiol 1 hr before sacrificing the rats.

The elution pattern of radioactivity of mixed proteins from experimental and control uteri from DEAE-cellulose column is shown in fig. 1. The pattern consists of several radioactive peaks, each containing a mixture of ^{14}C-labeled and ^{3}H-labeled protein from hormone stimulated and control uteri, respectively. Moreover, the $^{14}C/^{3}H$ ratio has a remarkably uniform value around 0.7 except for tubes 140–148 where the ratio reaches values of 1.0. This reflects a specific effect of 17β-oestradiol on the incorporation of [^{14}C]leucine only into the induced protein peak, while in the rest of the proteins, labeling is proportional to the concentration of the isotope in the incubation medium. Reversal of isotopes or use of different amino acid give comparable results, excluding the possibility of preferential uptake of one isotope by this protein peak. Fraction 140–148 is resolved into four main protein bands on polyacrylamide gel electrophoresis (fig. 2a); the $^{14}C/^{3}H$ values of these bands indicate that the synthesis of only one protein (band 3-IP) is selectively enhanced by 17β-oestradiol treatment. The electrophoretic mobility of band 3-IP is approximatively the same as that of rat serum albumin and appears essentially similar to that of the oestrogen-induced protein previously observed in total supernatant fraction [2]. Moreover, the similarity between band 3-IP and oestrogen-induced protein observed by others is indicated by experiments involving the use of actinomycin D. As shown in fig. 2b, the $^{14}C/^{3}H$ pattern obtained from actinomycin-treated uteri which had been stimulated with oestradiol for 1 hr and electrophoresed with control uteri does not contain the radioactive peak characteristic of oestrogen-induced protein that usually appears near rat serum albumin.

Due to high resolution of IP on polyacrylamide gel electrophoresis, further purification was carried out by this technique. The pattern of radioactivity and the $^{14}C/^{3}H$ values obtained in the preparative electrophoresis apparatus are given in fig. 3. The pattern which shows four main protein peaks is very similar to that previously observed with analytical gel. The

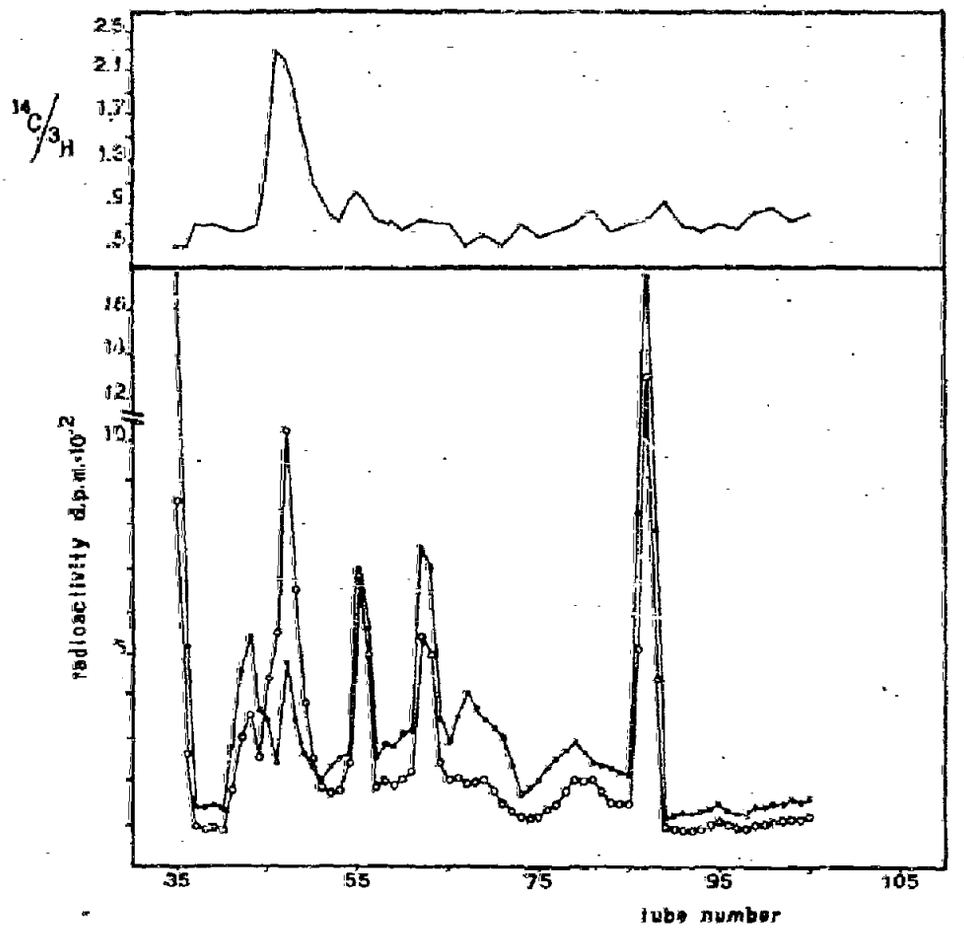

Fig. 3. Preparative gel electrophoresis of fraction containing oestrogen-induced protein from DEAE-cellulose chromatography. The sample (8.5 mg protein) in 40% sucrose was applied to a 2 × 10 cm column of 7.5% polyacrylamide. Fractions (2.5 ml) were collected and aliquots were used for the determination of ^{3}H-radioactivity (•---•---•) and ^{14}C-radioactivity (o---o---o). The upper curve indicates the $^{14}C/^{3}H$ ratio.

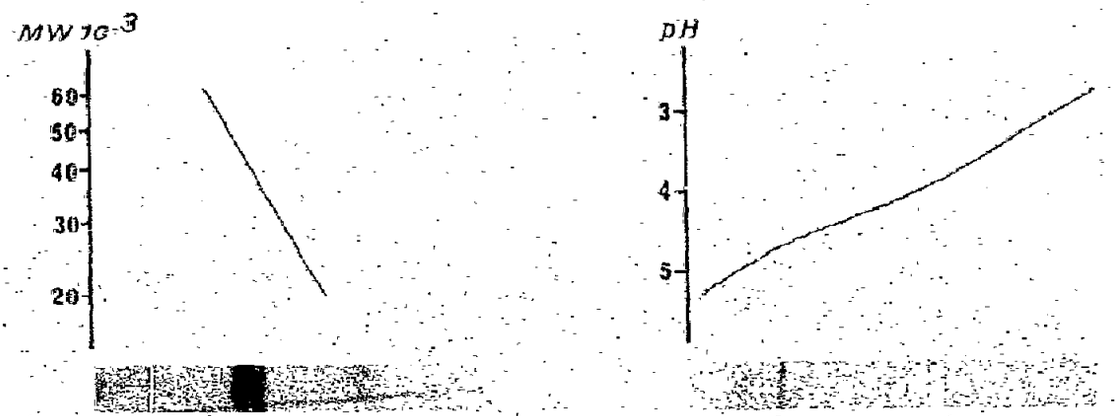

Fig. 4. Sodium dodecyl sulfate gel electrophoresis (left) and isoelectric focusing (right) of IP.

Table 1
Amino acid analysis of IP.

	(moles/100 moles total amino acid[§])
Lysine	6.51
Histidine	1.82
Arginine	4.35
Aspartic acid	9.45
Threonine	5.50
Serine	7.82
Glutamic acid	11.70
Proline	7.52
Glycine	8.86
Alanine	7.62
Valine	5.75
Methionine	1.92
Isoleucine	4.44
Leucine	8.05
Tyrosine	4.12
Phenylalanine	4.57

§ Values not corrected for hydrolytic losses.

determination of $^{14}C/^{3}H$ ratio in individual tubes again indicated the increase in the synthesis of a particular protein (tubes 40–44) under 17β-oestradiol treatment.

Because of the very small amount of IP recovered from the preparative gel column (approx. 200 μg from 500 uteri), only preliminary data on the physicochemical properties of the protein have been obtained, i.e., determination of molecular weight and isoelectric point, and amino acid composition. The results are shown in fig. 4 and table 1. Sodium dodecyl sulfate gel electrophoresis revealed a homogeneous polypeptide chain of 45,000 M.W. and a $^{14}C/^{3}H$ ratio of 2.6. The IP focuses also in a single band at isoelectric point of 4.7. This is a very strong indication of the homogeneity of the protein. The acidic nature of IP is evidenced by the isoelectric point value and by the ratio of acidic to basic amino acid residues of 1.66.

The physiological role of IP in rat uterus cannot be defined at the present time. It has been suggested that IP might play a role in the mechanism of gene expression thereby increasing the synthesis of RNA [8–16]. In this respect it would appear to be a KIP. Certainly, the prerequisite for solving problems concerning the functional significance of this molecule is the availability of the protein at a high degree of purity. The procedure we have developed offers such a possibility and should be regarded as a first step towards this goal.

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