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# EARLY TRANSCRIPTIONAL EVENTS INDUCED BY ESTRADIOL IN RAT UTERUS. PARTIAL PURIFICATION OF SPECIFIC PROTEIN FRACTION

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## SUMMARY

The specific protein fraction induced by estradiol in the rat uterus (IP) was purified by selective acid precipitation, DEAE cellulose chromatography, Sephadex G200 filtration and preparative cellogel electrophoresis.

The determination of specific phosphoprotein phosphatase activities and of the level of IP purification after the different preparation steps, confirmed Kaye's results and allowed to state that IP was not a phosphoprotein phosphatase.

The purified IP preparation did not display any detectable protein kinase activity, nor estradiol complexing ability.

The injection of cordycepin (3′ deoxyadenosine), an inhibitor of poly A synthesis, inhibits the synthesis of IP. This fact suggests that one of the earliest effects of estrogen is the production of Hn-RNA poly-A relative to IP. Cordycepin does not greatly affect the increase in ribosomal RNA observed under the effect of estradiol.

The blocking of IP by cordycepin and the lack of inhibition at the nucleolus level under the same conditions show that the two early effects of the action of estrogen on the immature rat uterus are not directly correlated.

## INTRODUCTION

The *in vivo* [1–4] and *in vitro* [3–7] induction of a specific fraction (IP) in the uterus of immature or ovariectomized rat is one of the earliest events brought about by estradiol. Whereas IP synthesis is detected 30 min after hormone administration [3–6], the synthesis of RNA [8–12] and proteins [13–15] are detectable only 1 or 2 h after injection of the hormone.

In the mature cycling rat, IP is synthesized by the various uterus tissues [16–17] and its synthetic level is closely related to the variations of endogenous estradiol and nuclear receptor levels [18–21].

Some correlations were established between IP induction and nucleolar activation by estradiol: cycloheximide blocks both IP synthesis [2] and nucleolar activation [5] induced by estradiol; in 5 day-old rats, estradiol fails to induce IP synthesis [15] and nucleolar activation [22].

These results allowed some hypothesis on the essential role of IP in the mechanism of estradiol action to be drawn. The hypothesis of the "Key intermediary protein" (IP would be responsible of nucleolar RNA synthesis, and possibly of pre-mRNA synthesis which would induce a general protein synthesis increase) suggested such a role for IP.

However as the direct inhibition by cycloheximide of nucleolar RNA polymerase was reported [23–24], we compared the effects of cordycepine, which is a major inhibitor of the poly A synthesis and the new messenger expression [25–29], both on nucleolar activation and on IP induction by estradiol.

About the nature of IP, purification was realized [30, 31]. A phosphoprotein phosphatase activity was demonstrated in purified IP fraction [31]. However this last result was cancelled recently [32]. We undertook a partial purification of IP and determined in parallel both IP level and phosphoprotein phosphatase activity.

## MATERIALS AND METHODS

All experiments were carried out on female Sprague–Dawley or Wistar rats. The values which are given for each type of experiments represent typical results which were reproduced at least three times.

### I. *IP purification*

1. *Treatment of animals.* The experiments were carried out on 90 immature 19–20 day-old Sprague–Dawley female rats.

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The animals received the following intra-peritoneal injections 1 h before sacrifice: 30 control animals (divided into 3 groups): 0.2 ml of 0.154 M NaCl + 10% ethanol. 60 estrogen-treated animals (divided into 6 groups): 0.2 ml of $10^{-4}$ M estradiol in 0.154 M NaCl + 10% ethanol.

The animals were killed by cervical luxation. After removal of adipose tissue, each group of 10 uteri was incubated for 90 min, under a $O_2/CO_2$ (95/5) atmosphere, in 3 ml of Eagle's minimal essential medium supplemented with: control: 400 $\mu$l [$^{14}$C]-serine (Amersham Radiochemical Centre, 174 mCi/mmol; 0.05 mCi/ml); estrogen treated: 400 $\mu$l [$^{3}$H]-serine (Amersham Radiochemical Centre, 1740 mCi/mmol; 0.25 mCi/ml).

The technique of double labeling allows the demonstration of IP [1]. After fractionation of the soluble proteins from rat uterus, the fractions whose synthesis are not affected by estradiol, are characterized by a fixed ratio $R = [^3H]/[^{14}C]$. The fractions containing IP are characterized by an excess $\Delta[^3H] = [^3H] - R.\,[^{14}C]$ and by an increased $\Delta R/R$ where $\Delta R = \Delta[^3H]/R.\,[^{14}C]$.

2. *IP purification.* All operations were carried out at 4°C.

At the end of the incubation period, the 90 uteri (30 control + 60 estrogen treated) were combined, rinsed three times with 100 ml of $T_1$ medium (2 mM EDTA; 2 mM $\beta$ mercaptoethanol pH 5.5) and ground in a mortar with 12 ml of $T_1$ medium + 2 g of sand. A 30 min centrifugation at 30,000 *g* produced a supernatant $S_1$ at pH 6.6 which was progressively acidified to pH 5.5 with 0.1 N AcOH. The precipitated proteins were eliminated by 30 min centrifugation at 30,000 *g*. The obtained supernatant was dialysed against $T_2$ (10 mM Tris–HCl, pH 7.5; 5 mM $MgCl_2$; 2 mM $\beta$ mercaptoethanol) during 3 h.

The resulting solution $S_2$ was then placed on DEAE cellulose column (16 × 1 cm.) equilibrated in $T_2$. The elution was carried out with 150 ml of $T_2$ and then with 400 ml of a linear gradient at a concentration of NaCl from 0 to 0.4 M. The value of $[^3H]/[^{14}C]$ ratio was determined for each fraction. The fractions with the most elevated values were combined and concentrated to 2.5 ml ($S_3$) by ultrafiltration using Diaflo cell and U M 10 membrane.

2 ml of $S_3$ were immediately placed on a G200 column (85 × 2.5 cm.) equilibrated in $T_2$. After filtration the $[^3H]/[^{14}C]$ ratio was determined for each fraction. The IP containing fractions were gathered and concentrated to 2.5 ml ($S_4$) by ultrafiltration.

The electrophoresis of $S_4$ on preparative cellogel blocks was carried out exactly as described by Somjen *et al.* [15, 33]: 0.25 ml of $S_4$ was applied to the cellogel block (12 × 6 × 0.25 cm.). After electrophoresis, the fluid of each strip was squeezed out through a 2 ml syringe. The $[^3H]/[^{14}C]$ ratio was again determined and the IP containing fractions were combined ($S_5$).

The purification of IP after the different steps was followed using electrophoresis on polyacrylamide [6] or cellogel [15, 33] (control of migration, determination of $\Delta R/R$ and $\Delta\,[^3H]/R\,[^{14}C]$).

II. *Phosphoprotein phosphatase assays*

The phosphatase activities of samples were based on the release of [$^{32}$P] orthophosphate from phosphorylated substrates according to the technique described by Maeno and Greengard [34]. A cAMP-dependent protein kinase extracted from calf uterus [35] was used to phosphorylate histones F II A or casein. The standard incubation medium contained in a final vol. of 2 ml: 50 mM NaOAc–HOAc buffer pH 6.0; 10 mM $Mg(OAc)_2$; 10 mM NaF; 4 $\mu$M cAMP; 6.6 $\mu$M [$\gamma^{32}$P]-ATP (15.5 Ci/mmol); 5 mg protein substrate; 450 $\mu$g of purified cAMP-dependent protein kinase. The reaction was carried out at 30°C for 1 h and stopped by the addition of 0.2 ml 10 mM ATP; 0.2 ml 20 mM $Na_4P_2O_7$ and 1 ml 50% trichloroacetic acid. After a 10 min centrifugation at 10,000 *g*, the pellet was dissolved in 2 ml water, and the obtained solution added with ATP, $Na_4P_2O_7$ and trichloroacetic acid as before. The resulting precipitate was centrifuged, and the process was repeated once more. The final pellet was solubilized in 2 ml water and dialysed against distilled water for 48 h.

The phosphoprotein phosphatase assays were performed at 30°C for 5, 10 or 30 min in a total vol. of 100 $\mu$l of 100 mM Tris–HCl buffer (pH 7.2) containing 2 mM dithiothreitol; 3 mM EDTA; 20 to 50 $\mu$g of phosphorylated histones F II A or casein, and 50 $\mu$l of the various samples diluted in $T_2$ so that less than 20% of the substrate was hydrolysed. Orthophosphate was extracted by the method of Plaut [36] as modified by Maeno and Greengard [34].

III. *Protein kinase assays*

The protein kinase activities of samples were based on the incorporation of [$^{32}$P]-orthophosphate, from [$\gamma^{32}$P]-ATP, into histones F II A or casein according to Kuo and Greengard [37] or Watisla *et al.* [38]. The activities were measured also in the presence of cyclic nucleotides (10 $\mu$M cAMP, 10 $\mu$M cGMP) and/or 10 mM $ZnCl_2$ (with histones only in order to inhibit phosphoprotein phosphatase activity).

IV. *Estradiol binding assays*

The determinations of estradiol binding activity were carried out with the technique described by Santi *et al.* [39].

The sum of specific + non-specific bindings was obtained after incubation of samples with 10 nM [$^{3}$H]-estradiol, 60 Ci/mmol, the non-specific binding after incubation of samples with 5 $\mu$M [$^{3}$H]-estradiol, 120 mCi/mmol.

V. *Cordycepin effect on [$^3H$] uridine incorporation in RNA*

These experiments were carried out on groups of 5–18 day-old rats. The animals received: an intraperitoneal injection of isotonic saline solution or

cordycepin (25 μg/g weight) followed one half-hour later by an injection of isotonic saline solution of estradiol (0.16 μg/g weight). The duration of estradiol treatment was one and a half hours. Each animal received an intra-peritoneal injection of 20 μCi of tritiated [5-$^3$H]-uridine (CEA, S.A. 23 Ci/mmol) one half-hour before being sacrificed.

The animals were sacrificed by cervical luxation. The uteri were removed and ground in a mortar with the use of sand in 1 ml of 0.05% EDTA. The RNA extraction technique was that using hot phenol and SDS [40, 41].

In control experiments cordycepin was replaced by actinomycin D (200 μg/animal).

VI. *Cordycepin and the specific protein fraction induced by estradiol*

1. *Treatment of the animals—Incubation of the uteri.* The experiments were carried out on immature 19–20 day-old rats.

The animals (3 or 4 per group) received two intraperitoneal injections of 0.2 ml, the first at one hour before sacrifice (control, cordycepin), the second at 30 min before sacrifice (control, estradiol).

| Groups | 1st injection | 2nd injection | Isotope incubation | [$^3$H]/[$^{14}$C] corresponds to |
|---|---|---|---|---|
| 1 | NaCl (1) | NaCl | $^{14}$C | Control |
| 2 | NaCl | NaCl | $^3$H | |
| 3 | NaCl | NaCl | $^{14}$C | Estradiol |
| 4 | NaCl | Estradiol (2) | $^3$H | |
| 5 | Cordycepin (3) | NaCl | $^{14}$C | Estradiol/ |
| 6 | Cordycepin | Estradiol | $^3$H | Cordycepin |

(1) 0.154 M ( + 10% ethanol for 2nd injection).
(2) $10^{-4}$ M in NaCl (0.154 M) + 10% ethanol.
(3) 5 mg/ml in NaCl (0.154 M).

The animals were sacrificed by cervical luxation. Adipose tissue adherent to the excised uteri was removed. Each group of uteri was incubated for 1 h under an $O_2/CO_2$ (95/5) atmosphere in 1 ml of Eagle's minimum essential medium which contained: odd groups: 100 μl of [$^{14}$C]-L-serine (Amersham Radiochemical Centre 174 mCi/mmol; 0.1 mCi/ml); even groups: 50 μl of [$^3$H]-L-serine (Amersham Radiochemical Centre 1500 mCi/mmol; 1 mCi/ml).

*Demonstration of the induced protein fraction (IP).* After completion of incubation the uteri of groups 1 and 2, 3 and 4, 5 and 6, were combined, rinsed successively in 3 × 50 ml of EDTA ($Na_2$) 0.05%, then ground in 0.5 ml of EDTA in the presence of 200 mg of sand. The homogenates were centrifuged for 20 min at 15,000 *g* and the supernatants obtained frozen in liquid nitrogen. The three types of supernatants were fractionated by electrophoresis on polyacrylamide gel and the radioactivities were determined after digestion of the sliced gels by Soluene.

## RESULTS

I. *Purification of IP*

IP, which constitutes a small part of the soluble proteins extracted from rat uterus, appears as one homogeneous fraction for each of the three purification techniques used (Figs. 1–3).

On DE 52, IP is eluted in NaCl, through concentrations between 0.21–0.24 M (fraction numbers 72–79). On G200, IP is eluted between 240–300 ml (fraction numbers 48–60). Its electrophoretic mobility on polyacrylamide gel or on cellulose acetate is 10–15% greater than that for BSA. These results agree with the parameters reported by others for IP: pHi in the order of 4.5–4.7 [33, 42, 43] and a molecular weight around 40–45 × $10^3$ [33, 42].

Figures 1–3 clearly demonstrate the progression realized in the purification of IP. The rate of purification of IP (measured by excess of Δ[$^3$H] in proportion to the total quantity of proteins contained in the fraction) steadily increased after each fractionation as did the maximal increase of $\Delta R/R$ (Table 1).

At stage $S_4$, the rate of purification obtained varied about 50–60. At stage $S_5$, the rate of purification was always clearly over 100 (evaluated by default, as the protein concentration could no longer be determined).

II. *Phosphoprotein phosphatase activity*

The phosphoprotein phosphatase activity at different stages of purification of IP is presented in Table 2. The specific phosphoprotein phosphatase activities diminished while the purification of IP progressed and the ratio of phosphoprotein phosphatase/IP was decreased much more rapidly. An important residual phosphatase activity remained at $S_4$. This activity, optimal at a pH near neutrality, and strongly inhibited by $Zn^{2+}$, is much more important with respect to phosphorylated histones F II A than to phosphorylated casein (Fig. 4); Iacobelli *et al.* [31] reported similar results (protamines > histones > phosvitin). At stage $S_5$, phosphoprotein phosphatase activity is very weak and the ratio of phosphoprotein phosphatase/IP represents less than 0.02% of the value at stage $S_1$.

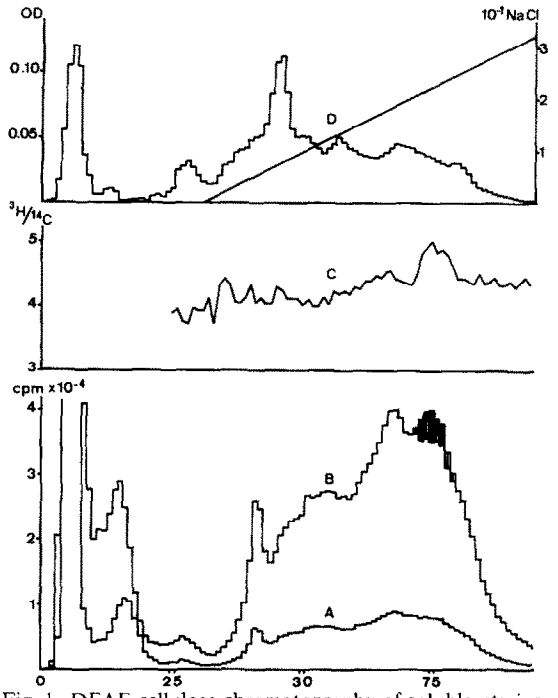


Fig. 1. DEAE cellulose chromatography of soluble uterine proteins from estradiol treated ($^3H$) and control ($^{14}C$) animals; A, [$^{14}C$]-profile (control animals); B, [$^3H$]-profile (estrogen-treated animals); C, [$^3H$]/[$^{14}C$]-ratio; D, O.D. at 280 nm. 5 ml fractions were collected and radioactivities were determined after mixing 0.5 ml aliquots of the fractions with 10 ml of Instagel Packard. ■ [$^3H$]-excess representative of IP.

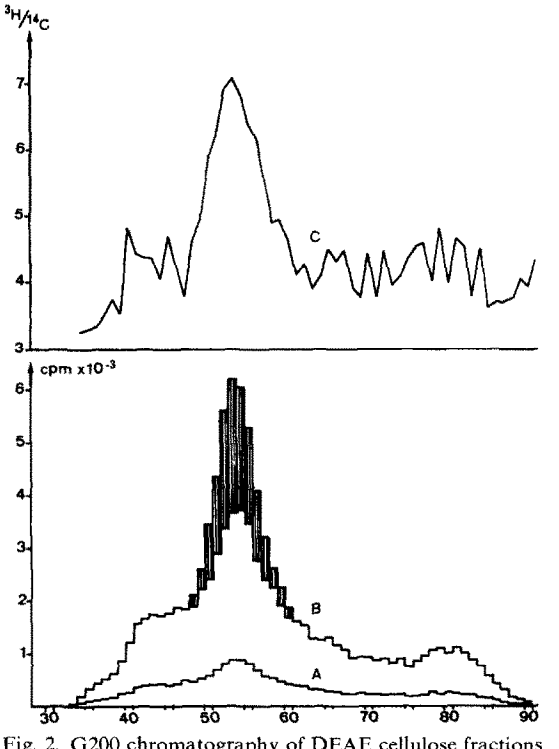


Fig. 2. G200 chromatography of DEAE cellulose fractions containing estrogen-induced protein; A, [$^{14}C$]-profile (control animals); B, [$^3H$]-profile (estrogen-treated animals); C, [$^3H$]/[$^{14}C$]-ratio. 5 ml fractions were collected. The radioactivities were determined as described in Fig. 1. ■ [$^3H$]-excess representative of IP.

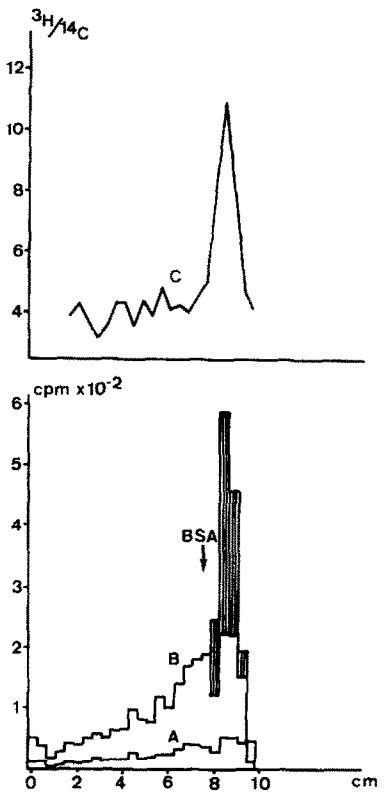


Fig. 3. Preparative cellogel electrophoresis of G200 fractions containing estrogen induced protein; A, [$^{14}C$]-profile (control animals); B, [$^3H$]-profile (estrogen-treated animals); C, [$^3H$]/[$^{14}C$]-ratio. After electrophoresis the cellogel block was sliced into strips (0.4 × 6 × 0.25 cm). Each 1/2 strip was treated by 1 ml of soluene 350 during 12 h at 60°C. After mixing with 10 ml of scintillation cocktail the radioactivities were determined. Arrow indicates the migration of BSA. ■ [$^3H$]-excess representative of IP.

Parallel measurements of IP and phosphoprotein phosphatase activity after fractionation of 250 µl samples of $S_2$, $S_3$ and $S_4$ by preparative electrophoresis on cellogel blocks, allow the distinction between IP and phosphoprotein phosphatase activity to appear more clearly (Fig. 5).

### III. *Other functions measured*

On the fraction $S_4$, we were not able to reveal a protein kinase activity with respect to the histones F II A or casein. Our studies made also in the presence of cyclic nucleotides (cAMP or cGMP) with or without Zn $Cl_2$ were negative.

The $S_4$ fraction does not contain a high affinity site for estradiol.

### IV. *Cordycepin effect on the specific protein fraction induced by estradiol (Fig. 6)*

The presence of IP was checked by electrophoresis in the supernatants from uteri of animals which were submitted to 3 types of treatments: "control" group, "estradiol" group, "cordycepin-estradiol" group.

Table 1. Purification of IP

| Fractions | Proteins mg | IP CPM[$^3$H] | IP CPM[$^3$H] / mg Protein | Purification | $\frac{\Delta R}{R}$ max |
|---|---|---|---|---|---|
| $S_1$ | 33.9 | 30,900 | 910 | 1 | |
| 95% $S_1 \rightarrow S_2$ | 18.6 | 27,300 | 1,470 | 1.6 | |
| 95% $S_2 \rightarrow S_3$ | 1.39 | 23,500 | 16,910 | 18.6 | (DE 52) 0.14 |
| 72% $S_3 \rightarrow S_4$ | 0.28 | 14,900 | 53,210 | 58.5 | (G 200) 0.69 |
| 10% $S_4 \rightarrow S_5$ | <0.01 | 960 | >96,000 | >100 | Electrophoresis 1.60 |

$S_1$: first supernatant obtained after homogenization of uteri.
$S_2$: fraction obtained after acidification to pH 5.5 and dialysis.
$S_3$: fractions No. 72 to 79 of DE 52 chromatography, reassembled and concentrated by ultrafiltration.
$S_4$: fractions No. 48 to 60 of G200 chromatography, combined and concentrated by ultrafiltration.
$S_5$: fluid of fractions No. 21–24 of preparative cellogel electrophoresis combined.

1. *Analysis of the control.* The fractions obtained after electrophoresis all presented an appreciably constant ratio $R = [^3H]/[^{14}C]$, thus permitting the elimination of any effect eventually resulting from the utilisation of two distinct radioactive samples.

Table 2. Phosphoprotein phosphatase activities and IP

| Fractions | PPPase(1) | Specific PPPase(2) | PPPase/IP(3) |
|---|---|---|---|
| $S_1$ | >200 | >5.9 | >100 |
| $S_2$ | 127 | 6.82 | 72 |
| $S_3$ | 4.70 | 3.39 | 3.1 |
| $S_4$ | 0.44 | 1.58 | 0.46 |
| $S_5$ | < 0.001 | | < 0.02 |

(1) Total phosphoprotein phosphatase activities of fractions expressed as nmol [$^{32}$P] orthophosphate released from [$^{32}$P] histones F II A during 1 h incubation at 30°C.
(2) S. A. of phosphoprotein phosphatase.
(3) Relative phosphoprotein phosphatase activities versus IP's contained.

2. *Effect of estradiol.* The electrophoresis permit the demonstration of IP. During the different experiments performed, the increase $\Delta R$ of the ratio $R$ varied

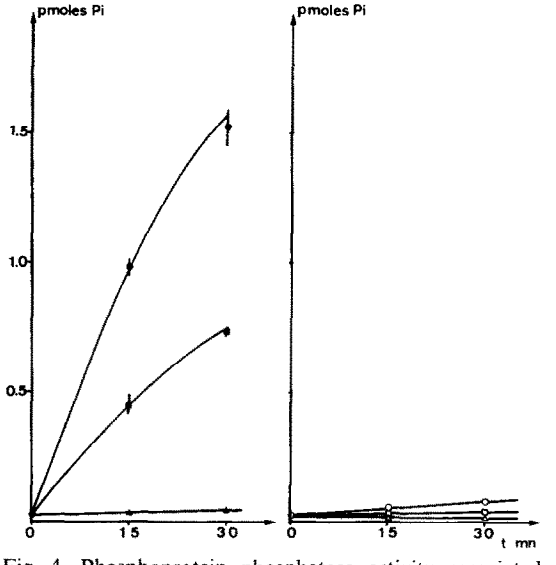


Fig. 4. Phosphoprotein phosphatase activity associated with partially purified IP:G200 step ($S_4$). The phosphoprotein phosphatase activity was measured by the amount of [$^{32}$P]-orthophosphate released from [$^{32}$P] labelled protein [31]. The [$^{32}$P]-labelled proteins were obtained as described by Maeno *et al.* [34]. Substrate concentration was 0.4 $\mu$M as phosphate for both histones F II A and casein. Incubations were carried out at 30°C in 100 $\mu$l containing: 0 $\mu$l of $S_4$ = △ [$^{32}$P]-casein; ▲ [$^{32}$P]-histones F II A. 10 $\mu$l of $S_4$ = □ [$^{32}$P]-casein; ■ [$^{32}$P]-histones F II A. 20 $\mu$l of $S_4$ = ○ [$^{32}$P]-casein; ● [$^{32}$P]-histones F II A.

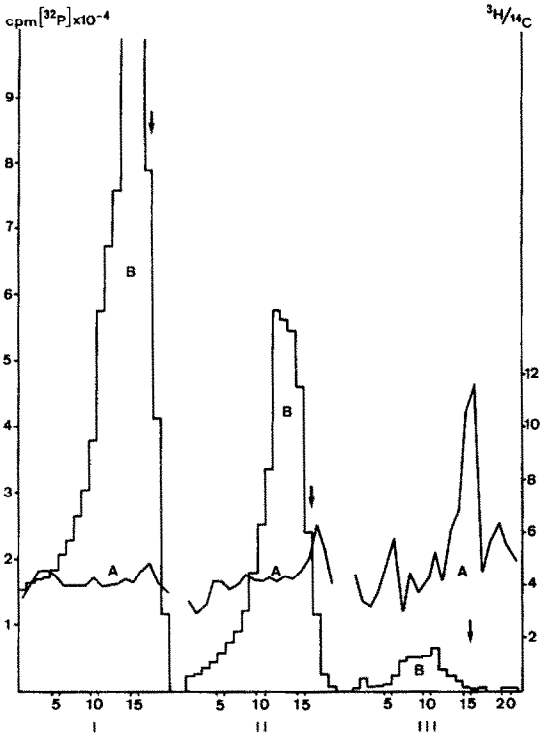


Fig. 5. IP's contain and phosphoprotein phosphatase activities of fractions obtained by preparative cellogel electrophoresis of samples corresponding with various steps of IP purification. I, $S_2$ (acid precipitation and dialysis); II, $S_3$ (DE 52 chromatography); III, $S_4$ (G200 chromatography). A, [$^3$H]/[$^{14}$C]-ratio; B, phosphoprotein phosphatase activities. After electrophoresis the cellogel blocks were sliced and the fluid of each strip was squeezed out. IP's contained were measured from 100 $\mu$l. Assays for phosphoprotein phosphatase activities were carried out at 30°C for: 10, 20 or 30 min in 100 $\mu$l incubation medium containing: 10, 20 or 50 $\mu$l electrophoresis fractions and [$^{32}$P]-histones F II A. Phosphoprotein phosphatase activities were expressed as CPM [$^{32}$P]-orthophosphate released by 50 $\mu$l electrophoresis fraction during 1 h incubation at 30°C. Arrows indicate positions of IP.

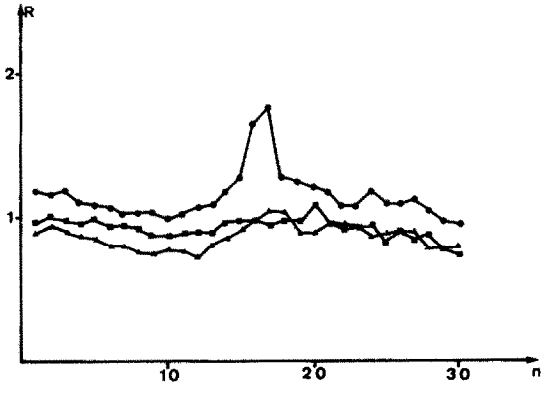


Fig. 6. Effect of cordycepin on the induction of IP by estradiol. Profiles of [$^3$H]/[$^{14}$C]-ratio along electrophoresis acrylamide gels. The soluble protein contents of uteri from animals subjected to various treatments (▲ control, ● estradiol, ■ estradiol/cordycepin) have been analyzed as described in Materials and Methods.

between 35 and 65%. The tritium surplus $\Delta$[$^3$H] varied, per uterus treated with estradiol, between 1500 and 2400 c.p.m. (counting output for $^3$H in the vicinity of 20%) and the ratio $\Delta$[$^3$H]/*R*. [$^{14}$C] varied between 0.25 and 0.40.

3. *Effect of estradiol in the presence of cordycepin (25 mg/kg).* Our results confirm those obtained by Sömjen *et al.* [7] concerning the action of cordycepin *in vitro*.

Cordycepin inhibits the formation of IP. At the doses considered, this inhibition, if not total, is at least very considerable (>85% in the different experiments performed).

V. *Cordycepin effect on [$^3$H]-uridine incorporation in RNA*

The S.A. of the uterus-extracted RNA are shown in Table 3. Estradiol clearly stimulated the global synthesis of RNA and this stimulation was not notably affected by cordycepin. This confirms the observations in electron microscopy [22] since the duration

Table 3. Effects of estradiol and cordycepin on the incorporation of [$^3$H]-uridine in RNA in the rat uterus

| Type of treatment: successive injections | [$^3$H]Uridine incorporation in total RNA (CPM/µgRNA) | | |
|---|---|---|---|
| | Experiment 1 | Experiment 2 | Experiment 3 |
| 1 isotonic solution / isotonic solution | 51 | 62 | 53 |
| 2 isotonic solution / estradiol | 112 | 125 | 107 |
| 3 cordycepin / isotonic solution | | 53 | 54 |
| 4 cordycepin / estradiol | | 128 | 114 |
| 5 actinomycin D / estradiol | 5 | | |

The animals (18 day-old female rats, 5 animals per group) received an initial intraperitoneal injection of isotonic solution (groups 1 and 2), or cordycepin (groups 3 and 4, 25 mg/kg body weight), or actinomycin D (group 5, 5 mg/kg body weight), followed one half hour later by an injection of isotonic solution (groups 1 and 3) or estradiol (groups 2, 4 and 5).

The animals received an injection of [$^3$H]-uridine (20 $\mu$Ci/animal) one half hour before being sacrificed, the sacrifice taking place 2 h after the initial injection. The specific activities of the RNA were determined as indicated in "Materials and Methods"

of treatment with estradiol used in our experiments (1 h 1/2) implies that the stimulation of RNA synthesis is for a large part nucleolar in origin [12].

It appears from these studies that cordycepin, which apparently does not affect the global synthesis of RNA, must have a selective role in the uterus which we have tried to specify at the level of poly A synthesis [44].

## DISCUSSION

The non implication of IP in many estrogen-dependent systems [45] is one of the most important limitations about the hypothesis of a Key role for IP in the mechanism of estradiol action. Indeed IP induction was confirmed only in the rat, and moreover only in the rat uterus or vagina.

However, in species other than the rat, it is possible that: (1) IP induction would take place with levels lower than in the rat; (2) IP properties (specially the acidic nature of IP) would be distinct from rat IP, making its characterisation less easy. Both hypothesis would make IP detection much more difficult than in the rat.

Although estradiol induces the synthesis of proteins associated with chromatin in the rat uterus [32, 46, 47], IP could not be demonstrated in the nucleus or the chromatin of these cells [32, 48]. We ourselves searched in vain for IP, after electrophoresis in a medium of sodium dodecyl sulfate, in the different subcellular fractions and notably in purified preparation of plasma membranes [49]. IP was found only in the soluble protein fraction.

It seems unlikely that IP is a phosphoprotein phosphatase. Our results are in agreement with those recently obtained by Kaye *et al.* [32]. The purified fractions of IP have neither an ability to bind cAMP or cGMP nor protein kinase activity, these results agree with those of Iacobelli *et al.* [31].

We did not find an aptitude to bind estradiol in the purified fraction of IP; Pennequin *et al.* [48] recently separated IP from the receptor of estradiol.

The main effect of cordycepin in the uterus, with the dose we used, is to inhibit the synthesis of poly A which is found at the extremity of HnRNA and mRNA [44].

Our results and those of other authors [7] agree on a strong inhibition of IP by cordycepin.

In contrast, the electron microscopy studies [22] and the study of radioactive precursor incorporation in RNA establish that the stimulation of the nucleolus by estradiol is not eliminated by cordycepin.

Thus, IP synthesis induction and nucleolar induction by estradiol react in a different manner to cordycepin. This allows us to suppose that there is no direct quantitative relationship between these two early manifestations of estrogenic activity.

The hypothesis that IP is a "Key intermediary protein" (KIP), upon which the nucleolar RNA-polymerase would depend [5] seems rather unlikely to us. Recently Pennequin *et al.* [48] suggested also that IP would not be the KIP implicated in the amplification of RNA synthesis.

The function of IP is still unknown. Thus we remain ignorant of its eventual role in the mechanism of action of estradiol in the rat uterus as well as its localization and site of cellular action. The efforts of different teams, notably that of Gorski [50] to isolate the messenger for IP should greatly contribute to the resolution of these problems.

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