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ESTRADIOL RECEPTOR IN UTERINE TISSUE FROM NEONATAL MICE. INFLUENCE BY CYCLIC AMP

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SUMMARY

The estradiol receptor in uterine tissue of neonatal mice has been studied. It is present neonatally and fulfills normal criteria as regards sedimentation behaviour, specificity etc.

The influence of dibutyryl-cyclic AMP (db-cAMP) on receptor characteristics has been studied using sucrose gradient centrifugation and dry mount autoradiography.

A consistent change in the cytosol receptor sedimentation behaviour is found when db-cAMP is included in the incubation medium of whole uteri preparations. A depression in the 8s receptor region is found simultaneously with an increase in the 4s region.

There is no uptake of tritiated estradiol as seen in autoradiograms from organ cultures of neonatal uterine cervix preparations, when these have been cultured for 48 h. When db-cAMP is included in the incubation medium, a pronounced uptake is seen in the sections.

Different possible points of action of cyclic AMP related to the findings described, are discussed.

INTRODUCTION

The interaction between estradiol and a high affinity, low capacity receptor protein in the cytosol is an early event in the action mechanisms of estradiol [1–3]. The hormone–receptor complex is transferred into the nucleus but different opinions exist concerning the mechanism related to this transfer [20, 21]. Recently lysosomes have been ascribed an important function both in the transport of the hormone-receptor complex to the nucleus and also as general activators necessary for an adequate genomic response [4, 5]. Cyclic adenosine -3′,5′-monophosphate (cAMP) has been claimed to act as a chemotactic principle [8], redistributing lysosomes in the cytoplasm to a position closer to the cell membrane where the organelles could pick up the steroid hormone [4].

When during the differentiation process of a target organ the estradiol receptor mechanism appears is not known, but the receptor is found in the uterus from newborn rats [9]. The müllerian epithelium in the cervico-vaginal region from the neonatal mice responds to estradiol both *in vitro* and *in vivo* when high doses are used [6, 10]. Moreover, the *in vitro* studies pointed to cAMP as being of importance for the estradiol effect [6]. In this study, we wanted to confirm the presence of a receptor protein for estradiol in the neonatal mouse cervico-vaginal anlagen and, above all, to look for possible cAMP effects on the receptor and uptake of estradiol.

MATERIALS AND METHODS

Animals

All animals used belong to a randomly bred NMRI strain. The animals were given water *ad libitum* and fed a standard pellet diet.

Biochemical studies on the estradiol cytosol receptor

The uterine horns from neonatal mice (1–5 days old) were dissected out under a dissecting microscope and freed from attaching tissue. Immediately after removal, the horns were placed in 5 ml ice cold buffer A (0·05 M Tris–HCl pH 7·4 at 4°C, and 0·002 M EDTA, disodium salt) containing 10^{-8} M ^{3}H estradiol [2,4,6,7,-^{3}H]-estradiol from New England Nuclear, S.A. 95 Ci/mmol). ^{3}H-estradiol was dissolved in 10% ethanol. This basic incubation medium was in different experiments supplemented with: (a) sodium salt of N^6, 0^2-dibutyryl adenosine-3′,5′-cyclic monophosphoric acid (db-cAMP, Sigma) dissolved in Parker 199, final concentration in the incubation medium 10^{-5} or 10^{-4} M db-cAMP; (b) cold estradiol (Sigma) dissolved in ethanol, giving a final concentration of 5×10^{-6} M cold estradiol; (c) [1,2-$^3H_2$]-5α-dihydrotestosteron (Radiochemical Centre, Amersham, 49 Ci/mmol) according the same schedule as given for estradiol in b; (d) In parallel control experiments the vehicle only was added. In order to study the difference between a target and non-target tissue, the thigh musculature from the same animals was chosen as an example of the latter.

The uterine horns of every experimental group were incubated in the same vial for 0·5–2 h, that is, due to the dissection time which was 1·5 h, the first removed uterine horn was incubated for 2 h and the last one for 0·5 h. The incubation medium was ice-cold. During the last 0·5 h the incubation took place in a cold room at +4°C where further processing took place. After this incubation, the uterine horns were dried on a filter paper and 30 of them belonging to the same experimental group were placed in 1·1 ml buffer A without estradiol or other additives. The tis-

sue was homogenized in a Potter–Elvehjem homogenizer, 15 complete strokes during 5 min. The homogenate was centrifuged at 140,000 *g* (average) in a B-60 IEC ultracentrifuge, rotor 460, for 60 min at 2°C. The supernatant (cytosol) 0·4 ml was layered onto preformed 5–20% sucrose gradient tubes, made in a gradient mixer. Gradient centrifugation took place in a B-60 IEC ultracentrifuge (SW rotor 488) for 22 h at 2°C. Bovine serum albumin (Sigma) was used as a marker for S value estimation. Fractions (about 0·56 ml) were collected by direct puncture of the gradient tubes. To each fraction was added 10 ml scintillation liquid (5 *g* PPO, 0·3 *g* POPOP in 1l toluene [11]. A Nuclear Chicago liquid scintillator, Mark 1, was used for determinations of radioactivity. The counting efficiency was 34%, estimated by external standard channels ratio. For estimation of the protein distribution throughout the gradient in some experiments, the optical density at 280 nm was recorded in a Gilford 240 continuous flow spectrophotometer. In some experiments, uterine horns were dissected out and kept in ice-cold buffer A for maximum 1·5 h, which was the time needed for dissection. These uteri were then homogenized and cytosol was prepared as described above. This cytosol was then incubated with ^{3}H-estradiol and db-cAMP (10^{-4} M) or cAMP (10^{-4} M), and layered onto sucrose gradients as described above.

Tissue culture

The cervical anlagen of newborn mice (within 24 h after birth) were dissected out under a dissecting microscope. The explants were placed on Millipore filters placed on top of pieces of Spongostan^R. The incubation took place at 37°C in Leighton tubes, two explants in every tube. The standard incubation medium (SIM, 2 ml in every tube) consisted of 70% glutamin enriched Eagle L medium (Difco, 0·3 ml of a 5% glutamin solution to 100 ml Eagle L), 20% fetal bovine serum (Microbiol. Assoc.), and 10% chicken embryo extract. Antibiotics: 60 U benzyl penicillin and 60 μg streptomycin sulphate per ml medium. Control explants were incubated in the SIM alone. In the experimental series, the medium was supplemented with sodium salt of N^6, O^2-dibutyryl adenosine-3′,5′-cyclic monophosphoric acid (db-cAMP, Sigma) or a combination of db-cAMP and estradiol-17β (Sigma). The hormone was dissolved in 96% ethanol, which was also added to the control cultures, final concentration 0·24%. The explants were cultured for 48 h. For details, see [6].

During the last hour of incubation, [2,4,6,7-H^3]-estradiol-17β (New England Nuclear, S.A. 95 Ci/mmol), dissolved in 10% ethanol was added to the incubation medium, giving a final concentration of 10^{-8} M. The explants were then washed in Tyrode solution for 0·5 h with three changes, and used for dry mount autoradiography.

Dry mount autoradiography

Autoradiograms were prepared according to the method of Stumpf *et al.*[12]. Tissue specimens, still attached to the Millipore filters, were mounted on minced liver on a tissue specimen holder and plunged into liquid propane cooled in liquid nitrogen. The mounted tissue was rapidly transferred to and stored in liquid nitrogen until further processing. Sections were cut at 2 μm in a Harris Wide Range Cryostat equipped with a IEC Model 3310 rotary microtome (Harris Manufacturing Co., Cambridge, Mass.). The sections were freeze-dried in a Thermovac^R cryopump, and postfixed in desiccated paraformaldehyde vapor at 60°C for 30 min in order to reduce chemography. The sections were mounted dry on slides precoated with Kodak AR 10 stripping film and exposed in dark boxes over silica gel for 30–40 days at −20°C. The film was developed in Kodak D 19 developer for 1 min. These sections were stained in methylgreen-pyronin, air dried, and mounted in a synthetic resin (Eukitt).

RESULTS

Biochemical studies of the cytosol receptor

The results are summarized in Fig. 1a and 1b. The radioactivity distribution curve reflecting the conditions in uterine horns incubated in buffer only (control) has its peak in the 8 S region. As indicated by the corresponding protein curve, most proteins are localized in a less heavier region, closer to the top of the gradient. Almost no radioactivity is recovered from the 8 S region when 5×10^{-6} M cold estradiol was added to the incubation medium. There is no binding of tritiated 5α-dihydrotestosterone in the 8 S region and no 8 S peak could be demonstrated when a non-target organ such as thigh muscles was analysed.

No significant changes are seen compared with the control experiments when 10^{-5} M db-cAMP is included in the incubation medium (Fig. 1b). However, most experiments pointed to a slightly higher retention of radioactivity both in the 8 S and less heavier regions. When the db-cAMP concentration is increased ten-fold (10^{-4} M), a pronounced and consistent change in the radioactivity pattern is found. In the 8 S region a pronounced decrease in radioactivity occurs while in the 4 *S region* an opposite effect is seen. In the latter region, higher amounts of radioactivity are recovered than in the control experiments. Generally, db-cAMP also seems to promote a higher uptake of estradiol. When cold estradiol in excess is added together with db-cAMP, there is no radioactivity found either in the 8 S region or in the 4 S region.

When the incubation was done with cytosol instead of whole uteri, no shift as described above was seen, either with cAMP or db-cAMP added to the incubation medium.

Autoradiographic studies

The results are illustrated in Fig. 2a and 2b. Figure 2a demonstrates a section from a control explant incubated for 48 h in the SIM alone. No grains indicating the presence of tritiated estradiol are observed

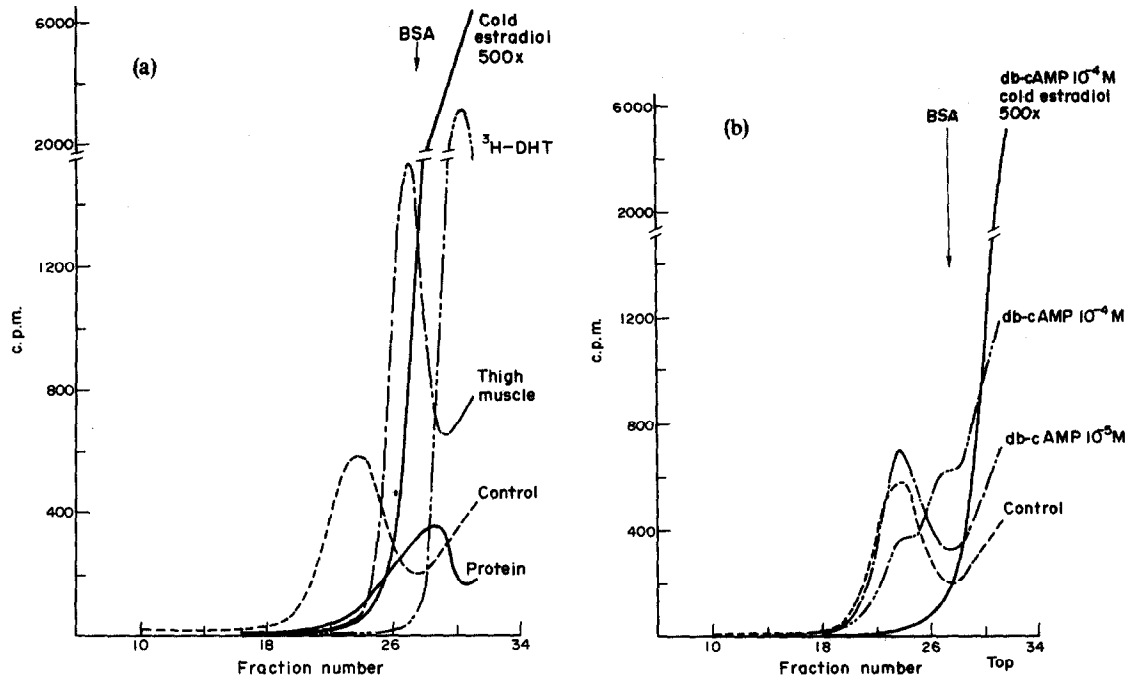

Fig. 1a. Sucrose gradient analysis of cytosol from uteri (and thigh muscle) of neonatal mice after the tissue having been incubated with tritiated steroids. The fractions to the right represents the top of the gradient, those to the left the bottom. BSA = 4·6 S.

Control (----): The uterus was incubated with 1×10^{-8} M ^{3}H-estradiol prior to gradient centrifugation of the cytosol. A distinct peak in the 8 S region is shown.

Protein (——): A plot of the O.D. for protein distribution of the uterine cytosol in the gradient (280 nm. arbitrary units). The bulk of protein is found in the 4 S region.

Cold estradiol 500 × (——): The uteri were incubated with 500 fold non-tritiated estradiol in addition to 1×10^{-8} M ^{3}H-estradiol. Thereby the 8 S peak was completely depressed.

^{3}H-DHT (-·-·-·-·-): Cytosol from uteri incubated with ^{3}H-DHT reveals no 8 S peak.

Thigh muscle (-·-·-): Thigh muscle incubated with 1×10^{-8} M ^{3}H-estradiol shows no 8 S peak after gradient centrifugation of the cytosol.

Fig. 1b. Control (----): Sucrose gradient analysis of the uterine cytosol from neonatal mice after incubation of the uteri with 1×10^{-8} M ^{3}H-estradiol.

db-cAMP 10^{-5} (-·-·-): The incubation mixture was supplemented with 1×10^{-5} M db-cAMP. No significant change in the radioactivity distribution pattern occurred.

db-cAMP 10^{-4} (-·-·-·-): When the incubation mixture was supplemented with this amount of db-cAMP a consistent change took place in the receptor distribution pattern.

over the section. Figure 2b demonstrates a section from an explant incubated in SIM with the addition of 10^{-4} M db-cAMP. A large number of grains are seen over the section.

In the stroma cells a preferential nuclear localization of the grains is obvious. In the epithelium, on the contrary, grains are localized over the nuclei but the difference between nuclear and cytoplasmic localization is not evident. The general concentration is higher over the epithelium than over the stroma.

DISCUSSION

The presence of an 8 S cytosol receptor for estradiol in the uterus from neonatal rats has been reported earlier [9]. In that study an increase in the number of estradiol-binding sites during the period 1–10 days after birth was found. After day 10, the number of binding sites per cell was constant until days 22–23.

Our studies lead us to conclude that a specific estradiol receptor is present in the uterus of neonatal mice as well. The presence of a saturable, highly specific estradiol receptor is shown by the fact that the 8 S cytosol peak is depressed when cold estradiol is added in excess, and that dihydrotestosterone is not bound in the 8 S region. The specificity is further stressed by the lack of radioactivity in the 8 S region in cytosol from thigh musculature. All these observations are in favour of the above conclusion.

In earlier papers we have reported on studies indicating a role of cAMP in the mechanism of action of estradiol when the production of an estradiol sensitive antigen, specific for the cervicovaginal epithelium, (CVA) was used [6]. Recently we have been able to demonstrate an increased activity of adenyl cyclase after estradiol injections into neonatal mice, using cytochemical methods for electron microscopy [32]. All these studies were carried out using the cervical and upper vaginal epithelium from neonatal mice. It would have been an advantage to be able to analyse the receptor characteristics in the same epithelia, but for technical reasons, such as tissue mass, etc. the

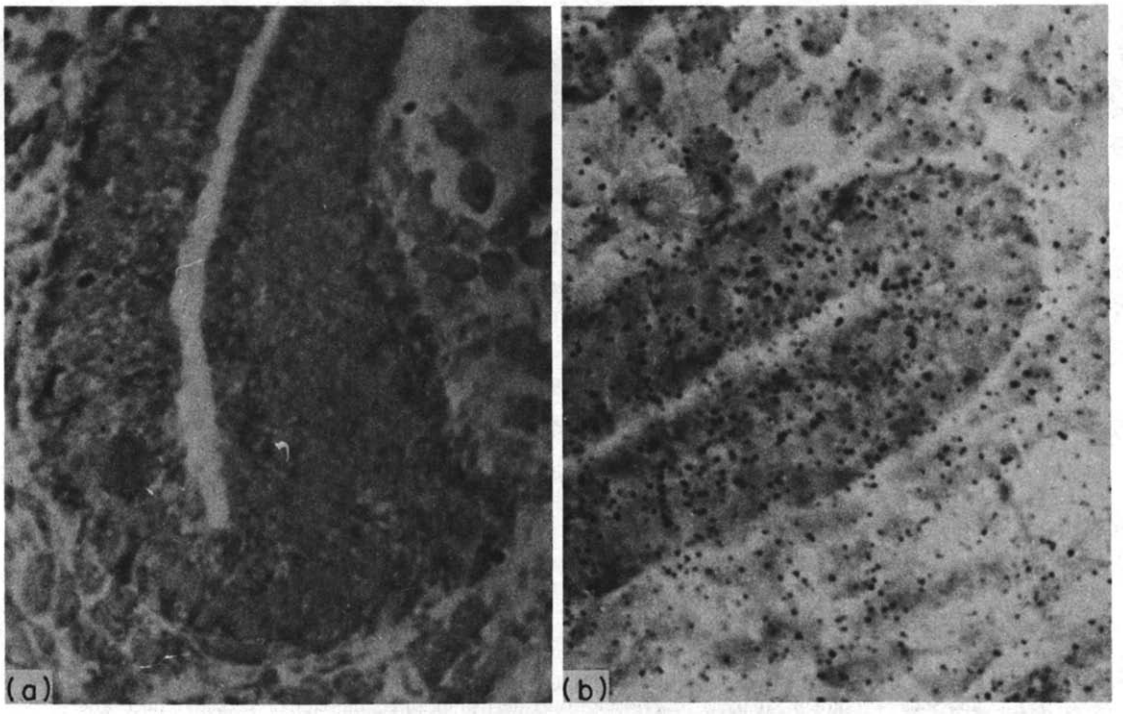

Fig. 2. Radioautograms of ^{3}H-estradiol in sections from, A: Control culture. B: db-cAMP treated culture. The difference in grain numbers is obvious. Magnification: × 1350

problems were so large that we preferred to use uterine tissue for studies on the receptor. The autoradiograms were made from the cervical region. In the following discussion we make the assumption that there are no qualitative differences between the receptor in the cervix and uterus proper although this has not been proved.

Cyclic AMP has been widely reported to be implicated as the "second messenger" in the mechanism of action of several polypeptide hormones and biogenic amines. Some reports have stressed that this cyclic nucleotide also may be a link in the mechanism of action of steroid hormones [4–6, 13, 14]. Testosterone stimulates adenylate cyclase in rat seminal vesicles [13] and the same enzyme is stimulated by hydrocortisone in asthmatic and non-asthmatic children and adults [14]. As mentioned above, cAMP seems to be involved in the production of CVA in the neonatal mice cervico-vaginal epithelium [6]. In contrast to these results there are other studies showing no increase in cAMP level after estradiol treatment [15, 16]. The present situation as regards the role of cAMP in steroid hormone action has been characterized "at best as equivocal" [17].

When control explants from the neonatal uterine cervix were cultured for 48 h as organ cultures no uptake of H^3-estradiol could be demonstrated in the autoradiograms when the isotope was added to the medium during the last hour of incubation. After addition of db-cAMP (10^{-4} M) as setting up the cultures a pronounced labelling was found both over the stroma and the epithelium. In the former compartment the labelling was mainly localized to the nuclei while in the epithelium no such preponderance was observed. Thus db-cAMP profoundly influences the uptake of the hormone. Further experiments are needed to clarify the mechanism behind this observation. An effect on the receptor level is possible in accordance with the difficulties in reproducing typical estradiol effects *in vitro* [31]. Peck *et al.* [30] reported on instability of the estrogen receptor under *in vitro* conditions, with about 85% loss during the first 2 h *in vitro* at 30–37°C.

Our receptor studies indicate a db-cAMP effect on the estradiol–receptor conditions. When db-cAMP was present in the incubation medium (ice-cold) there was a depression of the 8 S receptor peak in the radioactivity distribution curve with a simultaneously increase in the 4 S region. A peak in the latter region was never seen in control experiments.

There was no change in the sedimentation pattern when the cytosols were incubated with either cAMP or db-cAMP. This excludes a direct effect of the cyclic nucleotide on the receptor molecule and also might point to a role of membranes in this specific process.

The mechanism and importance of this change in the sedimentation characteristics is not easily evaluated. Recent reports [18, 19] have shown that the sedimentation behaviour can vary in relation to ionic strength and other factors in the medium. However, there seems to be agreement about the fact that in the transport of estradiol from the cell periphery to the nucleus there is a switch from the cytosol–receptor to a receptor associable with the nucleus. This

change in receptor protein characteristics could be due to hormone binding to the receptor [20], or it could be a result of the socalled receptor transforming factor [21]. It could also result from a conversion of receptor protein in the cell membrane [22]. Our results clearly favours the last hypothesis, that the change in hormone–receptor characteristics might be membrane dependent. All these alternatives could involve the action of cAMP. Cyclic AMP exerts most of its effects via controlling the activity of protein kinase resulting in phosphorylated substrates [23]. Cytosol from uterine horns contains cAMP dependent protein kinase [26, 27]. cAMP dependent protein kinase has also been shown to function at 0°C [28]. The findings by Arnaud *et al.*[24] are highly relevant in this connection. Their results point to a cAMP dependent phosphorylation of the estradiol–receptor, which results in an increased activity of the RNA polymerase. Moreover, the nuclear estradiol receptor seems to be an acidic protein, possibly containing phosphate groups [25]. The possibility of a phosphate contamination could not be ruled out.

Although stimulating an idea it is too premature to relate the appearance of the 4 S receptor in the presence of db-cAMP to a transformation of the cytosol receptor to a receptor associable with the nucleus. This is now being studied in our laboratory.

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REFERENCES

1. Jensen E. V. and DeSombre E. R.: *Ann. Rev. Biochem.* **41** (1972) 203–230.
2. Ellis D. J. and Ringold H. J.: In *The Sex Steroids* (Edited by K. W. McKerns). Appleton Century Crofts, New York (1971) 73–106.
3. Jensen E. V., Suzuki T., Kawashima T., Stumpf W. E., Jungblut P. W. and DeSombre E. R.: *Proc. natn. Acad. Sci., U.S.A.* **59** (1968) 632–638.
4. Szego C. M.: In *Advances in Cyclic Nucleotide Research* Vol. 1. (1972) 541–564. Raven Press, New York.
5. Szego C. M., Seeler B. J., Steadman R. A., Hill D. F., Kimura A. K. and Roberts J. A.: *Biochem. J.* **123** (1971) 523–538.
6. Kvinnsland S.: *Life Sci.* **12** (1973) 373–384.
7. Lafrenier R. T. and Singhal R. L.: *Steroids* **17** (1971) 323–329.
8. Konijn T. M.: In *Advances in Cyclic Nucleotide Res.* Vol. 1 (1972) 17–31. Raven Press, New York.
9. Clark J. H. and Gorski J.: *Science* **169** (1970) 76–78.
10. Forsberg J.-G. and Kvinnsland S.: *J. exp. Zool.* **180** (1972) 403–412.
11. Høisæter P. Å.: *Biochim. biophys. Acta* **317** (1973) 492–499.
12. Stumpf W. E.: *Acta endocr., Copenh.* **67** (1971) 205–222.
13. Thomas J. A. and Singhal R. L.: *Biochem. Pharmac.* **22** (1973) 507–511.
14. Logsdon P. J., Middleton E. Jr. and Coffey R. G.: *J. Allerg. clin. Immunol.* **50** (1972) 45–56.
15. Zor U., Koch I., Lamprecht S. A., Ausher J. and Lindnen H. R.: *J. Endocr.* **58** (1973) 525–533.
16. Korenman S. G., Sandborn B. M. and Bhalla R. C.: In *Receptors for Productive Hormones* (Edited by B. W. O'Malley and A. R. Means) (1973) 241–262. Advances in experimental medicine and biology Vol. **36.** Plenum Press, New York, London.
17. Mangan F. R., Pegg A. E. and Mainwaring W. I. P.: *Biochem. J.* **134** (1973) 129–142.
18. Chamness G. C. and McGuire W. L.: *Biochemistry* **11** (1972) 2466–2472.
19. Giannopoulos G. and Gorski J.: *J. biol. Chem.* **246** (1971) 2530–2536.
20. Jensen E. V., Mohla S., Brecher P. F. and DeSombre E. R.: In *Receptors for Productive Hormones* (Edited by B. W. O'Malley and A. R. Means) (1973) 60–77. *Advances in Experimental Medicine and Biology* Vol. **36.** Plenum Press, New York, London.
21. Bresciani F., Nola E., Sica V. and Puca G. A.: *Fedn. Proc.* **32** (1973) 2126–2132.
22. Jackson V. and Chalkley R.: *J. biol. Chem.* **249** (1974) 1627–1636.
23. Kuo J. F. and Greengard P.: *Proc. natn. Acad. Sci., U.S.A.* **64** (1969) 1349–?.
24. Arnaud M., Beziat Y., Borgna J. L., Guilleux J. C. and Mousseron-Canet M.: *Biochim. biophys. Acta* **254** (1971) 241–254.
25. King J. B., Gordon J. and Steggles A. W.: *Biochem. J.* **114** (1969) 649–657.
26. Puca G. A., Nola E., Sica V. and Bresciani F.: *Biochem. biophys. Res. Commun.* **49** (1972) 970–976.
27. Døskeland S. O., Kvinnsland S. and Ueland P.: *J. Reprod. Fest.* In press.
28. Døskeland S. O. and Ueland P.: Personal communication.
29. Sarff M. and Gorski J.: *Biochemistry* **10** (1971) 2557–2563.
30. Peck E. J., Delibero J., Richards R. and Clark J. H.: *Biochemistry* **12** (1973) 4603–4608.
31. Talwar G. P., Jailkhani B. L., Sharma S. K., Soponi M. L., Pandian M. R., Sundharadas G. and Rao K. N.: In *Control Processes in Multicellular Organisms* (Edited by G. E. W. Wolstenholme and J. Knight). Ciba Foundation Symposium (1970).
32. Åbro A. and Kvinnsland S.: *Histochemistry* **42** (1974) 333–344.