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PREFACE

During the past few years there has been considerable progress in understanding the biochemical changes brought about by the action of steroid hormones and the Biochemical Society is grateful to the contributors to this symposium for their very lucid accounts of these recent developments. The Society is also grateful to Professor, F. G. Young, F.R.S. and Dr. G. F. Marrian, F.R.S. who acted as chairmen for the two sessions of the symposium.

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CONTENTS

ESTROGEN ACTION: A STUDY OF THE NFLUENCE OF STEROID HORMONES ON GENETIC EXPRESSION

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INTRODUCTION

One of the most fascinating problems in molecular biology of eucaryote systems is the mechanism of action of steroid hormones. These substances, comprising a sizeable spectrum of cyclopentanophenanthrene derivatives, exhibit extraordinary cellular and organ selectivity as they elicit their steroid-specific responses. Some of the responses are achieved using predominantly the existing metabolic machinery of the cell; others are the culmination of striking inductive effects of the steroid on the genetic expression processes and depend on the synthesis of new ribonucleic acids and proteins. In the organized growth sequences of certain tissues the inductive effects of the steroid lead as well to distinctive patterns of differentiation among the cell progeny. Taken together, the steroids appear to operate at various levels in the control of cell physiology and at first glance might be assumed to act by distinctly different mechanisms. However, it is the purpose of this paper to point out that a common denominator of steroid hormone action may exist and is a target for investigation. Drawing from model studies on the mechanism of action of estrogens the following points in a concept of steroid hormone action are proposed and documented: (1) that cells, which are responsive to a steroid hormone, contain a receptor protein (or protein complex) which interacts specifically with certain structural features of the steroid; (2) that the receptor protein is further characterized by a remarkable propensity to enter into distinctive associations or aggregations with other macromolecules of the cell; (3) that these associations or complexes may play a role in the control of cell function by regulating the availability or functionality of certain macromolecules; (4) that the character, composition, and stability of these receptor complexes is modified by the binding of the steroid to the receptor protein; (5) that the metabolic response which ensues is determined by the way in which the altered availability or activity of a specific component of the complex affects limiting cellular processes, either metabolically or genetically (Mueller, 1970). The key feature of this concept of steroid hormone action

* Recipient of a Career Research Award from the National Cancer Institute, U.S. Public Health Service.

is that the receptor protein acts as the cornerstone or nucleation center in the formation of certain kinds of macromolecular aggregates and that the pattern and stability of the interaction is subject to rapid modification by allosteric effects brought about by the binding of the steroid to the receptor unit. These views derive from an over-view of estrogen action which is now presented.

GENERAL FEATURES OF ESTROGEN RESPONSES

Estrogens incite rapid and diverse responses in a variety of tissues. For example, they have been shown to cause: the suppression of gonadotrophin production and release by action on the hypothalamic-pituitary axis (DaLage, 1966; Flerkó, 1957; Schwartz, 1969), the growth of mammary tissues when combined with appropriate adrenal and pituitary hormones (Lyons, Li & Johnson, 1958; Turkington, 1968; Hilf, Michel & Bell, 1967), the accelerated growth and cornification of vaginal epithelium (Bigger & Claringbold, 1954; Ladinsky, Grunchow & Peckham, 1968), the synthesis of phospho-proteins in the liver (Greengard, Sentenac & Acs, 1965), the synthesis and accumulation of fat in adipose cells (Gassner *et al.*, 1958) and the growth and differentiation of uterine tissue (O'Malley *et al.*, 1969; Mueller, Herranen & Jervell, 1958; Oka & Schimke, 1969). Among these effects of estrogen the most dramatic and most amenable to biochemical analysis is the response of the uterus.

The administration of a single physiological dose of a natural estrogen rapidly converts the atrophic uterus of the immature or ovariectomized female rat into an actively growing organ. Within minutes histamine levels decline (Szego, 1965) and cyclic-AMP levels increase (Szego & Davis, 1967). As early as 1 hr after the administration of the hormone there is a generalized hyperemia of the tissue (McLeod & Reynolds, 1938), followed by a general imbibition of fluid throughout the different cellular layers of this tissue. The water uptake reaches a maximum, 4–6 hr after hormonal treatment (Astwood, 1938; Szego & Roberts, 1953). After 12 hr an increasing dry weight becomes measurable and a second surge of water imbibition occurs which correlates with the accelerated polymeric growth of that tissue (Mueller *et al.*, 1958; Telfer, 1953).

This rapid mobilization of growth processes is reflected in the composition of the uterus. As shown in Fig. 1 the phospholipid content is elevated in a striking manner during the first few hours of estrogen treatment and is antecedent to the gross accumulation of ribonucleic acid and protein. Characteristic of the growth of mobilization in a number of mammalian cells (Mueller *et al.*, 1958; Mueller, 1969), nuclear replication as measured by DNA content of the organ does not occur until 36 or more hours after the administration of estradiol. Thus the initial response is one of hypertrophy rather than hyperplasia—in fact hypertrophy appears generally to be a necessary step in the preparation for hyperplasia where hormonally restricted cells are concerned.

Whereas the compositional changes infer certain sequential effects of the hormone on the different synthetic pathways, incorporation studies with radioactive precursors demonstrate that each of these pathways (i.e. lipid, protein and RNA synthesis) was accelerated with little or no lag period after administration of estradiol (Mueller *et al.*, 1958). In recent confirmation of these earlier conclusions Hamilton (1968) and Means & Hamilton (1966) have found an increased labelling of nuclear RNA as early as 2 min. Similarly Barnes & Gorski (1970) have documented an estrogen induced synthesis of

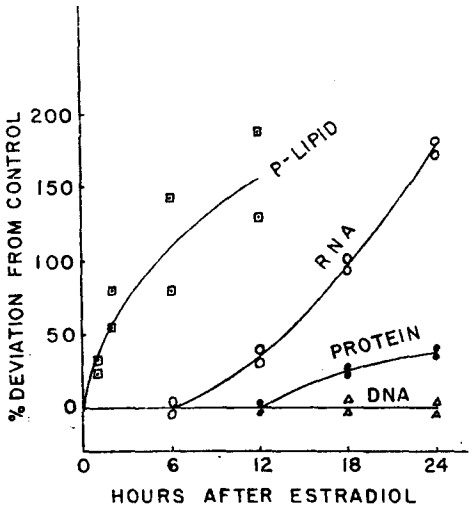

FIG. 1. Alterations in the composition of rat uteri following a single dose of estradiol (10 μg) injected at zero time. DNA was measured in μmoles of thymine/uterus. RNA was measured at μmoles of uridine and calculated as the ratio of uridine to thymine. Phospholipid was measured as μmoles of ethanolamine phosphate. All data are expressed as the per cent deviation from the control during the first 24 hr after hormone treatment. Reprinted from Aizawa and Mueller (1961).

a single electrophoretically resolved protein as early as 40 min following an injection of 17β estradiol. Since this induction was prevented when RNA synthesis was blocked, the addition of the hormone must have induced the synthesis of some RNA even earlier; this conclusion has been substantiated recently by carefully timed experiments with actinomycin D (DeAngelo & Gorski, 1970). Thus the activation of growth processes in the rat uterus by estradiol is correlated with an early acceleration of the mechanisms for genetic expression: the acceleration of RNA synthesis and the use of this RNA as templates in the synthesis of proteins.

The degree to which the early estrogenic effects depend on the synthesis of new RNA and proteins has been investigated using specific inhibitors. Experiments with puromycin demonstrated very clearly that blocking protein synthesis prevented the estrogenic acceleration of RNA and phospholipid synthesis (Fig. 2). Even the characteristic early imbibition of fluid by the

tissue was prevented (Mueller, Gorski &Aizawa, 1961). Similar results were obtained by Gorski & Axman (1964) using cycloheximide which inhibits protein synthesis by a completely different mechanism. Nicolette & Gorski (1964) also showed that cycloheximide prevents the early estrogenic stimulation of glucose incorporation into RNA, lipid and CO_2. Using labeled deoxyglucose this group also demonstrated that the estrogen-induced transport and phosphorylation of hexoses was prevented by cycloheximide (Gorski & Morgan, 1967). A further interesting feature of these experiments was that a

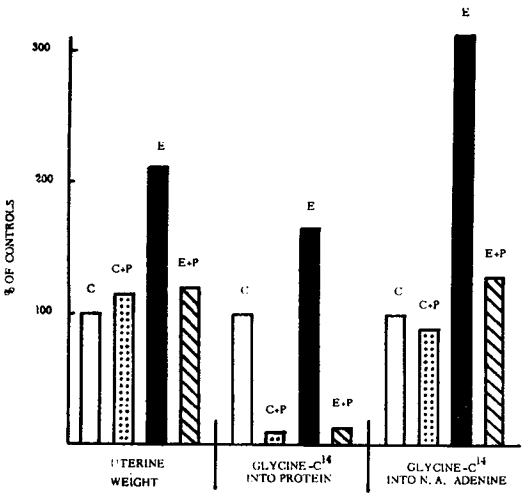

FIG. 2. The prevention of the estrogen response by treatment with puromycin. Four hr before killing (0 hr) rats received 10 μg estradiol (E) or a control solution (C). Puromycin (15 mg, P) was injected at 0, 1, 2, and 3 hr. All animals received glycine-2-C^{14} at 0, 1, 2, and 3 hr. The uteri were analysed for wet weight, incorporation of glycine-2-C^{14} into protein, and incorporation of glycine-2-C^{14} into nucleic-acid adenine. The data are plotted as % of the control values. Data are taken from Mueller *et al.* (1961).

delayed treatment with cycloheximide and puromycin caused a rapid loss of the estrogen-induced deoxyglucose transport and phosphorylation. From these experiments one must conclude that the expression of early estrogen action is highly dependent, if not completely dependent, on the continued synthesis of new protein and that the hormone action encompasses a means for activating this segment of metabolism.

In vivo experiments with actinomycin D also demonstrated that early estrogenic effects were highly dependent on the synthesis of RNA (Ui & Mueller, 1963). However, in these studies it was clear that not all of the hormonal response was prevented since a significant fraction of the water imbibition and the acceleration of phospholipid synthesis still occurred (Fig. 3) thereby suggesting an extragenomic action of the hormone. Adrenalectomy, as observed by Lippe & Szego (1965) and confirmed by Nicolette & Mueller (1966a) stages the animals in a way so that early effects of the hormone which

are independent of RNA synthesis are more prominent. In this case one can also see a limited acceleration of protein synthesis by estradiol in the presence of actinomycin D treatment. A further example of the actinomycin D insensitive response is revealed in the interesting experiments of Billing, Barbiroli & Smellie (1969). These authors showed that estrogen treatment stimulated the uptake of labelled ribonucleosides into the acid soluble fraction of the uterus; this stimulation, although lower than with estradiol alone, was none the less highly evident in the presence of actinomycin D and thus appears to

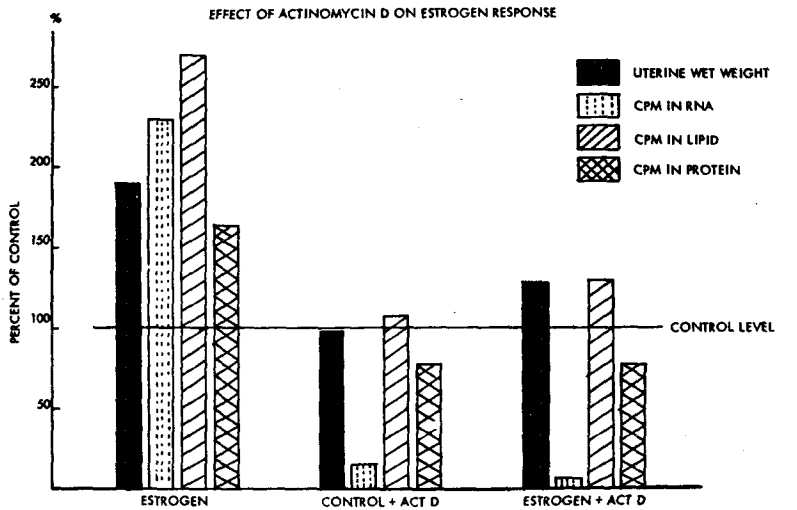

FIG. 3. The effects of actinomycin D on the early response to estrogen. Groups of three rats were injected with 375 μg actinomycin D or a control solution 30 min before receiving 10 μg of estradiol or a control solution. At 2 hr and 3 hr after the hormone treatment, uridine-H^3 (25 μC) and glycine-2-C^{14} (6 μC) were injected. The rats were killed 4 hr after the hormone treatment, and the uteri were removed and analyzed for wet weight, counts/min of H^3 into RNA, counts/min of C^{14} into protein and into mixed lipid fractions. The data are expressed as per cent of the control values. Reprinted from Ui & Mueller (1963).

be independent of RNA synthesis. Unfortunately the authors did not test the effects of cycloheximide or puromycin on the estrogen-induced uptake of ribonucleosides. In accord with the high dependency of other estrogen effects on the continued synthesis of protein, it is quite possible that these agents would have abolished the actinomycin insensitive portion of this response as well.

Taken together, the results from studies with inhibitors point out quite clearly that a major fraction of the early estrogenic response depends on the accelerated synthesis of RNA; however, the entire response is even more acutely dependent on the continued synthesis of protein. Accordingly a major aspect of the problem of estrogen action becomes one of understanding the role of protein synthesis in genetic expression and the elucidation of the way in which the hormone affects this role.

STUDIES ON THE ROLE OF PROTEIN SYNTHESIS IN THE ESTROGEN INDUCTION OF RNA POLYMERASE ACTIVITY

Gorski (1964) was first to demonstrate the elevation of a DNA-dependent RNA polymerase in the nuclei from estrogen treated rat uteri (Fig. 4), this result has been confirmed in many laboratories. As in the case of the other *in vivo* responses, the estrogenic induction of RNA polymerase activity was prevented by puromycin or cycloheximide treatment of the rats. Most surprising, however, was the observation (Gorski & Morgan, 1967; Gorski, Noteboom & Nicolette, 1965) that delayed treatment with puromycin (or

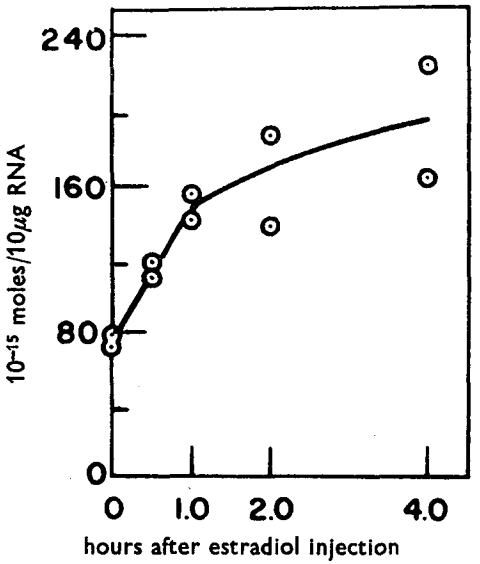

FIG. 4. The stimulating effect of estradiol pretreatment on the level of RNA polymerase in rat uteri. Reprinted from a paper by Gorski (1964).

cycloheximide) resulted in a rapid loss of the estrogen-induced polymerase activity (Fig. 5). Thus the maintenance of the estrogen-induced polymerase activity as well as the primary induction depends on the continued synthesis of protein.

In an attempt to explore the relationship of protein synthesis to the induction and function of the estrogen-induced polymerase activity our laboratory has studied the maintenance of this enzyme in the nuclei of uterine segments incubated *in vitro*. For these experiments uteri from control or estrogen-treated immature female rats are transferred to small flasks containing a modified Eagle's medium with 10% bovine serum (Nicolette & Mueller, 1966b) and incubated with 95% O_2–5% CO_2 gas phase at 37° or another temperature as indicated. At the indicated times the uteri are removed,

homogenized in Winnick and Winnick's buffer medium (Winnick & Winnick, 1960) and the nuclear fraction isolated by differential centrifugation. The RNA polymerase activity of these fractions are then assayed according to the method of Gorski (1964) as modified by Nicolette, Le Mahieu & Mueller (1968).

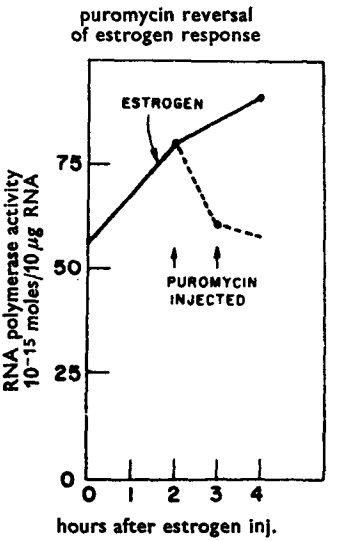

FIG. 5. The reversal by puromycin of the increase in RNA polymerase activity caused by estrogen. Estradiol injected at zero time into 21-day-old female rats. Puromycin (5 mg) was injected at 2 hr and in some groups again at 3 hr after estrogen. Rats were killed at time indicated in figure and RNA polymerase assayed in uteri. Uteri from five rats were pooled for assays at each time point. Data are expressed as 10^{-15} moles cytidine triphosphate-H^3 incorporated per 10 μg of RNA released by ribonuclease treatment. Reprinted from Gorski *et al.* (1965).

As shown in Fig. 6 the RNA polymerase activity of both control uteri and estrogen-induced uteri is maintained reasonably well during a 2-hr incubation of the uteri in tissue culture medium; in fact the nuclear polymerase survives well beyond 4 hr and even increases in activity in uteri incubated longer (Nicolette *et al.*, 1968; Nicolette, 1969). However, the addition of cycloheximide (or puromycin) to the tissue culture medium, at a level which blocks protein synthesis in the surviving uteri, depressed the RNA polymerase activity of the estrogen-treated uteri to the level of the controls. This response is largely accomplished within 30 min. Treatment of control uteri by cycloheximide (or puromycin) during the same interval had little or no effect on the nuclear RNA polymerase activity.*

* Similarly it was noted that the omission of essential amino acids from the Eagles medium also depressed the polymerase levels of estrogenized uteri incubated *in vitro*. Estrogenized uteri were transferred to fresh medium without cycloheximide, the level of RNA polymerase rose again in the ensuing hour.

Taken together these experiments illustrate the usual dependence of the estrogen-induced RNA polymerase on the continued synthesis of new protein under the conditions of treatment. These results, which are in accord with the effects of cycloheximide (or puromycin) in the living rat, also show that once registered, the estrogenic state persists in the surviving uteri through temporary restrictions of protein synthesis which inactivate or depress the estrogen-induced RNA polymerase.

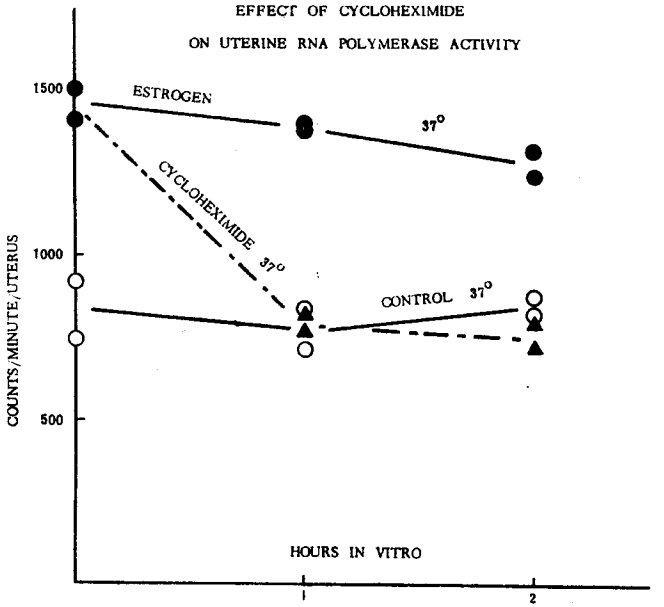

FIG. 6. Maintenance of estrogen induced RNA polymerase activity in uteri surviving *in vitro* and the effect of cycloheximide (25 μg/ml) (▲--▲) on this activity. Estradiol (5 μg) given *in vivo* 4 hr before *in vitro* incubation at 37°; controls (○——○) received no estradiol. RNA polymerase was assayed in nuclei isolated at the indicated times by the method of Gorski (1964). Reprinted from Nicolette and Mueller (1966b).

In the course of these experiments it was also noted that the estrogen-induced RNA polymerase activity of the surviving uteri was unusually sensitive to temperature. Whereas incubation of the uteri *in vitro* at either 37° or 0° permitted the maintenance of the RNA polymerase activity of both control and estrogenized uteri, incubation at 23° caused a rapid depression of the RNA polymerase activity of the estrogenized uteri to the control level. Incubation of control uteri at 23° had little or no effect on the RNA polymerase activity (Fig. 7), (Nicolette & Mueller, 1966*b*). Return of the surviving uteri to 37° was associated with a very rapid rise of the RNA polymerase activity in the estrogenized uteri. In fact the level of activity usually rose above the starting level of the tissue for a short time. Control uteri, which had been incubated *in vitro* at 23° for a 3-hr period, also responded with an

elevation of RNA polymerase activity on transfer to 37°; however, this response was always quantitatively less than the estrogenized uteri and transitory in character. In both tissues the striking acquisition of polymerase activity was prevented by the addition of cycloheximide (or puromycin) to the tissue culture medium (Fig. 8). In accord with the earlier results the delayed addition of cycloheximide also caused a striking depression of the estrogen-induced polymerase activity which was regained by the shift of the uteri back to 37°.

The observations in these experiments that the estrogen-induced RNA

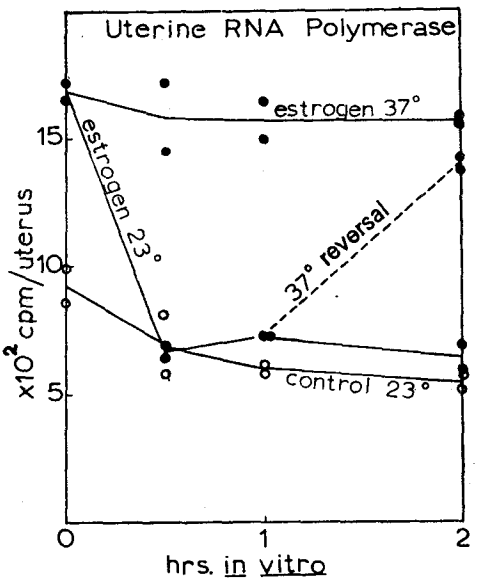

FIG. 7. The effect of *in vitro* incubation of oestrogen treated (●——●) and control (○——○) at 23° on the RNA polymerase activity in isolated nuclei. Polymerase activity of oestrogen-treated uteri incubated continuously at 37°, or at 37° after 1 hr at 23° (●– –●) were also determined. Reprinted from Nicolette & Mueller (1966b).

polymerase activity was maintained in uteri incubated at 37° or 0°, but lost in uteri incubated at 23° suggests that at 37° two opposing reactions might be taking place: one which leads to the inactivation of the estrogen-induced polymerase and a second which is responsible for the cyclic reactivation of this enzyme. In this situation the experimental observations could then be explained by postulating that the reactivation process is dominant in estrogenized uteri at 37° whereas the reaction leading to polymerase inactivation dominates at 23° In this view, control uteri differ from estrogenized uteri by having an inadequate reactivation process in all situations except under the intracellular conditions which prevail when the 23° treated control uteri are shifted to 37° for a short time (Fig. 8). A natural corollary of this concept is that an important aspect of estrogen action concerns the establishment or

facilitation of the reactivation mechanism. Accordingly the identification and characterization of processes which regulate the activity of the estrogen-induced RNA polymerase has become a high priority goal in our laboratory.

Considerable insight into this realm, particularly with respect to the role of protein synthesis, has been obtained through experiments with 2 mercapto-1-(β-4-pyridethyl) benzimidazole (MPB). Contrary to the report of Nakata & Bader (1969) this agent is an excellent reversible inhibitor of RNA synthesis in surviving rat uteri as well as in 4 strains of cells cultured in this laboratory

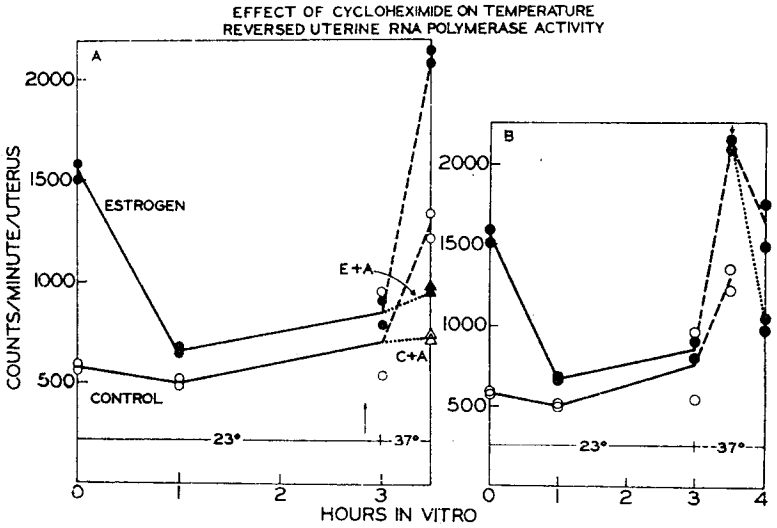

FIG. 8. The effect of cycloheximide on the temperature-induced reversal of uterine RNA polymerase activity. A: uteri from estrogen-treated (●——●) or control (○——○) animals incubated at 23° for 3 hr, then transferred to 37° (● – – ●) (○ – – ○). Ten min before transfer to 37°, 25 μg/ml cycloheximide added to appropriate flasks with estrogen-treated (● - - - ▲) (E + A) and control (○ - - - △) (C + A) uteri. Conditions and procedures as described in text. B: same experiment and symbols as A; cycloheximide added 30 min after transfer to 37° (● - - - - ●). Reprinted from Nicolette & Mueller (1966b).

(Mueller, LeMahieu, Nishigori & Kajiwara). When MPB is added to the tissue culture medium in which the estrogenized uteri are incubated *in vitro* it protects the estrogen-induced polymerase against the depressive effects of cycloheximide treatment. In experiments in which the level of estrogen-induced RNA polymerase has been depressed by pre-incubation of the uteri with cycloheximide the delayed addition of MPB causes a prompt reactivation of the enzyme (Fig. 9) (Mueller & LeMahieu, unpublished). A minor, but qualitatively similar effect is seen with the control uteri. When MPB is added along with cycloheximide at the onset of the incubation of the surviving uteri in tissue culture medium a rapid increase of the RNA polymerase activity is observed in the nuclei of both control and estrogenized uteri

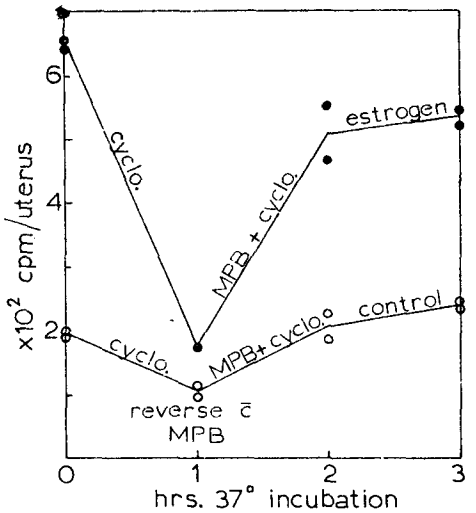

FIG. 9. Reversal of the cycloheximide depression of estrogen-induced RNA polymerase by 2-mercapto-1-(β-pyridethyl)benzimidazole (MPB). Estrogen-treated (●——●) and control uteri (○——○) were incubated in tissue culture medium containing cycloheximide (1·0 μg/ml); after 1 hr MPB (50 μg/ml) was added. Nuclei were isolated at the indicated times and assayed for RNA polymerase activity (Mueller & LeMahieu, 1970).

(Fig. 10). The surge of activity is particularly impressive in the control uteri whose level of RNA polymerase activity may reach the same level as that observed with estrogenized uteri. However, this situation is a transitory one in control tissues and soon subsides towards the initial control level.

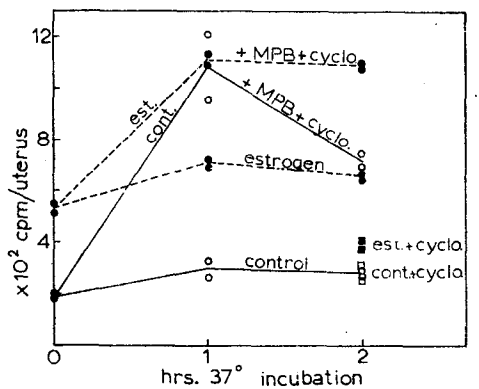

FIG. 10. Activation of nuclear RNA polymerase in surviving uteri incubated *in vitro* with MPB. Estrogen-treated and control uteri were incubated in tissue culture medium ± the addition of 1·0 μg/ml cycloheximide. MPB (50 μg/ml) was added to cycloheximide-treated uteri as indicated. RNA polymerase activity was measured in nuclei isolated from the surviving uteri after the indicated treatments (Mueller & LeMahieu, 1970).