

# Steroid regulation of monoamine oxidase activity in the adrenal medulla

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## ABSTRACT

Administration of different steroid hormones in vivo has distinct and specific effects on the MAO activity of the adrenal medulla. In an effort to reconstitute these effects in defined cells, we have isolated endothelial cells and chromaffin cells from the bovine adrenal medulla and tested each cell type for sensitivity to these steroids. As in the intact animal, we found that endothelial cell MAO activity was stimulated 1.5- to 2.5-fold by 10  $\mu$ M progesterone, hydrocortisone, and dexamethasone, inhibited by ca. 50% by 17- $\alpha$ -estradiol, but unaffected by testosterone. The type of MAO in the endothelial cells was found to be exclusively of the A type. The chromaffin cells had MAO B exclusively and were inert to treatment with dexamethasone. The mode of action of the various steroids on MAO A activity in endothelial cells seemed to be that of affecting the number of MAO molecules, as binding of [<sup>3</sup>H]pargyline, an MAO inhibitor, changed in proportion to changes in enzyme activity. Consistently, the kinetic parameters for MAO A showed changes in  $V_{\max}$  but not  $K_m$  under all conditions. The specificity of steroid action on MAO A activity was also supported by the fact that steroid-induced changes in total cell division ([<sup>14</sup>C]thymidine incorporation) and total protein synthesis ([<sup>14</sup>C]leucine incorporation) were seen after changes in MAO A. We conclude that the differential effects of steroids on MAO activity in the intact adrenal medulla can be reproduced in cultured adrenal medullary endothelial cells but not in chromaffin cells. Therefore we suggest that the action of these steroid hormones on the intact adrenal medulla may be restricted to the endothelial cell component of this tissue. —YODIM, M. B. H.; BANERJEE, D. K.; KELNER, K.; OFFUTT, L.; POLLARD, H. B. Steroid regulation of MAO activity in the adrenal medulla. *FASEB J.* 3: 1753-1759; 1989.

**Key Words:** steroid hormones • adrenal medulla • endothelial cells • chromaffin cells • kinetic parameter • monoamine oxidase • A-type MAO

STEROID HORMONES SUCH AS progesterone and glucocorticoids, and sex steroids such as estradiol and testosterone, have specific actions on the levels of monoamine oxidase in the adrenal gland and other tissues (1-10). Monoamine oxidase (MAO: [monoamine: oxygen oxidoreductase (deaminating) (flavin containing), EC 1.4.3.4]) occurs in two molecular forms, A and B, with specific and distinct pathophysiological importance. However, the relative influence of the steroid hormones on these two forms has been little studied (6, 9). MAO degrades biogenic amines in both central and peripheral tissues, and the different forms affect quite separate physiological states. For example, MAO A has importance for high blood pressure in response to indirectly acting sympathomimetic amine, whereas MAO B is important in movement disorders such as Parkinson's disease.

Previous work from our laboratory has shown that MAO B in the adrenal medulla is located only on the chromaffin cells (11), which has been confirmed by others (12). Because the adrenal medulla contains both MAO A and MAO B, we have proposed that the endothelial cells might be the locus of the latter enzyme in the whole tissue (13). We developed a method for culturing endothelial cells from the adrenal medulla (14) to test this hypothesis. In addition, we have attempted to determine the site of steroid action in the adrenal medulla by testing the action of the different steroids on MAO activity in pure cultures of the different cell types.

In this paper we report that adrenal medullary endothelial cells indeed show MAO A activity exclusively, and that different steroids affect the number of MAO A molecules in these cells. By contrast, few effects of these steroids could be detected from MAO B activity in cul-

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tured chromaffin cells. Furthermore, the differential action of the various steroid hormones on MAO activity of intact glands seems to parallel the actions of the same hormones on cultured endothelial cells. We interpret these data to indicate that the effects of steroids on MAO activity in the intact adrenal medulla are primarily mediated by direct actions of the hormones on MAO A in endothelial cells.

## MATERIALS AND METHODS

Dexamethasone, hydrocortisone, progesterone, testosterone, and 17- $\alpha$ -estradiol were purchased from Sigma (St. Louis, Mo.). [2- $^{14}$ C]5-Hydroxytryptamine creatinine bisulfate (56 mCi/mmol), [1- $^{14}$ C]phenylethylamine hydrochloride (60 mCi/mmol), [7- $^{14}$ C]tyramine hydrochloride (56 mCi/mmol), [methyl- $^{14}$ C]thymidine (50 mCi/mmol), and [U- $^{14}$ C]leucine (348 mCi/mmol) were obtained from Amersham (Arlington Heights, Ill.). [2- $^{14}$ C]dopamine hydrochloride (56 mCi/mmol) and [ $^3$ H]pargyline hydrochloride (25 Ci/mmol) were products of New England Nuclear (Boston, Mass.).

### Endothelial cell culture and treatment with steroid hormones

Capillary endothelial cells were cultured in 75-cm<sup>2</sup> flasks (Corning) at a concentration of  $3 \times 10^5$  cells per flask in 15 ml of minimal essential medium (MEM) supplemented with 10% fetal calf serum (heat inactivated), glutamine (2 mM), penicillin (50 units/ml), and streptomycin (50  $\mu$ g/ml) (14). Each group consisting of three flasks of endothelial cells was treated with 10  $\mu$ M dexamethasone, progesterone, hydrocortisone, testosterone, or 17- $\alpha$ -estradiol in the presence of 0.95% ethanol. At the end of each time point, flasks were collected, medium was removed, and the cell monolayer was washed with phosphate buffer saline (PBS), pH 7.4. The cells were scraped with a rubber policeman, washed in PBS, and finally suspended in 50 mM sodium phosphate buffer, pH 7.4. The cells were then frozen in a dry ice-ethanol mixture and thawed just before assaying for monoamine oxidase activity.

### Assay of monoamine oxidase activity

Monoamine oxidase type A and type B activities were measured according to the procedure described above (15) using [ $^{14}$ C]5-hydroxytryptamine bisulfate, [ $^{14}$ C]phenylethylamine hydrochloride, [ $^{14}$ C]tyramine hydrochloride, and [ $^{14}$ C]dopamine hydrochloride as substrates. In each case the frozen and thawed cell preparation was incubated at 37°C for 1 h in the presence of 20–400 mM radiolabeled monoamines in a total volume of 0.5 ml. At the end of each incubation period, the unchanged monoamine substrates were separated by applying the incubation mixture on 1-ml Amberlite CG-50 columns. The deaminated product eluted with water from the columns was assayed for radioactivity in Hydrofluor.

### Binding of [ $^3$ H]pargyline to monoamine oxidase

The binding of the irreversible MAO inhibitor pargyline (16, 17) was measured in order to estimate the number of MAO enzyme molecules. Endothelial cells were harvested after culturing for different times in the absence or presence of dexamethasone, progesterone, or 17- $\alpha$ -estradiol. The harvested cells were broken by freezing and thawing as described above and incubated with 5 mM [ $^3$ H]pargyline (3.0  $\mu$ Ci) in 50 mM sodium phosphate buffer, pH 7.4, for 90 min at 37°C in a total volume of 0.5 ml (16). The reactions were stopped with 1 ml of 5% perchloric acid. The precipitates were collected by filtration on 0.45- $\mu$ m Millipore filters and assayed for radioactivity in Hydrofluor.

### Chromaffin cells

Chromaffin cells were isolated from bovine adrenal medulla by an established procedure, purified from contaminating endothelial cells by differential plating, and grown under the same conditions as endothelial cells, except for the addition of cytosine arabinoside (10  $\mu$ M) (18).

### Cytokinetics of endothelial cells after steroid hormone treatment

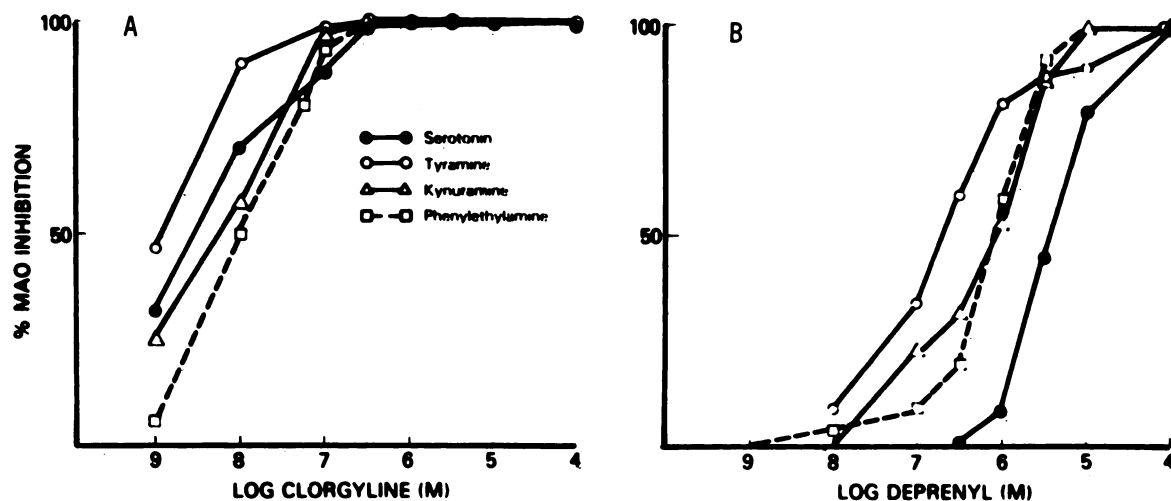
Capillary endothelial cells were trypsinized for 5 min at 37°C, suspended in minimal essential medium, and plated onto 24-well culture dishes (16 mm diameter) at a concentration of  $3 \times 10^3$  cells per well in 1.5 ml of medium as described by Banerjee et al. (14). The cells were cultured in the absence or presence of 1  $\mu$ M each of dexamethasone, progesterone, hydrocortisone, or 17- $\alpha$ -estradiol. At 24-h intervals, [ $^{14}$ C]thymidine and [ $^{14}$ C]leucine were added into parallel wells, and the labeling continued for another 24 h. At the end of the procedure, cells were washed in PBS, pH 7.4, trypsinized, and adjusted to 10% trichloroacetic acid. The precipitates were then collected on 0.45  $\mu$ m Millipore filters and assayed for radioactivity in Ready Solv.

Protein content of each sample was determined by Bradford's procedure using bovine serum albumin as the standard, as described for application to chromaffin tissue (19).

## RESULTS

### Characterization of monoamine oxidase activity in endothelial cells

The inhibitor specificity of endothelial cell MAO with selective inhibitors of MAO A and MAO B (20, 21) has been used before to characterize the type of monoamine oxidase present in various tissues. Indeed, the characteristics of MAO B for chromaffin cells has been exhaustively studied in terms of substrate and inhibitor specificity (11). However, it was necessary to use the same criteria to unambiguously delineate the MAO as



**Figure 1.** A) Clorgyline and B) (–)-deprenyl inhibition of MAO activity in frozen and thawed endothelial cells. The enzyme preparations were incubated with the inhibitors for 20 min and then assayed using  $10^{-3}$  M serotonin (●),  $10^{-3}$  M tyramine (○),  $2 \times 10^{-5}$  M l-phenylethylamine (□), and kynuramine (△) as substrates. Each curve represents the mean of four separate experiments for each substrate. Standard errors of the mean are excluded for the sake of clarity and are no more than 8–10%.

type A in the endothelial cell. As shown in Fig. 1B, l-deprenyl, the selective inhibitor of MAO B, was a poor inactivator of endothelial cell MAO when tyramine, phenylethylamine, and serotonin were used as substrates. The median inhibitory concentration ( $IC_{50}$ ) ranged from  $10^{-7}$  to  $5 \times 10^{-6}$  M. In addition, l-deprenyl exhibited a simple sigmoid curve with all three substrates, which indicated one type of enzyme (20).

Clorgyline, a specific inhibitor of MAO A (20), also showed a single inhibitory curve when tested with endothelial cells. But in contrast to l-deprenyl, it was a much more potent inhibitor of tyramine, phenylethylamine, and serotonin deamination. The  $IC_{50}$  values for clorgyline ranged from  $5 \times 10^{-9}$  M to  $5 \times 10^{-8}$  M (Fig. 1A). These data indicated that the monoamine oxidase in adrenal medullary endothelial cell was primarily type A, which is also supported by the kinetic parameters of the enzyme used to study the deamination reaction of a variety of monoamines (Table 1). These results hence gave credence to studies of hormone action on cells possessing virtually exclusive types of MAO.

#### Response of endothelial cell monoamine oxidase to steroids

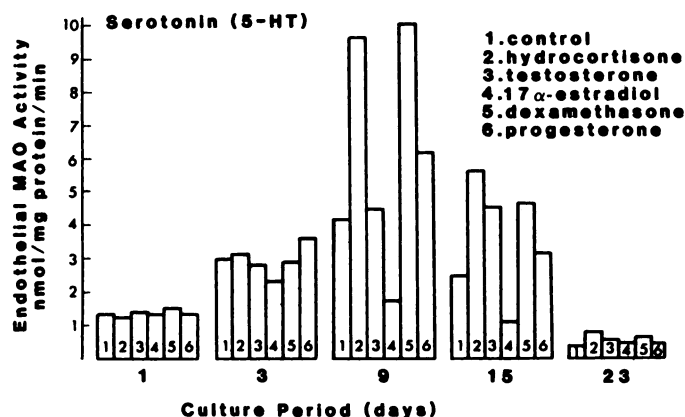
When the endothelial cells were maintained under normal culture conditions, the monoamine oxidase activity reached a maximum on the 8th day. Upon further culturing, the activity declined and became almost undetectable in cells cultured for 23 days. These changes can be followed by monitoring changes in bar number 1 throughout Fig. 2.

The activity was also followed with phenylethylamine, an MAO B substrate, to ensure that MAO B activity did not intrude into the system over the entire time course of the experiment. Little or no MAO B activity was detected at any time (data not shown). We then added various steroids and measured the enzyme activity in cells treated for 8 days or as many as 23 days. We observed that hydrocortisone and dexamethasone increased MAO A activity by two- to threefold more than that of the untreated controls. Testosterone and progesterone caused much smaller but still consistent increases in MAO A activity. However, treatment of

**TABLE 1.** Kinetic constants of endothelial cell MAO type A after exposure to steroid hormones<sup>a</sup>

| Substrates       | Control         |  | Dexamethasone   |  | 17- $\alpha$ -estradiol |  |
|------------------|-----------------|--|-----------------|--|-------------------------|--|
|                  | $K_m$ , $\mu$ M | $V_{max}$ , nmol · mg Pr <sup>-1</sup> · min <sup>-1</sup> | $K_m$ , $\mu$ M | $V_{max}$ , nmol · mg Pr <sup>-1</sup> · min <sup>-1</sup> | $K_m$ , $\mu$ M         | $V_{max}$ , nmol · mg Pr <sup>-1</sup> · min <sup>-1</sup> |
| Tyramine         | 400             | $0.43 \pm 0.04$  | 350             | $1.00 \pm 0.09$  | 380                     | $0.25 \pm 0.04$  |
| Serotonin        | 230             | $0.27 \pm 0.04$  | 210             | $0.77 \pm 0.06$  | 190                     | $0.12 \pm 0.02$  |
| Dopamine         | 416             | $0.26 \pm 0.03$  | 380             | $0.83 \pm 0.07$  | 410                     | $0.14 \pm 0.01$  |
| Phenylethylamine | 250             | $0.03 \pm 0.003$   | 270             | $0.09 \pm 0.01$  | 230                     | $0.02 \pm 0.003$   |
| Norepinephrine   | 430             | $0.17 \pm 0.02$  | — <sup>b</sup>  | —  | — <sup>b</sup>          | —  |

<sup>a</sup>For determination of  $K_m$  and apparent  $V_{max}$  values, endothelial cells were cultured for 8 days in the presence and absence of  $10 \mu$ M dexamethasone or 17- $\alpha$ -estradiol. The mitochondrial extracts were prepared and used as an enzyme source. The results are mean  $\pm$  SEM from four separate experiments. <sup>b</sup>Not tested.



**Figure 2.** The influence of culture time period and steroid hormones on endothelial cell MAO activity using serotonin as substrate. Endothelial cells were cultured in regular medium (as described in Materials and Methods) containing 10  $\mu$ M each of progesterone, hydrocortisone, testosterone, dexamethasone, or 17- $\alpha$ -estradiol. At the indicated times, cells were harvested and MAO activity was determined. Each point represents the mean from three flasks assayed in duplicate.

these cells with 17- $\alpha$ -estradiol caused a progressive decrease in MAO activity for the same period of time (Fig. 2).

#### Kinetics of endothelial cell monoamine oxidase

The changes in MAO A activity could be caused by either changes in the kinetic properties of the enzyme or simply in the number of enzyme molecules. Therefore, we measured kinetic constants for five different substrates in mitochondrial extracts of cells treated for 8 days with either 10  $\mu$ M of dexamethasone or 10  $\mu$ M of 17- $\alpha$ -estradiol (Table 1).

Neither dexamethasone nor 17- $\alpha$ -estradiol treatment modified the apparent  $K_m$  of the enzyme, contrasted to the untreated controls, when assayed with either serotonin (specific for type A), tyramine, and dopamine (substrates for both A and B types) or phenylethylamine (exclusively a B-type substrate) as substrates. However, the  $V_{max}$  values of the enzyme increased by 2- to threefold in dexamethasone-treated cells and were reduced 1.5- to twofold in 17- $\alpha$ -estradiol treated cells. These results indicated that the changes in enzyme activity observed in cells treated with either dexamethasone or 17- $\alpha$ -estradiol might indeed be related to changes in the amounts of MAO A in the cell.

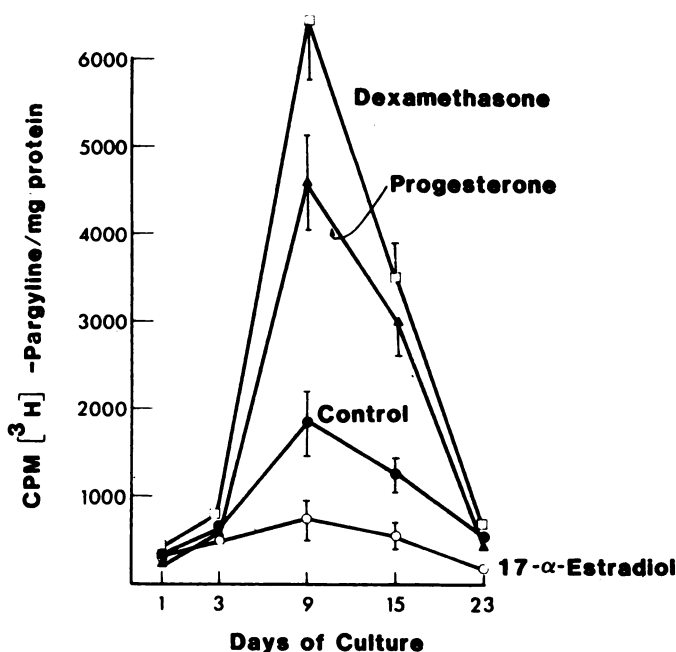
#### Titration of MAO with [ $^3$ H]pargyline

Irreversible suicidal inhibitors of MAO such as phenelzine (22), pargyline (16, 17), clorgyline, and deprenyl (16) interact with the enzyme by forming a stable adduct specifically and stoichiometrically to the covalently bound flavin adenine dinucleotide at the active center of the enzyme (23). The binding is time dependent, and when the enzyme is fully inhibited it has been found that 1 mol of inhibitor is bound per mole of the enzyme. This binding phenomenon can therefore be

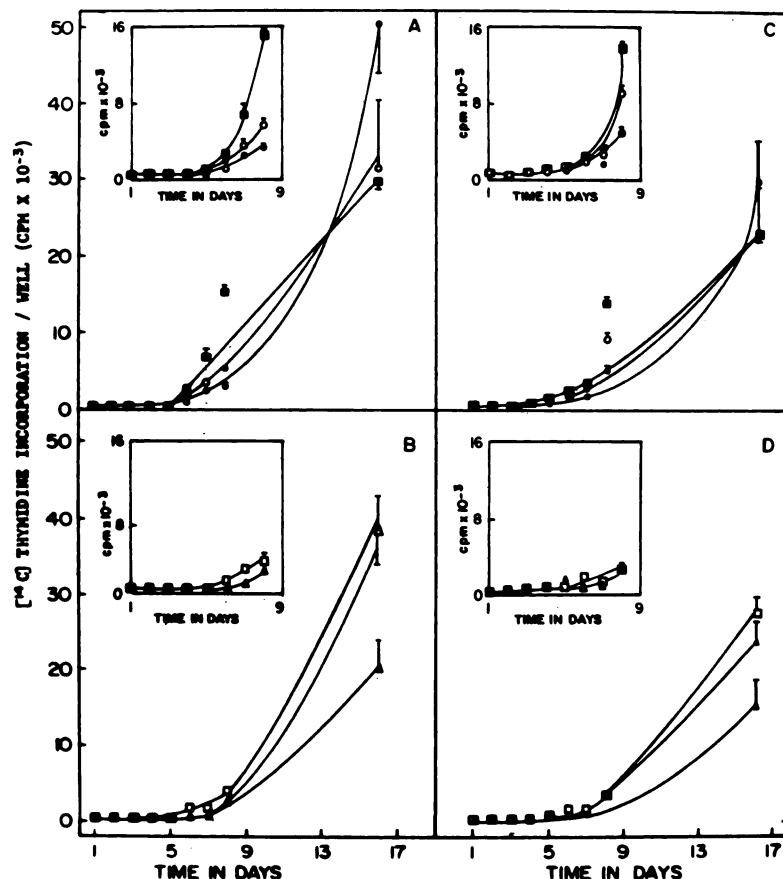
used to determine the relative amount of MAO present in a given preparation. As shown in Fig. 3, [ $^3$ H]pargyline binding to MAO increased 2- to threefold over the untreated controls in cells cultured for 8 and 15 days in the presence of progesterone and dexamethasone. As expected from the initial kinetic studies, identical treatment of cells with 17- $\alpha$ -estradiol decreased [ $^3$ H]pargyline binding to MAO by ca. 60% (Fig. 3). These data strongly support the concept that treatment of endothelial cells with progesterone and dexamethasone raises the number of MAO A molecules whereas 17- $\alpha$ -estradiol reduces the number of MAO A molecules.

#### Effect of steroid hormones on cellular protein and DNA synthesis

Both kinetic measurement and titration of the enzyme with [ $^3$ H]pargyline show rather convincingly that the presence of different amounts of MAO activity in endothelial cells after treatment with progesterone, hydrocortisone, testosterone, dexamethasone, or 17- $\alpha$ -estradiol greater than the untreated controls is probably caused by their differential effects on the enzyme production. This was further analyzed by [ $^{14}$ C]leucine and [ $^{14}$ C]thymidine incorporation into the protein and DNA of endothelial cells during steroid hormone treatment. As shown in Fig. 4A and Fig. 4B, [ $^{14}$ C]thymidine incorporation into endothelial cell cultures was stimulated to a different extent by the different steroids.



**Figure 3.** Titration of MAO A with [ $^3$ H]pargyline. Endothelial cells were grown in the absence and presence of various steroid hormones, as described in Fig. 2. At the indicated time intervals cells were removed, frozen-thawed, and then incubated with [ $^3$ H]pargyline for 90 min at 37°C. At the end of the incubation period the proteins were precipitated with 5% perchloric acid and collected on Millipore filters (0.45  $\mu$ m). The precipitates were washed twice with 0.05 M sodium phosphate buffer, pH 7.4, and analyzed for radioactivity in Hydrofluor. Each point is the mean from three flasks assayed in duplicate.



**Figure 4.** Effect of steroid hormones on cellular protein and DNA synthesis.  $2.2 \times 10^3$  cells were plated in 24-well plates in the absence and presence of  $1 \mu\text{M}$  of progesterone, hydrocortisone, testosterone, dexamethasone, or  $17\text{-}\alpha$ -estradiol and cultured at  $37^\circ\text{C}$  in a  $5\%$   $\text{CO}_2$  incubator. The cells were labeled for 24 h with  $[^{14}\text{C}]$ thymidine ( $0.2 \mu\text{Ci/well}$ ) (A, B) and in parallel with  $[^{14}\text{C}]$ leucine ( $0.5 \mu\text{Ci/well}$ ) (C, D). At the end of 24 h the cells were washed, harvested, precipitated in  $10\%$  TCA, filtered, and the radioactivity was analyzed in Ready Solv. Each point is the mean from three wells. Control (●), hydrocortisone (○), progesterone (■), dexamethasone (□), testosterone (▲), and  $17\text{-}\alpha$ -estradiol (△).

However, the increments showed a sustained rise between 9 and 17 days of culture. Moreover, as indicated by data depicted in Fig. 2, the most profound changes in MAO A levels occurred by day 8 and tended to decline thereafter. Similar data for  $[^{14}\text{C}]$ leucine incorporation are shown in Fig. 4C and Fig. 4D. These data show that the changes induced in endothelial cell MAO A by different steroids are relatively specific and do not occur solely in proportion to general metabolic changes induced by the same steroids. It is worth mentioning that the experiments described in Fig. 4 were undertaken to understand the metabolic behavior of the whole cell during steroid hormone activity. Because the cells were metabolically labeled with the radioactive precursors, we decided to reduce the steroid hormones concentration to  $1 \mu\text{M}$  as opposed to the  $10 \mu\text{M}$  that was used in the MAO activity measurements shown in Fig. 2.

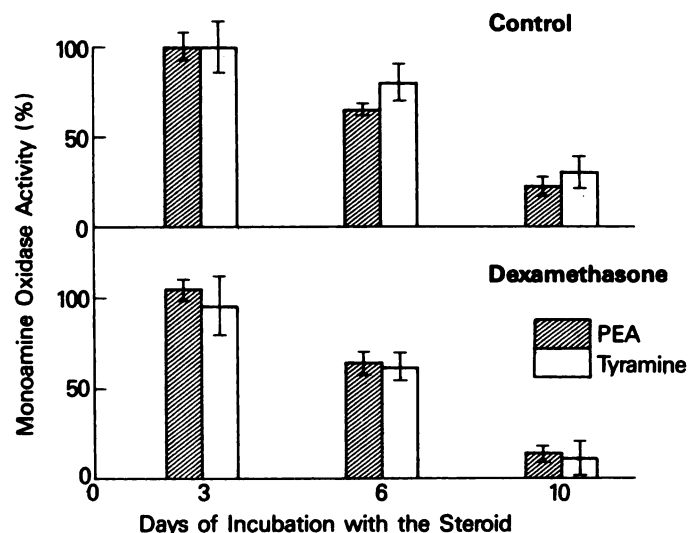
#### Influence of steroid hormones on MAO B activity of chromaffin cells

The effects of these steroids on the MAO B activity of cultured chromaffin cells was also examined. The activity was measured with PEA, a specific MAO B substrate, and tyramine, a substrate selective for both A

and B types of MAO. As shown in Fig. 5, dexamethasone ( $10 \mu\text{M}$ ) had no effect on the MAO activity measured with either substrate. Furthermore, over a period of 3–10 days, MAO B activity progressively declined, in marked contrast to the progressive increment in MAO A activity of endothelial cells. By contrast, the same amount of dexamethasone induced phenylethanolamine *N*-methyltransferase (EC 2.1.1.28) activity in these cultured chromaffin cells (24). Therefore, the chromaffin cells possess an active glucocorticoid receptor, but activation of this receptor has no measurable effect on MAO B.

#### DISCUSSION

The present study shows clearly that MAO activity in cultured bovine adrenal medullary endothelial cells is of the A type, and that different steroid hormones can cause increases or decreases in the number of MAO A molecules per cell. These results explain the previous *in vivo* experiments with rats, rabbits, and humans that indicated that steroid hormones such as progesterone,  $17\text{-}\beta$ -estradiol, and hydrocortisone have profound influence of MAO activity depending on age, sex, and the tissues examined (1–3, 7). These *in vivo* experi-



**Figure 5.** Influence of steroid hormone on MAO B activity of chromaffin cells. Chromaffin cells were isolated and cultured as described in Materials and Methods in the presence and absence of 10  $\mu$ M dexamethasone. At the indicated times, cells were harvested and MAO activity was determined. Each point is an average of three determinations.

ments have been difficult to interpret because no knowledge of the cell type or MAO type was available. Furthermore, it has not been possible to ascertain whether the observed hormone action on MAO activity was a direct effect or the result of interaction with other hormonal systems. However, the present studies of isolated and cultured endothelial cells and chromaffin cells from the adrenal medulla provide unambiguous information about the effects of steroids on the intact adrenal medulla.

For example, in the intact adrenal gland, progesterone, dexamethasone, and hydrocortisone cause increases in MAO activity. Similar effects are seen when the same steroids are added to cultured endothelial cells from the same tissue. By contrast, dexamethasone had no effect on chromaffin cell MAO activity, which involves an enzyme exclusively of the B type. Similarly, estradiol suppresses MAO activity in the intact gland and also suppresses MAO A activity in cultured adrenal medullary endothelial cells. One steroid, testosterone, had no effect on MAO activity in either the intact gland or the cultured endothelial cell. One simple interpretation of these data is that the influences of various steroids on the adrenal medulla in vivo are likely to be on MAO A activity of medullary endothelial cells exclusively. To our knowledge this is the first instance of a cytological dissection of different forms of MAO and their responses in specific tissues to different steroid hormones.

The mechanism by which various steroids affect MAO A activity in endothelial cells is also of interest. As seen from the data in Fig. 4, the effects of steroids precede the general onset of cell division and protein biosynthesis. Therefore, the effects seem to be specific to the MAO A system. MAO A is defined by a nuclear gene, but is finally placed on the outer mitochondrial membrane. The binding studies with pargyline clearly

show that additional MAO A molecules are synthesized coincidentally with acquisition of increased MAO A activity. We must conclude, therefore, that the process that controls the genesis and placement of new MAO A molecules is also subject to braking as well as acceleration, inasmuch as 17- $\alpha$ -estradiol can suppress both signals. In terms of total activity, 17- $\alpha$ -estradiol suppresses by ca. 50%. But in terms of changes in MAO A activity over the baseline, the reduction is ca. 90%. Similarly, in terms of MAO A molecules, the suppression by 17- $\alpha$ -estradiol is also of the same magnitude. The estradiol receptor does not distinguish between 17- $\alpha$ - and 17- $\beta$ -stereoisomers (25); therefore, response due to 17- $\alpha$ -estradiol is truly estrogenic. We must conclude that there is a complex and precise mechanism involving at least three different steroid hormone receptors in the endothelial cell that regulates MAO A genesis. The specific receptors for progesterone and estradiol in endothelial cells have previously been identified (26-28).

The nature of the cells in the adrenal medulla that respond to steroids is also of interest in terms of the physiology of the gland. The chromaffin and endothelial cells make up an archetypal endocrine system in which the endothelial cells are the pathway by or through which catecholamines from the endocrine cells gain access to the bloodstream. MAO B in the chromaffin cell hardly affects the principal catecholamines synthesized, stored, and secreted by the cell. By contrast, MAO A in the endothelial cells specifically catabolizes the chromaffin cell products, i.e., catecholamines. The present data, therefore, show that the modulation of MAO A activity in adrenal medullary endothelial cells by steroid hormones, including those from the adrenal cortex and other steroidogenic tissues, could control physiologically important mechanisms for regulation of global catecholamine homeostasis. [F]

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