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Monoamine Oxidases A and B Are Differentially Regulated by Glucocorticoids and "Aging" in Human Skin Fibroblasts

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SUMMARY

1. Two forms of monoamine oxidase (MAO A and MAO B) exist which, although similar in a number of properties, can be distinguished on the basis of their substrate specificity, inhibitor sensitivity, kinetic parameters, and protein structure. These properties were used to study the molecular mechanism(s) by which glucocorticoid hormones and "aging," known to alter MAO activity *in vivo*, regulated the expression of MAO A and MAO B in cultured human skin fibroblasts.

2. The addition of dexamethasone or hydrocortisone to cultures resulted in a dose- and time-dependent increase in total MAO activity, whereas the removal of hormone from cultures resulted in a time-dependent decrease in activity toward control levels.

3. The response to dexamethasone was affected by culture conditions such as serum concentration, feeding frequency, and cellular "age."

4. Cellular aging, in the absence of hormone, also resulted in increased levels of total MAO activity.

5. The effects of hormones and aging on total MAO activity appeared to be selective for MAO A. The 6- to 14-fold increases in total activity were paralleled by similar increases in the activity and amount of active MAO A but less than 2- to 3-fold increases in the activity and amount of MAO B.

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6. Altered synthesis or degradation of the active enzyme appeared to account for the effects of hormones, aging, and various culture conditions on MAO activity. Inhibitor sensitivity, substrate affinity, electrophoretic mobility, and molecular turnover number of either form of the enzyme were not altered during dexamethasone treatment or during cellular aging. However, rates of active MAO synthesis were affected by hormone treatment and feeding frequency, rates of active MAO degradation by serum concentration, and rates of active MAO synthesis or degradation by aging.

7. In summary, we have shown that glucocorticoids and cellular aging selectively affect the amount of MAO A at the level of active enzyme synthesis or degradation. Further, our finding that the expression of the two forms of MAO in human fibroblasts can be independently regulated supports the growing evidence that MAO A and MAO B are separate molecular entities.

INTRODUCTION

Regulation of biogenic amine metabolism is important in maintaining normal physiologic functions in the individual. One enzyme that plays a key role in this regulation is monoamine oxidase (MAO; monoamine:O_2 oxidoreductase; EC 1.4.3.4), a degradative enzyme that oxidatively deaminates monoamines to their corresponding aldehydes. Through its effects on neurotransmitter levels, MAO can affect many physiologic processes and behaviors mediated by monoaminergic pathways (Murphy and Kalin, 1980). Further, altered levels of MAO have been implicated in the etiology of several disorders affecting the nervous system (Giller, 1980).

Biochemical, physicochemical, and physiologic studies have demonstrated the existence of two forms of MAO, designated MAO A and MAO B. Both are integral proteins of the outer mitochondrial membrane (Greenawalt and Schnaitman, 1970) embedded in a phospholipid structure required for activity (Huang and Faulkner, 1980; White and Stine, 1984). The two forms of MAO appear to be glycoproteins (Tipton and Della Corte, 1979; Houslay and Marchmont, 1980) composed of two subunits similar in molecular weight, only one of which contains a covalently bound FAD cofactor (Minamiura and Yasunobu, 1978; Salach and Detmer, 1979). However, studies using purified enzymes have been difficult and questions still remain about the structure of MAO A and MAO B.

Despite their similarities, the two forms of MAO can be distinguished on the basis of substrate specificity and inhibitor sensitivity (see Fowler *et al.*, 1978). The A form preferentially deaminates serotonin and norepinephrine and is selectively inhibited by low concentrations of clorgyline; the B form preferentially deaminates benzylamine and phenylethylamine and is selectively inhibited by low concentrations of deprenyl; and both forms of the enzyme readily deaminate dopamine, tryptamine, and tyramine and are inhibited by similar concentrations of pargyline. The two forms of active enzyme also differ in their pH optima (Gabay *et al.*, 1976), sensitivity to various compounds and treatments (White and Tansik, 1979), dependence on phospholipids (Huang and Faulkner, 1980; White and Stine, 1984), degree of glycosylation (Houslay and Marchmont, 1980), immunoreactivity (Denney *et al.*, 1982; Pintar *et al.*, 1983), and

distribution in the mitochondrial membrane (Russell *et al.*, 1979). In addition, the two forms of MAO have been physically separated by ion-exchange (Pearce and Roth, 1984) and immunoaffinity (Denney *et al.*, 1982) chromatography and the flavin-containing subunits have been shown to differ in their molecular weight and one-dimensional peptide maps by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (Callingham and Parkinson, 1979; Brown *et al.*, 1980; Cawthon and Breakefield, 1983).

In addition to physicochemical differences, the two forms of MAO appear to be independently regulated. The ratio of MAO A to MAO B activity varies dramatically among different tissues (Lewinsohn *et al.*, 1980), cultured cell lines (Hawkins and Breakefield, 1978), and mitochondrial populations (Mitra and Guha, 1978). Within a single tissue, the two forms of MAO also have been found to differ in their concentration (Edwards and Pak, 1979; Fowler *et al.*, 1980), half-life (Feldman *et al.*, 1980), expression during ontogenetic development and aging (see Discussion for references), and response to various hormone treatments (see Discussion for references).

Although the biochemical and physicochemical properties of MAO A and MAO B have been studied extensively in a variety of tissues and cultured cells, much less is known about the physiologic factors that affect activity. Numerous studies have shown that hormones such as glucocorticoids, sex steroids, and thyroid hormones alter MAO activity in animals and humans; however, the direction and magnitude of the hormone response can vary with the age, sex, and hormonal status of the individual, the tissue examined, and the means used to alter hormone levels (see Sourkes, 1979). Similarly, the observed changes in MAO activity that occur during aging have been difficult to interpret. In most studies, MAO activity increases between late infancy and old age; however, the magnitude of the increase and the observed developmental pattern can vary with the sex or strain of the individual and the tissue examined (see Youdin and Holzbauer, 1976; Lewinsohn *et al.*, 1980).

In our laboratory, we have used cultured human skin fibroblasts as a model system to study the regulation of MAO by hormones and aging since fibroblasts are the only source of human tissue readily obtainable from living individuals which (1) express both MAO A and MAO B activities (Roth *et al.*, 1976; Groshong *et al.*, 1977), (2) contain receptors for various steroid hormones such as androgens and glucocorticoids (Bauknecht, 1977; Bruning *et al.*, 1979), (3) respond to hormonal stimuli with the accumulation of hormone–receptor complexes inside the nucleus (Bruning *et al.*, 1979) and changes in chromatin structure (Johnson *et al.*, 1979), and (4) display signs of cellular "aging" or senescence with successive passaging in culture (Hayflick, 1980).

In preliminary studies, several hormones known to alter MAO activity *in vivo* were examined for their effects on activity in human skin fibroblast cultures (Edelstein and Breakefield, 1981). Of the hormones tested, only the synthetic glucocorticoid dexamethasone stimulated MAO activity more than 30–40% at "physiologic" concentrations. We also have shown that levels of MAO activity in human fibroblasts increases 3- to 10-fold as a function of increasing donor age (Breakefield *et al.*, 1980) and cellular "age" (Edelstein *et al.*, 1978). In the present study, we have examined the conditions that affect the response of total MAO activity to dexamethasone treatment and have examined the effects of dexamethasone and cellular aging on various

properties of the active forms of MAO A and B, including (1) rates of enzyme synthesis and degradation, (2) inhibitor sensitivity, (3) substrate affinity and maximum reaction velocity, (4) electrophoretic mobility, (5) enzyme concentration, and (6) molecular turnover number.

MATERIALS AND METHODS

Materials

Disposable plastic tissue culture flasks were purchased from Corning (Corning, NY); tissue culture dishes from Falcon (Oxnard, CA); fetal calf serum (FCS) from Flow Laboratories (Rockville, MD) or Irvine Scientific (Santa Ana, CA); Dulbecco's modified Eagle's medium (DMEM; H-21) and 100 × antibiotic–antimycotic solutions from GIBCO (Grand Island, NY); and components for MCDB 105 medium (MCDB; McKeehan *et al.*, 1978) from Calbiochem (San Diego, CA), Schwartz–Mann (Orangeburg, NY), or Sigma (St Louis, MO). Thin-layer silica-gel sheets (20 × 20 cm; No. 6061) and X-Omat R film were purchased from Eastman Kodak (Rochester, NY); En^{3}Hance, Liquifluor, and Protosol from New England Nuclear (Boston, MA); and reagents for sodium dodecyl sulfate–polyacrylamide electrophoresis (SDS-PAGE) from BioRad Laboratories (Richmond, CA) or Sigma. The radiochemicals [1,2(*n*)-^{3}H]dexamethasone (20 Ci/mmol), [G-^{3}H]tryptamine hydrochloride (1.0–2.5 Ci/mmol), and [^{14}C]methylated molecular weight (MW) marker protein mixture (14,300–200,000 MW; 8–32 μCi/mg protein) were purchased from Amersham–Searle (Arlington Heights, IL); and [phenyl-3, benzyl-^{3}H]pargyline hydrochloride (6.86 Ci/mmol) from New England Nuclear. Clorgyline was a gift from May and Baker, Ltd. (London, England), and deprenyl was a gift from Dr. J. Knoll (Sammelweis University of Medicine, Budapest, Hungary). Dexamethasone, hydrocortisone, pargyline, trypsin, and tryptamine were purchased from Sigma; catalase and *N*-2-hydroxyethylpiperazine -*N*′-2-ethanesulfonic acid (HEPES) from Calbiochem; and all other chemicals from J. T. Baker Chemical Co. (Phillipsburg, NJ), Fischer Scientific Co. (Pittsburgh, PA), or Mallinckrodt Chemical Works (St. Louis, MO).

Cell Lines

The human diploid fibroblast line HF27 was established in our laboratory from a skin biopsy collected by Dr. Elliot Gershon (National Institute of Mental Health, Bethesda, MD) from the upper hip of a 38-year-old Caucasian male, as described (Edelstein *et al.*, 1980). The human fibroblast lines designated NF were established by Dr. Robert Harper (Temple University, Philadelphia, PA) from the papillary (NF70A) and reticular (NF708) dermal layers of a single skin biopsy obtained from the abdomen of a 45-year-old Caucasian female, as described (Harper and Grove, 1979). Viable frozen stocks of all cell lines were prepared and stored in liquid nitrogen, as described (Edelstein *et al.*, 1980).

Cell Culture

Fibroblasts were routinely grown as monolayers on 75- or 150-cm^2 flasks in antibiotic-free DMEM medium supplemented with 5% (v/v) fetal calf serum (DMEM–5% FCS) and maintained at 37°C in a humidified atmosphere of 5% CO_2–95% air. Stock flasks were fed at 6- to 8-day intervals with DMEM–5% FCS and subcultured (at a split ratio of 1:8) every 3–4 weeks by low-temperature trypsinization (McKeehan, 1977) using isotonic HEPES-buffered saline (HBS) containing 0.5% (w/v) trypsin. To avoid the changes in MAO activity that occur with cellular "aging" (Edelstein *et al.*, 1978), stock cultures were passaged only 4 to 9 times (unless indicated otherwise) since cultures passaged more than 10 times showed signs of aging such as decreased growth rate, decreased density at confluency, and flatter cell morphology (Hayflick, 1980).

Hormone Treatment

Stock cultures were subcultured onto 100- or 150-mm dishes using a split ratio of 1:11 and fed every 7–9 days with DMEM–5% FCS, as described above. Seven to ten days after reaching confluency, monolayers were rinsed twice with HSB, incubated with serum-free MCDB containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml Fungizone for 24 hr, then exposed to dexamethasone or hydrocortisone, as indicated. For *concentration curves*, cultures were exposed to MCDB containing no hormone or various concentrations (1 n*M* to 10 μ*M*) of dexamethasone or hydrocortisone for 5 days without feeding. For *time courses*, cultures were exposed to MCDB or DMEM–5% FCS containing no hormone or 50 n*M* dexamethasone with or without feeding every 3 days over a 12-day period. For *hormone withdrawal*, cultures were exposed to MCDB containing no hormone or 50 n*M* dexamethasone for 5 days without feeding, rinsed twice with HBS, then exposed to MCDB without hormone or feeding for an additional 11 days. For *recovery after irreversible inhibition*, cultures were preincubated with MCDB containing 10 n*M* clorgyline and 1 μ*M* deprenyl for 2 hr, rinsed twice with isotonic phosphate-buffered saline (PBS), then exposed every 1–4 days to MCDB with or without 50 n*M* dexamethasone over a 25-day period. For *all other experiments*, cultures were exposed to MCDB containing no hormone or 50 n*M* dexamethasone for 5–9 days without feeding.

Stock solutions (10 m*M*) of hormones were prepared in absolute ethanol, stored at −20°C, and diluted in the appropriate medium at least 1 day prior to use. Dexamethasone was relatively stable under the conditions used for storage as well as the conditions used for hormone treatment. In fact, less than 5% of the hormone was degraded after fibroblast cultures were incubated with MCDB containing 12.5 n*M* [1,2(*n*)-^{3}H]dexamethasone for 16 days, as judged by thin-layer chromatography of the medium on silica-gel sheets (Edelstein, 1984).

As indicated above, MCDB 105 medium was used for all incubations prior to and during hormone treatment that required serum-free medium. This medium was specifically formulated for growth of human fibroblasts in the presence of $< 0.05\%$ (v/v) serum (McKeehan *et al.*, 1978). In fact, fibroblast cultures could be maintained at least 3–4 weeks in serum-free MCDB, whereas cultures survived less than 1 week in serum-free DMEM (unpublished data). In contrast to the differences between the two media

on fibroblast survival, MAO activity was similar whether fibroblasts were subcultured and maintained for 4 days in MCDB containing 1–10% (v/v) FCS or in DMEM containing 1–10% (v/v) FCS (unpublished data).

Harvesting of Cell Monolayers

To harvest cells, monolayers were rinsed with PBS and scraped off dishes, as described (Cawthon and Breakefield, 1983). For enzyme assays, cells were collected by centrifugation at 10,500*g* (Brinkman 3200 microfuge) for 30 sec and pellets were stored at −65°C. For subcellular fractionation, cells were collected by centrifugation at 1100*g* (Beckman J-21 centrifuge) for 10–20 min at 4°C and used immediately (see below).

Isolation of Crude Mitochondrial Fractions

Crude mitochondrial fractions were isolated by differential centrifugation, as described by Costa and Breakefield (1979), with the following modifications: (1) unbroken cells and nuclei were collected by centrifugation at 1950*g* (Beckman J-21 centrifuge) for 15–20 min; (2) mitochondria were collected by centrifugation at 14,600*g* for 45 min; and (3) mitochondrial pellets were resuspended in 50 m*M* Tris–HCl, pH 7.5 (Tris buffer), at a concentration of 0.3–0.9 mg protein/ml. For enzyme assays, mitochondrial fractions were stored at −65°C prior to use. For [^{3}H]pargyline binding, mitochondrial fractions were used immediately after isolation.

Monoamine Oxidase Assay

Monoamine oxidase activity in cell homogenates and crude mitochondrial fractions was measured by toluene extraction, as described by Bonnefil *et al.* (1981), with the following modifications: (1) cell homogenates (10–170 μg protein) were incubated with 10 m*M* sodium phosphate buffer (pH 7.4) containing 34 μM [^{3}H]tryptamine hydrochloride and 10 μM ascorbic acid; (2) mitochondrial fractions (5–12 μg protein) were incubated with Tris buffer containing 37 μM [^{3}H]tryptamine and 40 μM ascorbic acid and reactions were stopped by the addition of 50 μl of 4 *N* HCl; and (3) deaminated products were extracted by shaking for 10 min. All enzyme activities were expressed as picomoles of tryptamine deaminated per minute per milligram of protein.

Due to substrate inhibition (unpublished data), concentrations of tryptamine higher than 40 μM were not used. Since subsaturating concentrations of tryptamine had to be used, the activities determined by this assay were only 54% (MAO B) to 76% (MAO A) of maximum. Except for the determination of molecular turnover numbers (see Table IV), activities were not corrected for subsaturating concentrations of tryptamine. To estimate maximum activity (V_{max}), the values for observed activity (v), tryptamine concentration ($[S]$) and substrate affinity (K_m; see Table III) were substituted into the Michaelis–Menton equation, rearranged to the form

$$V_{max} = \frac{(v)(K) + (v)([S])}{[S]}. \quad (1)$$

Determination of MAO A and MAO B Activities

Levels of MAO A and MAO B activities were determined by measuring the sensitivity of tryptamine deamination to inhibition by selective MAO inhibitors. Briefly, biphasic dose–response curves were established by preincubating cell homogenates for 15 min (NF70A and NF70B) or 30 min (HF27) at 37°C with serial dilutions of clorygline (10^{-16} to 10^{-3} *M*) or deprenyl (10^{-12} to 10^{-4} *M*) prior to measurement of MAO activity. By definition, activity inhibited by low concentrations of clorgyline (e.g., 10^{-11} to 10^{-9} *M*) was designated type A, while the remaining activity, inhibited only by high concentrations of clorgyline (e.g., 10^{-5} *M*), was designated type B. Conversely, activity inhibited by low concentrations of deprenyl (e.g., 10^{-8} *M*) was designated type B, while the remaining activity, inhibited only by high concentrations of deprenyl (e.g., 10^{-4} *M*), was designated type A.

To inhibit selectively only MAO A or only MAO B activity, cell homogenates or crude mitochondrial fractions were preincubated with a single concentration of inhibitor, as indicated in the experiments below. To assure that only the desired form of MAO would be measured, concentrations were selected which not only inhibited the undesired form of MAO but also partially inhibited the form of MAO being quantitated. At the concentrations of deprenyl or clorgyline used in these experiments, greater than 90% of the uninhibited levels of MAO A in control cultures as well as MAO A and MAO B in dexamethasone-treated cultures would be measured, whereas only 65–75% of the uninhibited levels of MAO B in control cultures would be measured (Edelstein, 1984).

Determination of Kinetic Parameters

Kinetic parameters were determined by measuring MAO activity in cell homogenates (4–16 μg cellular protein) using five to seven concentrations of [^{3}H]tryptamine ranging from 1 to 70 μM. Prior to the addition of substrate, cell homogenates were preincubated for 20–40 min at 37°C with 0.1 μM deprenyl or 10 n*M* clorgyline to determine kinetic parameters for MAO A or MAO B, respectively. Values for the substrate affinity (K_m) and maximum reaction velocity (V_{max}) were calculated using direct linear plots, as described by Cornish-Bowden and Eisenthal (1974).

Determination of Steady-State Activities, Rate Constants, and Half-Lives

Several methods based on the kinetics of change in enzyme activity from one steady state to a new steady state (e.g., hormone exposure, hormone withdrawal, recovery after inhibition) were used to determine steady-state activities, rate constants, and half-lives of MAO in control and dexamethasone-treated cultures. Each of these methods carries with it several assumptions which have been verified experimentally for MAO (Gordis and Neff, 1971; Lyles and Callingham, 1974; Della Corte and Callingham, 1977; Luine and McEwen, 1977; Nelson *et al.*, 1979) and many other enzymes (see Schimke and Doyle, 1970). For a complete discussion of the theories and assumptions behind each of the methods and the derivation of Eqs. (2)–(4) described below, see Schmike and Doyle (1970) and Gordis and Neff (1971).

In the first approach (Approach 1), the rate constants in dexamethasone-treated cultures were determined by following the rise in MAO activity from control levels toward new steady-state levels after exposure to dexamethasone. This approach assumed that the rate of MAO synthesis increased rapidly to a new constant rate following exposure to dexamethasone and that the observed increases in MAO activity represented *de novo* enzyme synthesis. Conversely, the rate constants in control cultures were determined by following the decay of MAO activity from elevated levels towards control levels following the removal of hormone from dexamethasone-treated cultures. This method assumed that the actions of dexamethasone terminated immediately upon its removal and that the rate of MAO degradation in dexamethasone-treated cultures following hormone withdrawal was similar to that in control cultures.

In the second approach (Approach 2), the rate constants in control and dexamethasone-treated cultures were determined by following the return of activity in the absence and presence of dexamethasone, respectively, from inhibited levels (e.g., the activity remaining after preincubation with inhibitors) back toward control and dexamethasone steady-state levels once inhibitors were removed. This approach assumed that (1) the inhibitors themselves did not affect rates of active MAO synthesis and degradation, (2) low levels of MAO activity did not alter the mechanism of dexamethasone action, (3) the return of MAO activity represented *de novo* protein synthesis, (4) the approach of MAO activity back toward steady-state levels followed first-order kinetics, and (5) the net rate for recovery of activity was solely a function of the first-order rate constant of degradation.

Using the data obtained from the experiments described above, the steady state and first-order rate constants of degradation (k_d) were calculated, as described below, from the equation describing the exponential change in MAO activity from one steady state to a new steady state,

$$[\mathrm{MAO}_t] = [\mathrm{MAO}_{ss}][1 - e^{-k_d t}] + [\mathrm{MAO}_0][e^{-k_d t}]; \tag{2}$$

the zero-order rate constant of synthesis (k_s) from the equation

$$k_s = k_d \times \mathrm{MAO}_{ss}; \tag{3}$$

and the half-life ($t_{1/2}$) from the equation

$$t_{1/2} = 0.693/k_d; \tag{4}$$

where MAO_t is the activity at time t, MAO_0 is the initial steady-state activity, and MAO_{ss} is the new steady-state activity.

Equation (2) was fit directly to the data by nonlinear regression analysis using a modification of the iterative regression program of Duggleby (1981). Bisquared weighting was used to detect and reduce the effects of outliers, e.g., observations deviating markedly from Eq. (2). Proportional weighting was also used to improve further the fit of Eq. (2) to data obtained from the inhibition experiments described above (Approach 2). No additional weighting, however, was required for the data obtained from the hormone exposure and withdrawal experiments (Approach 1). Once the observed activities were properly weighted and initial estimates of the steady state and degradative rate constant were made, final values for the MAO_{ss} and k_d (which were

largely independent of the initial estimates of these parameters) were calculated and substituted into Eqs. (3) and (4) to calculate the k_s and $t_{1/2}$, respectively.

Binding of [^{3}H]Pargyline

Crude mitochondrial fractions were incubated with [^{3}H]pargyline under conditions that maximally labeled both MAO A and MAO B but kept background and nonspecific labeling of other proteins to a minimum (Edelstein, 1984). Briefly, mitochondria were incubated with 1.25–1.50 μM [^{3}H]pargyline (3–6 pmol pargyline/μg mitochondrial protein) for 4 hr at 37°C in a final volume of 200–600 μl Tris buffer. Prior to labeling, samples were preincubated with 1.0 μM deprenyl or 0.1 μM clorgyline for 15 min at 37°C to label selectively the flavin-containing subunits of MAO A and MAO B, respectively. To remove unbound label, samples were diluted with 800 μl Tris buffer, held on ice for 15–20 min, and centrifuged at 10,500g (Brinkman 3200 microfuge) for 20 min at 4°C. The supernatants were discarded and the labeled mitochondrial pellets were stored at −20°C.

Electrophoretic Analysis

[^{3}H]Pargyline-labeled mitochondrial fractions were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), as described by Cawthon and Breakefield (1983), with the following modifications: (1) mitochondrial proteins (100–125 μg) were resuspended in 80–100 μl of loading solution containing 0.5% (v/v) β-mercaptoethanol and solubilized by heating for 4–6 min at 85–90°C; (2) molecular weight (MW) marker lanes were loaded with 3 μg of catalase (58,000 MW) and 180 dpm (83 pCi) each of the [^{14}C]methylated proteins phosphorylase *b* (100,000 MW), bovine serum albumin (69,000 MW), ovalbumin (46,000 MW), and carbonic anhydrase (30,000 MW); (3) electrophoresis was carried out using a 2 × 15 × 0.15-cm stacking gel and a 17 × 15 × 0.15-cm resolving gel for 22 hr at 50 V (constant voltage), then 4 hr at 70–80 V (constant voltage), until the tracer dye reached the bottom of the resolving gel; and (4) gels were destained in 10% (v/v) acetic acid and 30% (v/v) methanol for 3–5 hr.

The qualitative distribution of radioactivity was determined by treating gels for fluorography with En^{3}Hance (as recommended by New England Nuclear), exposing dried gels to preflashed X-Omat R film for 2–3 months at −65°C, and developing fluorograms on an RP X-Omat Kodak processor. The quantitative distribution of radioactivity was determined by cutting individual lanes from stained and dried gels into 2-mm slices in the MW region between 55,000 and 70,000, rehydrating slices with 50 μl distilled water for 30 min at room temperature, digesting slices with 4.5 ml toluene containing 5% (v/v) Protosol and 7.5% (v/v) Liquifluor for 48 hr at 45°C, and measuring eluted radioactivity by liquid scintillation spectrometry.

Protein Determinations

Protein concentrations were determined by the method of Bradford (1976) using methanol in place of ethanol. All samples were assayed in duplicate within the linear

range of the assay using bovine serum albumin as a standard. Protein values were expressed as milligrams of protein per sample assayed for calculating specific activities or as micrograms of protein per square centimeter of growth surface area for calculating the total protein per dish.

RESULTS

Effects of Varying Glucocorticoid Concentration on Total MAO Activity

To extend our previous findings on the effects of the synthetic glucocorticoid dexamethasone on MAO activity in human fibroblasts (Edelstein and Breakefield, 1981) and determine whether this hormone could be used as a model of glucocorticoid action, cultures of HF27 were exposed to various concentrations of dexamethasone or the naturally occurring glucocorticoid hydrocortisone (Fig. 1). Both dexamethasone and hydrocortisone increased the total MAO activity in a dose-dependent manner. A

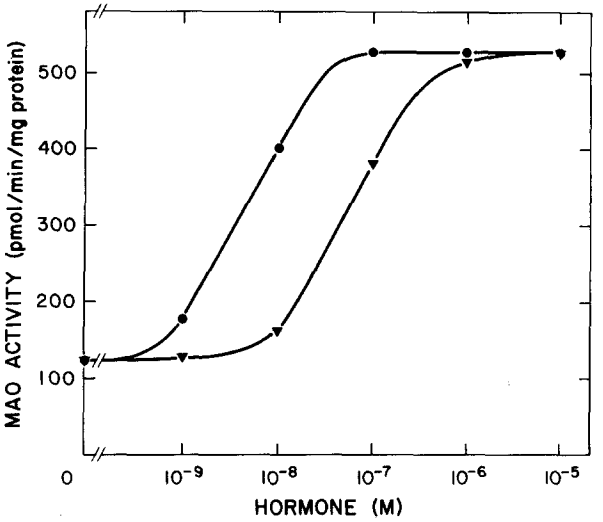

Fig. 1. Effects of varying glucocorticoid concentration on total MAO activity. Cultures of HF27 were grown in serum-containing medium (DMEM–5% FCS) until they were 7–10 days past confluency. Monolayers were rinsed twice with isotonic saline (HBS), preincubated with serum-free medium (MCDB) for 24 hr, then exposed to serum-free medium containing no hormone (control) or various concentrations of dexamethasone (●) and hydrocortisone (▼) for 5 days without feeding. Cultures were harvested and assayed for total MAO activity using 37 μM [^{3}H]tryptamine, as described in Materials and Methods. Results are expressed as pmol tryptamine deaminated/min/mg protein. Each point represents one homogenate assayed in triplicate at two protein concentrations (values differed $< 10\%$). Total cellular protein per dish remained constant over the range of hormone concentrations tested in the dexamethasone-treated (8.84 ± 0.65 $\mu g/cm^2$) and hydrocortisone-treated (8.58 ± 0.79 $\mu g/cm^2$) cultures as well as in the control cultures (9.92 ± 1.61 $\mu g/cm^2$).

5-day exposure to high concentrations (1–10 μM) of either hormone resulted in levels of MAO (525.4 $\pm$ 2.1 pmol/min/mg protein) that were four times control levels (132.2 $\pm$ 19.5 pmol/min/mg protein). However, dexamethasone was considerably more potent than hydrocortisone. Whereas half-maximal and maximal stimulation of MAO activity were observed at concentrations of 5 and 50 nM dexamethasone, respectively, similar increases in MAO activity required 10- and 20-fold higher concentrations of hydrocortisone, respectively.

Time Course of Response to Dexamethasone Under Different Culture Conditions

In the above studies, fibroblasts were exposed to hormone in the absence of serum and feeding. To determine whether these culture conditions were optimal for dexamethasone treatment and whether changes in culture conditions would affect the response to hormone, cultures of HF27 were exposed to medium with or without dexamethasone for various times using three different treatment schedules (Fig. 2). Levels of MAO remained fairly constant over the course of the experiment in cultures exposed to the different treatment schedules in the absence of dexamethasone (data not shown). In contrast, the kinetics and the magnitude of the increase in MAO activity in dexamethasone-treated cultures were affected by the treatment schedule used during hormone exposure. In cultures exposed to dexamethasone with serum and feeding, activity increased rapidly over the first 3 days and approached steady-state levels that were three times those of control cultures after 6 to 9 days of hormone treatment. In cultures exposed to dexamethasone without serum but fed, activity increased rapidly over the first 6 to 9 days and began to approach steady-state levels that were 8.8 times those of control cultures after 9–12 days of hormone treatment. In cultures exposed to dexamethasone without serum or feeding, activity increased rapidly over the first 9–12 days and began to approach steady-state levels that were 14 times those of control cultures only after 12 days of hormone treatment.

The differences in the response to dexamethasone under the various culture conditions could be totally accounted for by differences in the rates of synthesis and degradation of MAO (Table I, Experiment 1). For example, the presence of serum affected primarily MAO degradation. Although no significant difference in the rate of MAO synthesis was observed between cultures exposed to dexamethasone with feeding using serum-free and serum-containing medium, there were differences in the degradative rate constant and half-life of MAO. The k_d in the presence of serum was three times higher than that observed in the absence of serum, whereas the $t_{1/2}$ in the absence of serum was three times higher than the value observed in the presence of serum. Such differences would account for the threefold higher steady-state activities in dexamethasone-treated cultures not exposed to serum as compared to those exposed to serum. In contrast to the effects of serum, feeding appeared to affect primarily MAO synthesis. No differences in the degradative rate or half-life of MAO were observed between cultures exposed to dexamethasone with and without feeding in the absence of serum. However, the synthetic rate constant in cultures that were fed was only 70% of the k_s observed in cultures that were not fed. Such findings would thus account for the 30% lower steady-state activities in cultures that were fed as compared to those that were not fed.

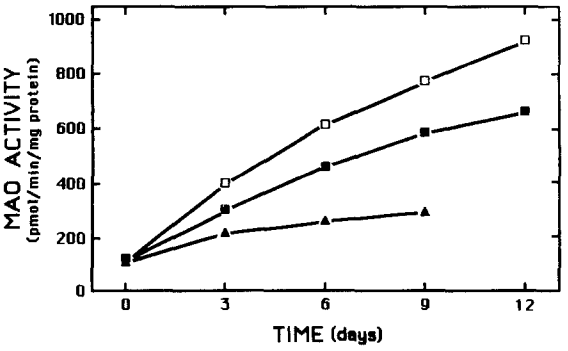

Fig. 2. Time course of response to dexamethasone under different culture conditions. Cultures of HF27 were grown, rinsed with saline, and preincubated with serum-free medium, as described in the legend to Fig. 1. On Day 0, monolayers were exposed to medium containing no hormone (control) or 50 n*M* dexamethasone, as follows: Schedule 1—cultures were exposed to serum-free medium without feeding (□); Schedule 2—cultures were exposed to serum-free medium with feeding every 3 days (■); and Schedule 3—cultures were exposed to serum-containing medium with feeding every 3 days (▲). Cultures were harvested periodically over 9–12 days and assayed in parallel for MAO activity. Each point represents one homogenate assayed in triplicate at two protein concentrations (values differed <15%). Control steady-state activities (calculated by averaging the activity in cultures not exposed to hormone over the course of the experiment) under the different schedules were 110.7 ± 18.5 pmol/min/mg protein (Schedule 1), 114.4 ± 12.2 pmol/min/mg protein (Schedule 2), and 104.5 ± 12.0 pmol/min/mg protein (Schedule 3). Dexamethasone steady-state activities (calculated as described in Materials and Methods) under the different schedules were 1410.3 ± 50.6 pmol/min/mg protein (Schedule 1), 1008.7 ± 77.4 pmol/min/mg protein (Schedule 2), and 308.4 ± 11.0 pmol/min/mg protein (Schedule 3). Over the course of the experiment, total cellular protein per dish in cultures exposed to serum-free medium remained constant in control (8.93 ± 1.55 $\mu g/cm^2$) or dexamethasone-treated (7.51 ± 1.10 $\mu g/cm^2$) cultures that were not fed (Schedule 1) and in control (8.53 ± 0.77 $\mu g/cm^2$) and dexamethasone-treated (8.93 ± 0.84 $\mu g/cm^2$) cultures that were fed (Schedule 2), whereas protein in cultures exposed to serum-containing medium increased from 10.21 to 17.23 $\mu g/cm^2$ in control cultures and from 10.21 to 21.52 $\mu g/cm^2$ in dexamethasone-treated cultures.

Effects of Dexamethasone Withdrawal on Total MAO Activity

Since dexamethasone does not break down and is not metabolized under the conditions used for hormone exposure (Edelstein, 1984), fibroblasts were exposed to dexamethasone continuously for up to 12 days regardless of the feeding schedule used in the above studies. To determine whether the continual presence of dexamethasone in the culture medium was necessary to maintain increased levels of MAO activity, cultures of HF27 were exposed to medium with or without dexamethasone for 5 days

Table I. Effect of Dexamethasone on Steady-State Activities, Rate Constants, and Half-Lives of Total MAO

	Condition[a]				Rate constant[b]		
Experiment	FCS	Fed	Dex	Steady state[b]	k_d	k_s	$t_{1/2}$
Experiment 1							
Hormone exposure							
Schedule 1	−	−	+	1410.3	0.081	114.9	8.5
Schedule 2	−	+	+	1008.1	0.081	81.6	8.6
Schedule 3	+	+	+	308.4	0.246	76.0	2.8
Experiment 2							
Hormone withdrawal	−	−	−	130.0	0.085	11.1	8.1
Hormone exposure	−	−	+	1410.3	0.081	114.9	8.5
Experiment 3							
Recovery after inhibition	−	+	−	34.5	0.065	2.2	10.7
	−	+	+	85.3	0.064	5.4	10.9

[a] Cultures of HF27 were exposed to serum-free (−FCS) or serum-containing (+FCS) medium supplemented with no hormone (−) or 50 n*M* dexamethasone (+) and not fed (−) or fed (+), as described in the legends to Fig. 2 (hormone exposure), Fig. 3 (hormone withdrawal), and Fig. 4 (recovery after inhibition).

[b] Steady-state activities and rate constants were calculated by fitting the data in Figs. 2–4 to Eq. (2) by nonlinear regression, as described in Materials and Methods. Steady-state activities are expressed as pmol tryptamine deaminated/min/mg protein, rate constants of degradation (k_d) as days^{-1}, rate constants of synthesis (k_s) as pmol tryptamine deaminated/min/mg protein/day, and half-lives ($t_{1/2}$) as days.

prior to incubation with medium lacking hormone. The effects of hormone withdrawal on MAO activity were then followed over the next several days (Fig. 3). In cultures never exposed to hormone, activity remained constant throughout the withdrawal period (130.0 ± 27.9 pmol/min/mg protein). In contrast, activity in cultures exposed to dexamethasone decreased over the 11-day period following hormone removal from values that were four times control levels (476.4 pmol/min/mg protein) to values that were less than twofold above control levels (235.1 pmol/min/mg protein) after a short lag of approximately 1 day.

Effects of Dexamethasone on the Recovery of Total MAO Activity After Irreversible Inhibition

The experiments described to this point examined the effects of dexamethasone on existing pools of active MAO molecules. To determine the effects of dexamethasone on newly synthesized active MAO molecules, we used the experimental protocol originally described by Gordis and Neff (1971). Cultures of HF27 were preincubated with the irreversible MAO inhibitors clorgyline and deprenyl to inactivate existing pools of active MAO. Inhibitor concentrations and preincubation times were chosen such that only 85–90% of the initial MAO activity was inhibited since it was essential that no free inhibitor remained which could inactivate newly synthesized MAO molecules. Once inhibitors were removed, the recovery of MAO activity—presumably representing *de novo* synthesis of the active enzyme—was followed in the absence and presence of dexamethasone over the next 25 days (Fig. 4).

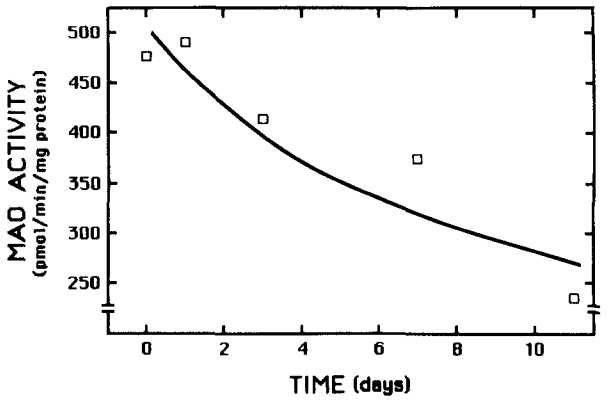

Fig. 3. Effects of dexamethasone withdrawal on total MAO activity. Cultures of HF27 were grown and exposed to serum-free medium containing no hormone (control) or 50 n*M* dexamethasone for 5 days, as described in the legend to Fig. 1. On Day 0, cultures were rinsed twice with saline, then exposed to serum-free medium without hormone or additional feeding. Cultures were harvested periodically over the next 11 days and assayed in parallel for MAO activity. Each point represents one homogenate assayed in triplicate at two protein concentrations (values differed $< 15\%$). Total cellular protein per dish remained constant over the withdrawal period in both control (9.15 ± 1.62 $\mu g/cm^2$) and dexamethasone-treated (9.05 ± 0.55 $\mu g/cm^2$) cultures.

Activity increased almost immediately after inhibitors were removed. By 4 hr, activity had already increased 11 and 21% in the absence and presence of dexamethasone, respectively. The more rapid recovery of MAO activity in dexamethasone-treated cultures as compared to control cultures, already apparent by 4 hr, was seen at all times examined. In fact, over a period of 25 days, activity increased almost 11-fold, from 7.7 to 81.5 pmol/min/mg protein, in the presence of dexamethasone but only 4-fold, to 30.9 pmol/min/mg protein, in the absence of hormone. Even though the recovery of activity in the presence of dexamethasone appeared more rapid than in the absence of hormone, MAO levels at the end of this 25-day period were nearly back to the steady-state levels observed prior to inhibition in both dexamethasone-treated cultures (85.3 pmol/min/mg protein) and control cultures (34.5 pmol/min/mg protein).

The discrepancy between the extremely low steady-state levels observed in this experiment and the 4-fold (control cultures) to 12-fold (dexamethasone-treated cultures) higher steady-state levels observed in previous experiments (see Table I, Experiment 2) most likely reflected differences in growth and culture conditions. In the present study, the total cellular protein per dish decreased exponentially over the course of the experiment to levels that were 63% (control cultures) and 44% (dexamethasone-treated cultures) of those observed immediately following the removal of inhibitors (Edelstein, 1984). In addition, cultures were fed quite frequently—every 1 to 2 days over the first part of the experiment and every 3 to 4 days throughout the rest of the experiment. Since MAO activity has been shown to decrease with decreased cell

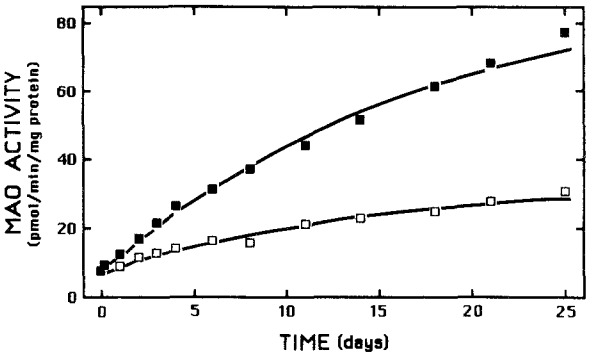

Fig. 4. Effects of dexamethasone on the recovery of total MAO activity after irreversible inhibition of existing pools of active enzyme. Cultures of HF27 were grown, rinsed with saline, and preincubated with serum-free medium, as described in the legend to Fig. 1. Monolayers were exposed to serum-free medium containing 10 n*M* clorgyline and 1 μM deprenyl for 2 hr to inhibit irreversibly existing pools of MAO molecules, then rinsed twice with phosphate-buffered saline (PBS) to remove unbound inhibitor. On Day 0, cultures were exposed to serum-free medium containing no hormone (□) or 50 n*M* dexamethasone (■) with feeding every 1–4 days. Cultures were harvested immediately prior to preincubation with inhibitors, immediately after removal of inhibitors, and periodically over the next 25 days, then assayed in parallel for MAO activity. Each point represents the average of two experiments in which homogenates were assayed in triplicate at two protein concentrations (values differed $< 20\%$). Activity prior to incubation with inhibitors was 60.4 pmol/min/mg protein, while activity immediately after removal of inhibitors was 7.7 pmol/min/mg protein. Steady-state activities in the absence of inhibitors (calculated as described in Materials and Methods) was 34.5 ± 2.7 pmol/min/mg protein in control cultures and 85.3 ± 6.7 pmol/min/mg protein in dexamethasone-treated cultures. Total cellular protein per dish decreased exponentially over the 25-day recovery period from 6.79 ± 0.19 to 4.29 ± 1.71 $\mu g/cm^2$ in control cultures and from 6.70 ± 0.31 to 2.95 ± 0.44 $\mu g/cm^2$ in dexamethasone-treated cultures.

density and increased feeding frequency (Edelstein *et al.*, 1978; Breakefield *et al.*, 1981; unpublished data), the recovery of MAO activity observed after inhibition would be counterbalanced by the decreases in MAO activity due to decreased protein content and increased feeding, thus resulting in lower steady-state activities.

Effects of Dexamethasone on the Synthesis, Degradation, and Half-Life of Total MAO

In the various experiments described above, we found that (1) steady-state activities were higher in the presence of dexamethasone than in its absence, (2) the continued presence of dexamethasone was required to observe these effects, and (3) the recovery of MAO activity after irreversible inhibition of existing pools of active MAO molecules was more rapid in dexamethasone-treated cultures than in control cultures.

To determine the basis for these effects of dexamethasone on MAO activity, two approaches were used to examine whether dexamethasone affected the rate of synthesis or degradation of active MAO molecules (Table I). In the first approach (Experiment 2), relative rate constants and half-lives of MAO in control or dexamethasone-treated cultures were determined from changes in MAO activity following the removal of hormone from dexamethasone-treated cultures (see Fig. 3) or the addition of hormone to control cultures (see Fig. 2), respectively. In the second approach (Experiment 3), rate constants were determined from changes in MAO activity following irreversible inhibition of existing pools of active MAO molecules in control and dexamethasone-treated cultures (see Fig. 4).

Dexamethasone had no affect on the rate of degradation or turnover of active enzyme—the k_d and $t_{1/2}$ in dexamethasone-treated cultures were similar to those in control cultures, regardless of the approach used to determine rate constants. In contrast, the rate of active enzyme synthesis in the presence of hormone was 2.4-fold (Experiment 3) to 10.4-fold (Experiment 2) higher than the respective rates observed in the absence of hormone. Thus, using two different experimental approaches, the above results indicated that the observed increases in MAO activity after exposure to dexamethasone resulted primarily from an increased rate of synthesis of active MAO molecules rather than from a change in the rate of degradation or half-life of MAO.

The difference in the absolute values of the rate constants, in particular the k_s, observed between the different experimental protocols most likely reflected the different feeding schedules and cell densities used in the two sets of studies. For example, although not as dramatic as the above differences, studies on the effects of culture conditions on relative rate constants (Experiment 1) indicated that cultures exposed to dexamethasone without feeding (as in Experiment 2) had higher k_s values than cultures exposed to dexamethasone with feeding (as in Experiment 3) but there was no difference in their k_d values.

Effects of Dexamethasone on MAO A and MAO B Activities

Since cultured human fibroblasts express two types of MAO activity, it was possible that the increased rate of total MAO synthesis observed after hormone treatment was due to the selective effects of dexamethasone on one form of the enzyme but not the other form. To test this possibility, levels of MAO A and MAO B activity in control and dexamethasone-treated cultures from three different fibroblast lines were determined from dose–response curves established using the selective MAO A inhibitor clorgyline. Regardless of the total activity (which varied from 44.8 to 162.1 pmol/min/mg protein) or the ratio of MAO A to MAO B activity (which varied from 0.8 to 13.0) prior to hormone treatment, the observed increases in total activity after exposure to dexamethasone could be accounted for by selective increases in MAO A activity in all three fibroblast lines (Table II). Exposure of HF27 cultures to dexamethasone for 7–8 days resulted in a 27-fold increase in MAO A activity but essentially no change in MAO B activity. Similarly, papillary (NF70A) and reticular (NF70B) fibroblast cultures exposed to dexamethasone for 6 days had levels of MAO A that were 7–10 times those in control cultures but levels of MAO B that were similar to those observed in control cultures.

Table II. Effects of Dexamethasone on Levels of MAO A and MAO B Activities in Different Human Fibroblast Lines

		Type of activity[a] (pmol/min/mg protein)	
Cell line	Dex	MAO A	MAO B
HF27[b]	−	27.8	34.7
	+	753.3	41.1
NF70A[c]	−	150.5	11.6
	+	1073.9	12.6
NF70B[c]	−	33.6	11.2
	+	332.0	11.4

[a] Fibroblast cultures were exposed to serum-free medium with no hormone (−) or 50 n*M* dexamethasone (+) for 6–8 days, harvested, and assayed for MAO activity, as described in the legend to Fig. 1. Activities for the two forms of MAO were determined from dose–response curves established by preincubating homogenates with serial dilutions of clorgyline for 15 or 30 min at 37°C prior to assay, as described in Materials and Methods.

[b] Values for HF27 represent the average of two experiments in which homogenates were assayed in quadruplicate at one protein concentration for each inhibitor concentration used (values differed <10%).

[c] Values for NF70A and NF70B represent the average of three experiments in which homogenates were assayed in duplicate at one protein concentration for each inhibitor concentration used (values differed <25%).

Effects of Dexamethasone on MAO A and MAO B Kinetic Parameters

To study further the effects of dexamethasone on the two forms of MAO, substrate affinities (K_m) and maximum reaction velocities (V_{max}) for MAO A and MAO B in control and dexamethasone-treated cultures of HF27 were determined using selective inhibitors (Table III). The selective increases in MAO A activity observed in the previous experiments were paralleled by 10-fold increases in the V_{max} for MAO A but only by 3-fold increases in the V_{max} for MAO B after a 7-day exposure to dexamethasone. In contrast, no change in the apparent K_m of MAO for tryptamine was observed for either form of the enzyme. The discrepancy between the effects of dexamethasone on the V_{max} for MAO B in the present study and the activity of MAO B in the previous study (see Table II) most likely reflected an underestimation of MAO B activity in the control cultures used in the present study, as discussed in Materials and Methods.

Effects of Dexamethasone and Cellular "Aging" on the Number of Active MAO A and MAO B Molecules

Previous studies from our laboratory indicated that cellular "aging" affected MAO activity in a manner similar to dexamethasone (Edelstein *et al.*, 1978; unpublished data). For example, levels of total MAO activity increased three- to eightfold as

Table III. Effects of Dexamethasone on the Kinetic Parameters of MAO A and MAO B

Type of activity[a]	Dex	Kinetic parameter[b]	
		K_m (μM)	V_{max} (pmol/min/mg)
MAO A	−	10.7	77.7
	+	11.0	764.8
MAO B	−	27.6	66.9
	+	30.3	219.8

[a] Cultures of HF27 were exposed to serum-free medium with no hormone (−) or 50 nM dexamethasone (+) for 7 days and harvested, as described in the legend to Fig. 1. Homogenates were preincubated with 0.1 μM deprenyl or 10 nM clorgyline prior to the measurement of MAO A or MAO B activity, respectively, using tryptamine concentrations ranging from 1 to 70 μM, as described in Materials and Methods.

[b] Substrate affinities (K_m) and maximum reaction velocities (V_{max}) were calculated by the direct linear plot method of Cornish-Bowden and Eisenthal (1974). Results are the averages of two experiments in which homogenates were assayed in triplicate at one protein concentration for each tryptamine concentration used (values differed $<5\%$ for the K_m and $<25\%$ for the V_{max}).

fibroblasts approached senescence with successive passaging in culture. Further, these increases in total activity were paralleled by increases in the V_{max} but not by changes in the apparent K_m when kinetic parameters for total MAO were determined in the absence of inhibitors. Therefore, to study further the mechanism by which dexamethasone increased MAO activity and determine whether cellular aging increased MAO activity by a similar mechanism, the amounts of active MAO A and B molecules were quantitated using inhibitors which bind irreversibly and stoichiometrically to the flavin-containing subunits of active MAO A and MAO B molecules but not to other flavoproteins (for review, see Fowler *et al.*, 1981). Briefly, crude mitochondrial fractions isolated from early (nonsenescent) and late (senescent) passages of control and dexamethasone-treated cultures of HF27 were incubated with [^{3}H]pargyline to label the flavin polypeptides of MAO, which were then separated by SDS-PAGE and quantitated. To label either both forms of MAO, MAO A, or MAO B, samples were preincubated, respectively, with either no inhibitor, deprenyl, or clorgyline prior to incubation with [^{3}H]pargyline.

As in previous studies (Edelstein and Breakefield, 1981), no major differences in the Coomassie blue protein staining pattern of SDS–polyacrylamide gels were observed between mitochondrial proteins from control and dexamethasone-treated cultures, nonsenescent and senescent cultures, or uninhibited and inhibitor-treated samples (data not shown). However, differences were observed in the distribution and amount of radioactivity detected by fluorography (Fig. 5). Due to the length of exposure needed to detect the labeled flavin polypeptides isolated from nonsenescent control cultures by fluorography, it was difficult to resolve completely the [^{3}H]pargyline-labeled flavin polypeptides corresponding to MAO A (63,000 MW) and MAO B (60,000 MW) in several of the gel lanes. Despite this difficulty in resolution, it was clear that dexamethasone treatment and cellular aging increased the amount of label associated with the MAO A and MAO B flavin polypeptides but did not alter

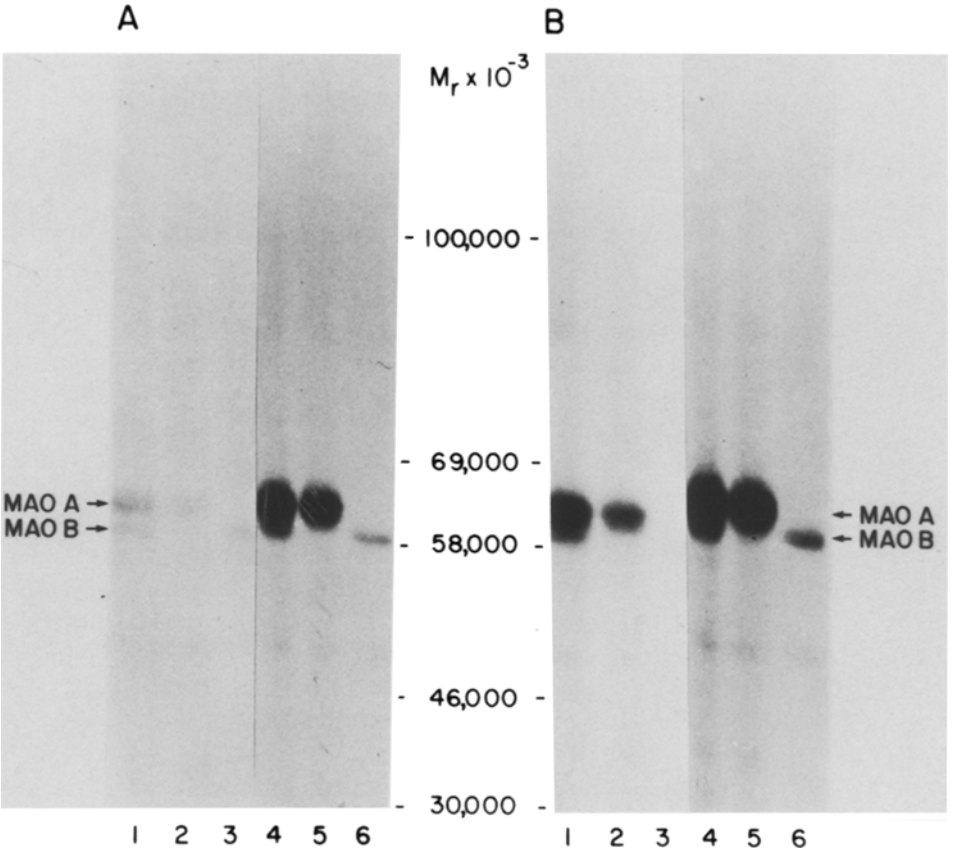

Fig. 5. SDS–polyacrylamide gel electrophoresis of [^{3}H]pargyline-labeled crude mitochondrial fractions from nonsenescent and senescent cultures of control and dexamethasone-treated fibroblasts. Nonsenescent (passage 6; A) and senescent (passage 11; B) cultures of HF27 were grown and exposed to serum-free medium containing no hormone (Lanes 1–3) or 50 n*M* dexamethasone (Lanes 4–6) for 9 days, as described in the legend to Fig. 1. Crude mitochondrial fractions were isolated and preincubated with no inhibitor (Lanes 1 and 4), 1.0 μM deprenyl (Lanes 2 and 5), or 0.1 μM clorgyline (Lanes 3 and 6) for 15 min at 37°C prior to labeling with 1.25–1.5 μM [^{3}H]pargyline for 4 hr at 37°C. Labeled mitochondria were washed by centrifugation, solubilized, and subjected to SDS-PAGE, as described in Materials and Methods. Gels were then treated for fluorography, dried, and exposed to preflashed X-ray film for 90 days at −65°C. The fluorogram shown is from one of three similar experiments. The positions of the flavin-containing polypeptides associated with MAO A and MAO B activity are indicated by arrows.

their electrophoretic mobilities. Further, the increase in labeling appeared to be greater for the 63,000 MW band than for the 60,000 MW band. Since concentrations of clorgyline and deprenyl were used which not only blocked labeling of the desired form of MAO but partially decreased labeling of the form being quantitated (see Materials and Methods), the amount of label associated with these two bands in the presence of inhibitors (Lanes 2, 3, 5, and 6) was less than that detected in their absence (Lanes 1 and 4).

The amount of [^{3}H]pargyline associated with each of the labeled bands in Fig. 5 was quantitated by determining the radioactivity in gel strips sliced from the region of

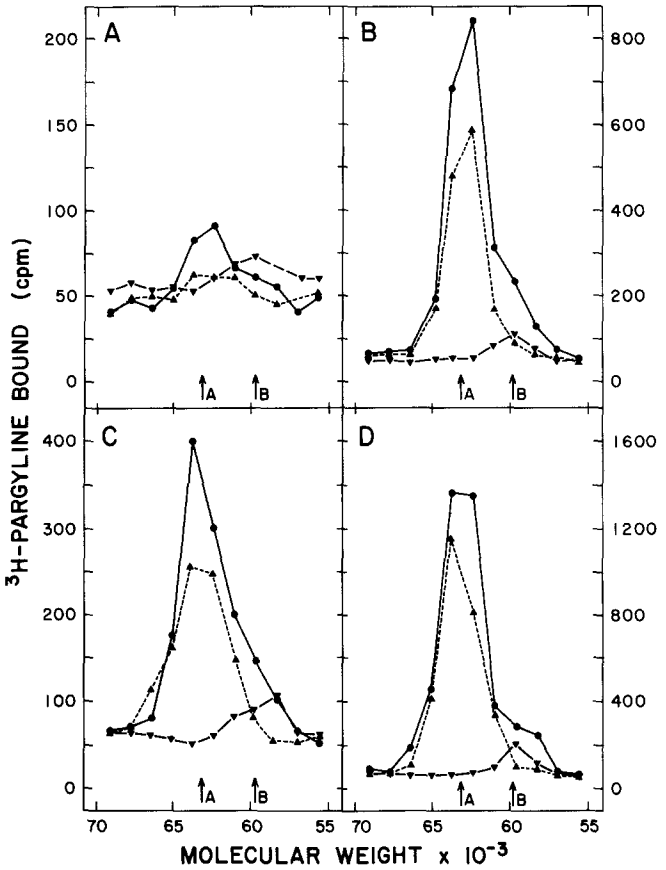

Fig. 6. Quantitative distribution of radioactivity after SDS-PAGE of [^{3}H]pargyline-labeled mitochondria from nonsenescent and senescent cultures of control and dexamethasone-treated fibroblasts. Crude mitochondrial fractions were isolated from nonsenescent control (A), nonsenescent dexamethasone-treated (B), senescent control (C), or senescent dexamethasone-treated (D) fibroblasts, preincubated with no inhibitor (●—●), deprenyl (▲– – –▲), or clorgyline (▼—▼), labeled with [^{3}H]pargyline, and subjected to SDS-PAGE, as described in the legend to Fig. 5. After electrophoresis, gels were sliced into 2-mm strips and the radioactivity in each gel slice was determined, as described in Materials and Methods. The results shown are from one of two similar experiments. The data in each of the panels were plotted on different scales to depict clearly the positions of the radioactive peaks associated with MAO A and MAO B (indicated by arrows).

the gel containing the labeled flavin polypeptides. When radioactivity was plotted against molecular weight (Fig. 6), a broad peak spanning the MW region from 65,000 to 58,000 was observed in samples preincubated without inhibitors (e.g., total MAO), whereas peaks spanning the MW region from 65,000 to 60,000 or the MW region from 61,500 to 58,000 were observed in samples preincubated with deprenyl (e.g., MAO A) or clorgyline (e.g., MAO B), respectively. When the radioactivity associated with each of

Table IV. Effects of Dexamethasone and Cellular Aging on Activity, Concentration, and Molecular Turnover Number of MAO[a]

Type of activity	Dex	V_{max}[b]		[MAO][c]		MTN[d]	
		Early	Late	Early	Late	Early	Late
		(pmol/min/mg)		(fmol/mg)		(mol/mol/min)	
MAO A+B	−	95.4	522.4	241	1340	396	390
	+	799.7	1715.0	2546	4759	314	360
MAO A	−	68.4	383.2	148	892	459	429
	+	695.2	1487.5	1571	3415	443	436
MAO B	−	36.4	75.3	79	160	459	469
	+	64.6	143.9	146	323	441	446

[a] Crude mitochondrial fractions were isolated from nonsenescent (early; passage 6) and senescent (late; passage 11) cultures of HF27 exposed to serum-free medium with no hormone (−) or 50 n*M* dexamethasone (+) for 9 days, as described in the legend to Fig. 5.

[b] Estimated maximum velocities (V_{max}) were calculated by correcting MAO activities in mitochondrial fractions for subsaturating tryptamine concentrations, as described in Materials and Methods. Results are expressed as pmol tryptamine deaminated/min/mg mitochondrial protein. Each value represents the average of two experiments in which mitochondrial fractions were assayed in triplicate at one protein concentration (values differed $<25\%$).

[c] Enzyme concentrations ([MAO]) were calculated from the data shown in Fig. 6. The radioactivity in each peak was summed, corrected for nonspecific binding using the counts in the gel slices immediately surrounding the observed peaks, converted to fmol [^{3}H]pargyline specifically bound, and expressed as fmol active MAO/mg mitochondrial protein. Each value represents the average of two experiments (values differed $<10\%$).

[d] Molecular turnover numbers (MTN) were calculated by dividing the estimated maximum velocity by the enzyme concentration (e.g., V_{max}/[MAO]). The resulting values are expressed as mol tryptamine deaminated/mol active MAO/min.

the peaks in Fig. 6 was quantitated and expressed as the femtomoles of enzyme per milligram of mitochrondrial protein (Table IV), we found that the amount of total active enzyme increased 10.6-fold in nonsenescent cultures and nearly 3.6-fold in senescent cultures after a 9-day exposure to dexamethasone. These increases in total MAO were paralleled by similar increases in the amount of active MAO A but less than twofold increases in the amount of active MAO B. Cellular aging itself, in the absence of hormone, also affected the amount of active enzyme. The amount of total active MAO in senescent cultures was almost sixfold higher than the amount in nonsenescent cultures. Further, the increase in total active MAO was paralleled by a similar increase in the amount of active MAO A but less than a twofold increase in the amount of active MAO B.

These studies suggested that the increases in total MAO activity observed after dexamethasone treatment or during cellular aging resulted from selective increases in the number of active MAO molecules. To provide further support for this hypothesis, molecular turnover numbers—a measure of catalytic activity per mole of enzyme—for total MAO, MAO A, and MAO B were calculated from measurements of the estimated maximum activity and the amount of active MAO in parallel mitochondrial preparations (Table IV). Exposure to dexamethasone and cellular aging did not alter turnover numbers for total MAO or for either form of the enzyme. However, turnover numbers for MAO A (442 ± 13) and MAO B (454 ± 13), although similar under all the conditions tested, were approximately 20% higher than those for total MAO

(357 ± 37). As discussed previously, this discrepancy most likely reflected an underestimation of the amount of [^{3}H]pargyline-labeled flavin polypeptides in samples preincubated with selective inhibitors.

DISCUSSION

A number of physiologic factors, such as hormones and aging, alter levels of MAO activity *in vivo* (see Sourkes, 1979; Sullivan *et al.*, 1980). However, the regulation of MAO at the molecular level in living organisms has been difficult to study since the observed enzyme levels are the result of many different metabolic processes. As an alternative to *in vivo* studies, we have used cultured human skin fibroblasts as a model system for studying the regulation of MAO activity. In earlier studies, we found that levels of MAO activity in fibroblasts varied as a function of culture conditions, cellular "age", donor age, and fibroblast type (Edelstein *et al.*, 1978; Breakefield *et al.*, 1980; Breakefield *et al.*, 1981; unpublished data). In the present studies, we have extended these findings to include the effects of glucocorticoids and have examined in detail the basis by which glucocorticoids and cellular aging regulate MAO activity.

Both the synthetic glucocorticoid dexamethasone and the naturally occurring glucocorticoid hydrocortisone increased total MAO activity in a time- and dose-dependent manner, with maximal stimulation occurring at concentrations similar to those required for maximal stimulation of other glucocorticoid-inducible proteins in various cell culture systems (Arinze *et al.*, 1978; Grieninger *et al.*, 1978; Johnson *et al.*, 1979; Rosner and Cristofalo, 1981). In addition, maximal stimulation was observed at concentrations similar to those of circulating glucocorticoids in human plasma, e.g., 100–500 n*M* (Brien, 1980).

The effects of dexamethasone on MAO activity were modulated by a number of factors. For example, steady-state activities in cultures after hormone treatment were 3.3 times higher when cultures were exposed to dexamethasone in the absence of serum than when exposed in the presence of serum and 1.4 times higher when cultures were exposed to dexamethasone without feeding then when exposed with feeding. In addition, the increase in MAO activity after hormone treatment were 2.5 times higher in nonsenescent cultures than in senescent cultures. As in the present studies, others have found that the response to glucocorticoids in various cell culture systems varies with serum concentration (Lundgren, 1977; Arinze *et al.*, 1978; Grieninger *et al.*, 1978, Oikarinen *et al.*, 1983), feeding frequency (Lundgren, 1977; Johnson *et al.*, 1979), and cellular age (Phillips *et al.*, 1984). Although the mechanisms responsible for these culture-dependent variations in hormone response are not known, several studies suggest that they may reflect changes in the concentration of glucocorticoid receptors. In cultured human fibroblasts, for example, receptor levels have been found to vary with serum concentration (Bauknecht, 1977), cell density (Ponec *et al.*, 1980), phases of the cell cycle (Hoshi *et al.*, 1982), and cellular age (Rosner and Cristofalo, 1981).

Several findings in the present study indicated that the observed increases in MAO activity after dexamethasone treatment resulted from an increased synthesis of the active enzyme. First, the removal of hormone from dexamethasone-treated cultures,

which presumably results in a rapid return to control rates of synthesis (see Schmike and Doyle, 1970), resulted in a slow decay of elevated levels of MAO activity back toward control levels. Second, the recovery of MAO activity after irreversible inhibition of existing pools of active enzyme, which presumably represents *de novo* synthesis (see Gordis and Neff, 1971), was more rapid in the presence of dexamethasone than in the absence of hormone. Third, and most convincing, the rate constant for synthesis of the active enzyme was higher in the presence of dexamethasone than in the absence of hormone, whereas no difference in the rate constant of degradation or half-life was observed when relative rates of MAO synthesis, degradation, and turnover were determined by two independent methods. The effects of dexamethasone on MAO synthesis in cultured human fibroblasts were not unique. In fact, increased rates of enzyme synthesis have been found to account for the stimulation of a number of enzyme activities by glucocorticoids in various cell culture systems (see Schimke and Doyle, 1970; Lan *et al.*, 1984) and the stimulation of MAO activity by glucocorticoids (Della Corte and Callingham, 1977) and thyroid hormones (Lyles and Callingham, 1974) in rat heart.

Because cultured human skin fibroblasts express both MAO A and MAO B activities (Roth *et al.*, 1976; Groshong *et al.*, 1977), exposure to dexamethasone could increase MAO activity by altering the synthesis of one or both forms of the enzyme. Since increased rates of synthesis in the absence of altered rates of degradation would result in increased levels of activity as well as increased amounts of active enzyme, the above hypothesis was tested by comparing levels of MAO A and MAO B activity with the amount of active MAO A and MAO B, respectively. Exposure to dexamethasone appeared to selectively affect MAO A. The increases in total MAO activity observed after hormones treatment were paralleled by similar increases in the activity and amount of active MAO A but by little change in the activity and amount of active MAO B, suggesting that dexamethasone treatment increased primarily the rate of MAO A but not MAO B synthesis.

Selective changes in MAO A activity were not unique to hormone treatment. Cellular aging also appeared to selectively affect levels of MAO activity. The increases in total MAO activity that occurred as fibroblast cultures were passaged and approached senescence were paralleled by similar increases in the activity and amount of active MAO A but by much smaller changes in the activity and amount of active MAO B. Such findings suggested that cellular aging affected the rate of synthesis or degradation of MAO A. Although we did not compare the relative rate constants of MAO synthesis and degradation in nonsenescent and senescent fibroblast cultures, results from studies on aging in rat heart (Lyles and Callingham, 1974; Della Corte and Callingham, 1977) and rat brain (Nelson *et al.*, 1979) suggest that the increases in MAO activity observed during cellular aging may result from a decreased rate of MAO degradation.

In contrast to the observed changes in the amount of active MAO, no changes in inhibitor sensitivity, substrate affinity, electrophoretic mobility, or molecular turnover number were observed for either form of the enzyme after hormone exposure or during cellular aging. Such findings argued against the possibility that the observed increases in MAO activity resulted from the induction of a new form of MAO or a change in the catalytic efficiency of the enzyme and further supported the hypothesis that the

observed increases in MAO activity resulted from selective changes in the synthesis or degradation of MAO A.

Similar to our findings in cultured fibroblasts, selective increases in MAO A activity have been observed in various rat and human tissues during the menstrual cycle (Mazumder *et al.*, 1980); after castration (Illsley and Lamartiniere, 1980), hypophysectomy (Holzbauer and Youdim, 1983), and chemical thyroidectomy (Vaccari *et al.*, 1983); after treatment with progesterone (Mazumder *et al.*, 1980) or thyroxine (Lyles and Callingham, 1974, 1979); and during aging (Lyles and Callingham, 1974, 1979; Fowler and Callingham, 1979; Illsley and Lamartiniere, 1980; Shih, 1979; Lewinsohn *et al.*, 1980). However, depending on the tissue and region examined, selective decreases in MAO A activity also have been observed after hypophysectomy (Holzbauer and Youdim, 1983) or chemical thyroidectomy (Lyles and Callingham, 1979); after treatment with dexamethasone (Holzbauer and Youdim, 1983), estrogen (Luine and McEwen, 1977; Chevillard *et al.*, 1981; Vaccari *et al.*, 1981), or testosterone (Illsley and Lamartiniere, 1980; Vaccari *et al.*, 1981); and during aging (Nelson *et al.*, 1979; Benedetti and Keane, 1980; Lewinsohn *et al.*, 1980; Leung *et al.*, 1981). In contrast to MAO A, selective changes in MAO B activity after hormone treatment (Chevillard *et al.*, 1981) and during aging (Fowler *et al.*, 1980; Benedetti and Keane, 1980; Lewinsohn *et al.*, 1980; Leung *et al.*, 1981) have been observed in relatively few studies.

Although the basis for the selective effects of hormone treatment and aging on MAO activity were not examined in great detail in most of these studies, their findings when taken together suggested that the selective increases in MAO activity resulted from selective changes in the amount of active enzyme due to selective changes in its synthesis or degradation. For example, several studies found that the selective changes in MAO activity were paralleled by changes in the proportion of MAO A and MAO B activity (Lyles and Callingham, 1974, 1979; Leung *et al.*, 1981) and selective changes in the V_{max} (Lyles and Callingham, 1974; Fowler and Callingham, 1979; Nelson *et al.*, 1979; Benedetti and Keane, 1980; Fowler *et al.*, 1980; Vaccari *et al.*, 1981, 1983), concentration of active enzyme (Fowler and Callingham, 1979; Nelson *et al.*, 1979; Fowler *et al.*, 1980), and relative rate constants (Lyles and Callingham, 1974; Luine and McEwen, 1977; Nelson *et al.*, 1979). In addition, several studies found that selective changes in MAO activity were not associated with changes in the substrate affinity (see references for V_{max} above), inhibitor sensitivity (Lyles and Callingham, 1974; Nelson *et al.*, 1979; Leung *et al.*, 1981), heat sensitivity (Lyles and Callingham, 1974), isoelectric point (Shih, 1979), and molecular turnover number (Fowler and Callingham, 1979; Nelson *et al.*, 1979; Fowler *et al.*, 1980).

In contrast to the present study, only a few of the above studies examined more than one or two properties of the enzyme (Lyles and Callingham, 1974; Fowler and Callingham, 1979; Nelson *et al.*, 1979; Fowler *et al.*, 1980). Further, none of these studies demonstrated that hormones or aging selectively affected MAO activity in a single cell type expressing both forms of the enzyme in the absence of other *in vivo* factors and metabolic processes, nor did they demonstrate that hormones and aging selectively affected the amounts, but not the electrophoretic mobility, of the two forms of MAO after physical separation of the MAO A and MAO B flavin-containing subunits by SDS–polyacrylamide gel electrophoresis.

The selective effects of hormones and aging on the multiple forms of MAO were not unique. Similar selective effects have been observed for multiple forms of other proteins. For example glucocorticoids increase acid phosphatase type I but decrease type III activity in rabbit red cells (Szajerka and Kwiatkowska, 1984), increase myosin V_1 but decrease myosin V_3 expression in rat heart (Sheer and Morkin, 1984), and decrease type III but not type I collagen synthesis in mouse fibroblasts (Verbruggen and Abe, 1982). Similarly, aging decreases α-mannosidase types I and II but increases type III activity in rat epididymis (Dutta and Majumder, 1984), increases hexosaminidase A but not B activity in rat liver (Oberkotter *et al.*, 1980), and increases enolase-2 but not enolase-1 expression in rat heart (Rothstein *et al.*, 1980).

Several different mechanisms could account for the selective effects of glucocorticoids and aging on the expression of the multiple forms of MAO and the other proteins described above (see Darnell, 1982; Nevins, 1983). For example, as shown for a number of glucocorticoid-inducible and developmentally regulated genes, the selective increase in MAO A expression could result from a selective increase in the rate of transcription of the structural gene(s) for MAO A but not MAO B, due to differences in specific DNA binding sites for glucocorticoid receptors or other regulatory factors (Lan *et al.*, 1984; Moore *et al.*, 1985; von der Ahe *et al.*, 1985), DNA methylation patterns (Cooper, 1983; Moore *et al.*, 1985), chromatin structure (Zaret and Yamamoto, 1984), or chromosome location (Feinstein *et al.*, 1982) of the MAO A and MAO B structural gene(s). Alternatively, the increased expression of MAO A could reflect a selective increase in the concentration or translation of the mature cytoplasmic mRNA for MAO A since glucocorticoids and aging can induce specific factors which affect transcription (Cavanaugh *et al.*, 1984; Mather *et al.*, 1984) and translation (see Cordell *et al.*, 1982, for references), regulate posttranscriptional processing of primary RNA transcripts such as polyadenylation of the 3′ end (Lan *et al.*, 1984) and splicing (Anderson and Klessig, 1984; Mather *et al.*, 1984), and affect the stability of mature cytoplasmic mRNAs (Lan *et al.*, 1984; Vannice *et al.*, 1984). Finally, the increased expression of MAO A could reflect differences in posttranslational processing of the polypeptides for MAO A and MAO B since peptide cleavage, glycosylation, compartmentation, and phospholipid synthesis are regulated by glucocorticoids (Firestone *et al.*, 1982; Grove *et al.*, 1983) and change during aging (Hayflick, 1980). Cloning of the structure gene(s) and isolation of the mRNAs for MAO A and MAO B, which are currently in progress, should help distinguish among these various explanations and elucidate the relationship between MAO A and MAO B at the DNA, mRNA, and protein levels.

The present studies support the accumulating evidence that the two forms of MAO are separate molecular entities and demonstrate that MAO A and MAO B expression in cultured human fibroblasts can be regulated independently. Further, several lines of evidence suggest that the selective effects of glucocorticoids and aging on MAO A activity may have important physiologic and behavioral correlates in humans since (1) the two forms of MAO have different distributions in the brain (Shih, 1979; Fowler *et al.*, 1980; Levitt *et al.*, 1982) and other tissues (Lewinsohn *et al.*, 1980), different affinities for endogenous neurotransmitters (White and Tansik, 1979), and different effects on neuronal function, physiology, and behavior (Murphy and Kalin, 1980); (2) many neuronal functions and behaviors are regulated by glucocorticoids

(McEwen, 1979) and are altered during aging (Samorajski, 1977); (3) abnormal levels of circulating glucocorticoids and other signs of adrenocortical system dysfunction increase with advanced age (Sartin *et al.*, 1980) and are observed in a number of neurologic and psychiatric disorders associated with altered levels of MAO activity (Shah and Donald, 1984); and (4) neurologic and psychiatric symptoms increase with advanced age (Samorajski, 1977; Meites, 1983) and are observed in disorders of adrenocortical hyperfunction (e.g., Cushing's syndrome, steroid psychosis) and hypofunction (e.g., Addison's disease) (Smith *et al.*, 1972; Hall *et al.*, 1979). Thus, elucidation of the mechanisms(s) by which hormones and aging regulate MAO activity could be important in understanding the etiology of a number of neuropsychiatric disorders and in designing more effective treatments.

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