Biochemical Pharmacology, Vol. 34, No. 7, pp. 1087–1092, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

IMIDAZOLE ANTIMYCOTICS: INHIBITORS OF STEROID AROMATASE

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(*Received* 17 *May* 1984; *accepted* 19 *July* 1984)

Abstract—Miconazole and clotrimazole, members of a class of imidazole agents which have broad spectrum antimycotic activity, were shown to be potent inhibitors of steroid aromatase activity of human placental microsomes. The I_{50} values for the inhibition of aromatase activity by miconazole, clotrimazole, ketoconazole, and aminoglutethimide were 0.6, 1.8, 60 and 44 μM respectively. The most effective compound, miconazole, exhibited competitive kinetics with respect to androstenedione, the aromatase substrate. The apparent inhibitory constant (K_i) was 55 nM, under assay conditions where the apparent K_m for androstenedione was 220 nM. The inhibition of aromatase activity by miconazole was shown to be reversible by dilution. Miconazole was a relatively poor inhibitor of the cholesterol side chain cleavage activity of a placental mitochondria-enriched fraction, while both clotrimazole and ketoconazole markedly inhibited this mitochondrial monooxygenase activity. Spectrophotometric studies revealed that miconazole bound to the cytochrome P-450 component of the placental microsomal aromatase complex and had negligible effect on NADPH-cytochrome *c* (P-450) reductase activity. These results strongly support direct interaction of miconazole with microsomal cytochrome P-450 in human placental microsomes with high affinity resulting in the inhibition of aromatase activity.

Miconazole, clotrimazole and ketoconazole are imidazole antimycotic agents effective against a wide range of fungal pathogens. Their structures are illustrated in Fig. 1. These drugs effectively inhibit ergosterol biosynthesis in yeast cells by inhibiting the sterol 14α-demethylase, a microsomal cytochrome P-450-dependent system [1]. This inhibition causes an accumulation of 14α-methylsterols which incorporate into the bilayer of fungal membranes. The presence of the 14α-methylsterol is believed to disrupt the close packing of acyl chains of phospholipids resulting in limited fungal growth [2]. Imidazole antimycotic agents have been shown recently to inhibit a number of cytochrome P-450-dependent steroidogenic enzyme activities in gonadal, hepatic and adrenal tissues [3–5]. The conversion of androstenedione to estrogens in steroidogenic tissues is

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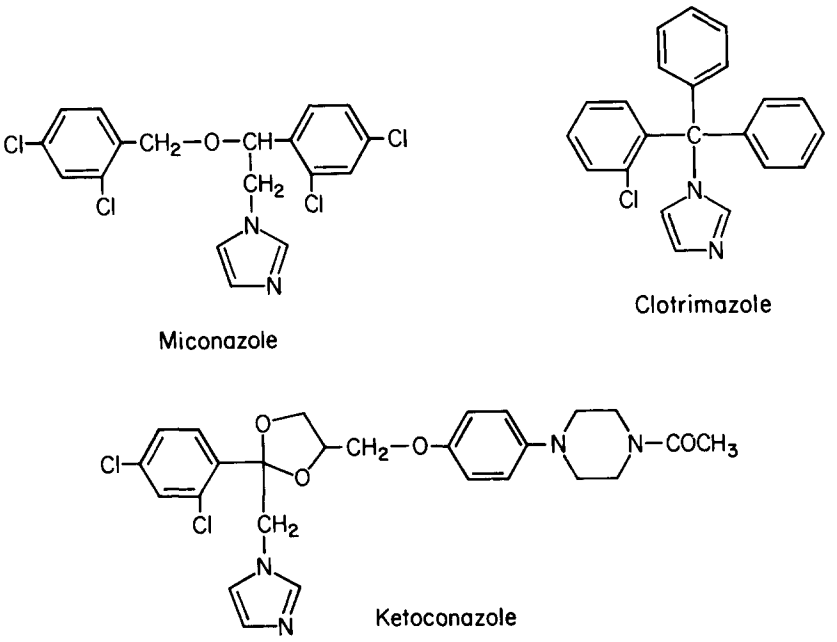

Fig. 1. Chemical structures of miconazole, clotrimazole and ketoconazole.

catalyzed by a cytochrome P-450-dependent monooxygenase commonly referred to as the aromatase enzyme [6]. The aim of the present research was to evaluate the effectiveness of these imidazole drugs in inhibiting steroid aromatase activity of human placental microsomes and to determine whether they also directly interact with placental cytochrome P-450.

MATERIALS AND METHODS

Chemicals

Miconazole nitrate and clotrimazole were obtained from the Sigma Chemical Co. (St. Louis, MO). Ketoconazole was supplied by Janssen Life Products Inc. (Piscataway, NJ). Trilostane (4α,5-epoxy-17β-hydroxy-3-keto-5α-androstane-2α-carbonitrile) and a racemic mixture of aminoglutethimide were provided by Sterling Winthrop Inc. (Rensselaer, NY) and Ciba-Geigy Inc. (Summit, NJ) respectively. [1β-^{3}H]Androst-4-ene-3,17-dione was synthesized from [1,2-^{3}H-(N)]androst-4-ene-3,17-dione by the base-catalyzed tritium hydrogen exchange technique as described previously [7]. [1,2-^{3}H-(N)]Androstenedione (50 Ci/mmole) and [7α-^{3}H] pregnenolone (12 Ci/mmole) were supplied by the New England Nuclear Corp. (Boston, MA). Pregnenolone antiserum was OP-2 from Pantex (Santa Monica, CA). All other chemicals were of reagent grade quality purchased from scientific supply houses.

Preparation of placental microsomes and mitochondria

The procedure was based on that described previously [8]. Term human placentae were obtained aseptically at the time of caesarean section and were transferred immediately to the laboratory. Small pieces of trophoblastic tissue were minced and then homogenized in sucrose (0.25 M) using initially a Waring blender and then a Teflon–glass homogenizer. The homogenate was centrifuged at 200 *g* for 10 min. The 200 *g* supernatant fraction was centrifuged at 10,000 *g* for 15 min to sediment a mitochondria-enriched fraction. After centrifugation at 20,000 *g* for 10 min, the supernatant fraction was centrifuged at 100,000 *g* for 1 hr to sediment a microsome-enriched fraction. The microsomal fraction was washed by suspension in KCl (0.15 M) and again centrifuged at 100,000 *g* for 45 min. The mitochondria-enriched fraction was suspended in sucrose (0.25 M) and centrifuged at 10,000 *g* for 10 min. Protein content was measured by the method described by Lowry *et al.* [9] using bovine serum albumin as a standard.

Enzyme assays

Aromatization of androstenedione. Human placental microsomes (0.025 mg) in potassium phosphate buffer (0.1 M), pH 7.4, were incubated with [1β-^{3}H]androstenedione (0.5 μCi, 300 pmoles) and $NADP^+$ (100 nmoles) in a total reaction volume of 0.2 ml. The incubation mixture contained an NADPH-regenerating system consisting of glucose-6-phosphate (5 mM) and glucose-6-phosphate dehydrogenase (0.2 units). The reaction was initiated by the addition of androstenedione and proceeded for 30 min at 37°. In preliminary experiments we established that the reaction rate was linear with time under these assay conditions. The assays were terminated by the addition of trichloroacetic acid (0.8 ml; 12.5%, w/v) to denature the protein. After the addition of chloroform (5 ml) and centrifugation, aliquots (0.8 ml) of the aqueous phase were mixed with an equal volume of an activated charcoal suspension (5%, w/v; Norit A). After centrifugation at 700 *g* for 15 min, aliquots (0.8 ml) of the supernatant fraction were transferred to scintillation vials, and the radioactivity was quantified by liquid scintillation spectrometry. This assay method is based on the specific loss of tritium from the 1-β position of tritiated androstenedione to form ^3H_2O during the process of aromatization [6].

Side chain cleavage of cholesterol. Human placental mitochondria-enriched fraction (1.5 mg protein) was incubated in a sucrose (0.25 M) solution, pH 7.4, containing KCl (20 mM), triethanolamine (15 mM), potassium phosphate (10 mM) and $MgCl_2$ (5 mM) in a total volume of 1.5 ml. Trilostane (19 μM) was added to inhibit the conversion of pregnenolone to progesterone. The assay was initiated by the addition of isocitrate (10 mM) and incubated at 37°. After 0, 20 and 40 min, aliquots (0.3 ml) were removed and added to ice-cold water (2.2 ml) to terminate the reaction. Pregnenolone formation from endogenous mitochondrial cholesterol was quantified by radioimmunoassay as described previously [10]. The imidazole compounds were determined not to cross-react with the antiserum under the assay conditions employed.

Reduction of cytochrome c. NADPH cytochrome *c* reductase activity of human placental microsomes was determined spectrophotometrically at ambient temperature with an Amino DW-2a dual beam spectrophotometer using the electron acceptor, cytochrome *c* and a millimolar absorptivity of 21 $mM^{-1}cm^{-1}$ at 550 nm as described previously [11]. The control rate was 14.1 nmoles cytochrome *c* reduced $\cdot min^{-1} \cdot (mg\ protein)^{-1}$ and was linear for at least 1 min.

Difference spectra of placental microsomal cytochrome P-450 with miconazole

Human placental microsomes were suspended to a total protein concentration of 6 mg/ml in buffer containing KCl (150 mM), Tris–chloride (50 mM) and $MgCl_2$ (10 mM), pH 7.5, at 22° and placed in the sample and reference cuvettes of an Aminco DW2a split beam spectrophotometer. After recording a base line, a difference spectrum was recorded after the addition of miconazole (5 μM), dissolved in ethanol, to the sample cuvette and an equivalent amount of ethanol added to the reference cuvette. In one experiment, androstenedione (10 μM) was added to both the sample and reference cuvettes. Miconazole (5 μM) was then added to the sample cuvette only, and the difference spectrum was recorded. The total amount of ethanol added to the microsomal suspension during the course of the titrations did not exceed 0.1% (v/v).

Reversibility of the inhibition of aromatase activity

The reversibility of miconazole inhibition of aromatase activity was determined by suspending human placental microsomes at a protein concentration of 0.25 mg/ml in the assay buffer described above. The microsomes were incubated at 37° in the presence of miconazole (0.5 μM) and NADPH for 15 min. The reaction was stopped by diluting with 5 vol. of cold KCl (0.15 M) and centrifuging the diluted reaction mixture at 100,000 *g* for 60 min to sediment the microsomes. The microsomes were then suspended into the original volume of fresh assay buffer, and the aromatase activity was determined as described above. A set of microsomes not treated with miconazole were taken through a similar procedure to serve as a control.

RESULTS

Effects of miconazole, clotrimazole, ketoconazole and aminoglutethimide on human placental microsomal aromatase activity

To determine the relative potency of the imidazole antimycotics and aminoglutethimide to inhibit human placental microsomal aromatase activity, we determined the activity in the presence of increasing amounts of these compounds. In Fig. 2, a plot of inhibitor concentration versus aromatase activity is presented. Miconazole and clotrimazole were found to be potent inhibitors of aromatase activity while aminoglutethimide and ketoconazole were much weaker inhibitors. The concentrations of miconazole, clotrimazole, ketoconazole and aminoglutethimide required to achieve 50% inhibition (I_{50}) of aromatase activity were 0.6, 1.8, 60 and 44 μM respectively.

The kinetic constants and the type of inhibition were determined from a Lineweaver–Burk plot of aromatase activity in the presence of various amounts of substrate and inhibitor. The apparent K_m for androstenedione and the apparent K_i for miconazole were determined from a linear regression analysis of

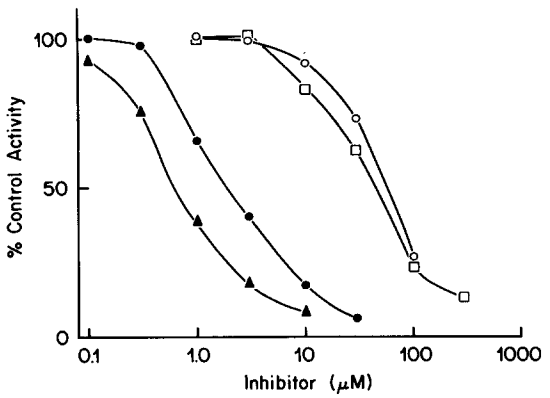

Fig. 2. Dose–response curves for the inhibition of aromatase activity by miconazole (▲), clotrimazole (●), ketoconazole (○), and aminoglutethimide (□). Placental microsomes were used to evaluate the effect of these drugs on the aromatization of androstenedione (1.5 μM). Control activity was 65 pmoles estrogen produced·min^{-1}·(mg protein)$^{-1}$.

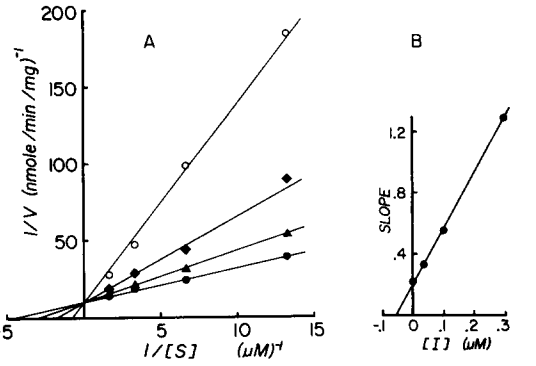

Fig. 3. (A) Lineweaver–Burk plot for miconazole inhibition of placental aromatase activity. Miconazole was tested under initial velocity conditions with less than 10% conversion of the substrate to product. The velocity is expressed as nmoles estrogen formed per mg microsomal protein per min. The miconazole concentrations used were zero (●), 0.03 μM (▲), 0.10 μM (◆), and 0.30 μM (○). Each point is the mean of four determinations with variation between samples of less than 3%. (B) Plot of the slopes of the lines shown in A versus inhibitor concentration. The intercept at the abscissa gives the apparent K_i.

the data points. As shown in Fig. 3, the apparent K_m for the substrate was 220 nM, and miconazole showed competitive inhibition with an apparent K_i value of 55 nM.

Reversibility of the miconazole inhibition of placental microsomal aromatase activity

A number of inhibitors of aromatase activity have been reported to function as "suicide substrates" [12]. By this mechanism, the inhibitor acts by binding to the hemoprotein and in the presence of NADPH is subsequently converted to a reactive intermediate. This intermediate then acts on the enzyme to form irreversibly a catalytically incompetent protein. To determine if miconazole acts in a similar manner, we analyzed the aromatase activity of microsomal samples that had been pretreated with miconazole and NADPH. The procedure is related in detail in Materials and Methods. The data are presented in Table 1. It is apparent that miconazole was effectively removed from the enzyme preparation by the washing/centrifugation procedure, and thus the inhibition of aromatase activity by miconazole was reversible.

Binding difference spectra of placental microsomal cytochrome P-450

Imidazole and a variety of analogues containing this functionality are known to bind and inhibit liver cytochromes P-450 [13]. The presence of an imidazole group on miconazole was suggestive that this compound could inhibit aromatase activity through a direct interaction with the cytochrome P-450 component of this enzyme complex. To determine if miconazole bound to the aromatase cytochrome P-450, spectral binding studies were performed using human placental microsomes. Figure 4A shows the difference spectrum resulting from the addition of miconazole (5 μM) to human placental microsomes in the sample cuvette. The broad trough between

Table 1. Effect of miconazole pretreatment on placental microsomal aromatase activity

Conditions	Activity*
(1) Miconazole-pretreated microsomes	1077 ± 121
(2) Miconazole-pretreated microsomes plus miconazole (0.5 μM)	480 ± 43
(3) Control microsomes	1102 ± 23
(4) Control microsomes plus miconazole (0.5 μM)	509 ± 13

* Expressed as the mean ± S.E.M. of four determinations in pmoles per mg microsomal protein per 30 min. The experimental details are given in Materials and Methods.

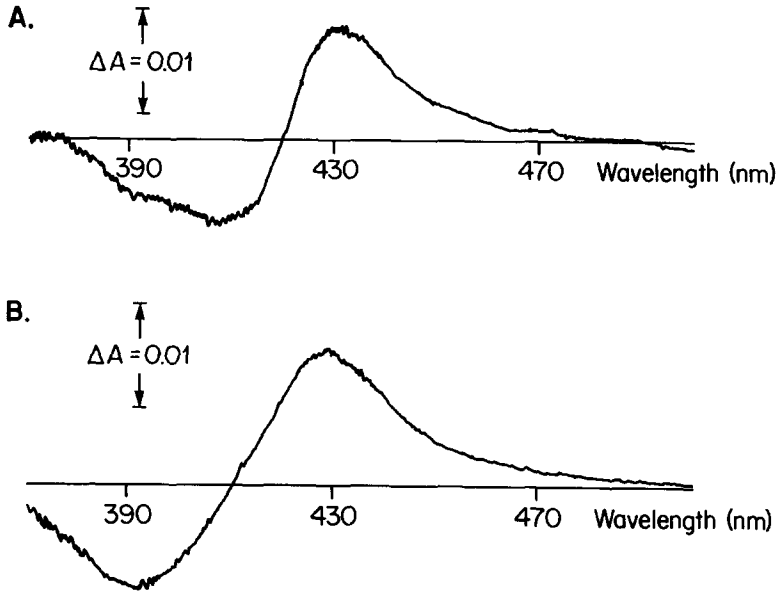

Fig. 4. Spectral perturbation of human placental microsomal cytochrome P-450 by miconazole. Placental microsomes were suspended to a total microsomal protein concentration of 6 mg/ml. (A) The difference spectrum was recorded after the addition of miconazole (5 μM) to the sample cuvette. (B) The difference spectrum was recorded after the addition of androstenedione (10 μM) to both the sample and reference cuvettes and then miconazole (5 μM) to the sample cuvette.

390 and 410 nm, the absorbance maximum at 430 nm, and zero crossing at approximately 421 nm are indicative of a type II difference spectrum [14]. This is suggestive of an interaction of a nitrogenous ligand with the heme-iron of the aromatase, presumably an imidazole nitrogen from the bound miconazole. The zero crossing occurring at 421 nm is indicative that the cytochrome P-450 being titrated with miconazole was predominately in a low spin ($S = \frac{1}{2}$) resting state [14].

In preliminary studies we observed that the addition of androstenedione (10 μM) to placental microsomal cytochrome P-450 resulted in a type I spectral perturbation, as demonstrated previously [6], indicative of a low to high spin state change. Further additions of androstenedione resulted in no further spectral change, which was suggestive that 10 μM steroid was sufficient to saturate the enzyme. To compare the relative spectral interactions of androstenedione and miconazole for the placental microsomal P-450, androstenedione (10 μM) was initially added to the microsomes in both the sample and reference cuvettes. The subsequent addition of miconazole (5 μM) to the microsomes in the sample cuvette resulted in the production of the expected type II spectral change (see Fig. 4B). The narrowing of the trough and the shift of the zero crossing from 421 nm to approximately 411 nm, however, is indicative that miconazole was binding to a cytochrome P-450 which was initially in a high spin state, presumably the aromatase P-450 bound to its substrate, androstenedione. Thus, miconazole (5 μM) competes for both the substrate-free and the substrate-bound forms of the enzyme, to form a low-spin inhibitor complex.

Effect of imidazole antimycotics on human placental mitochondrial cholesterol side chain cleavage activity

To determine the specificity of these compounds in inhibiting a different cytochrome P-450-dependent

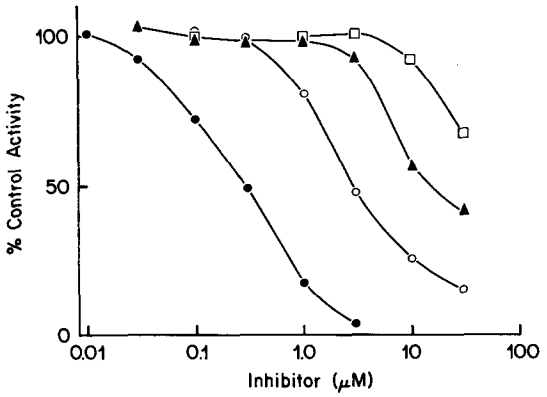

Fig. 5. Dose–response curves for the inhibition of human placental mitochondrial cholesterol side chain cleavage activity by miconazole (▲), clotrimazole (●), ketoconazole (○), and aminoglutetimide (□). The control activity was 4 nmoles pregnenolone produced·(mg protein)$^{-1}$·(20 min)$^{-1}$. Each data point was the mean of three determinations which differed by less than 5% from each other.

reaction in the placenta, we determined their inhibitory potencies on placental mitochondrial cholesterol side chain cleavage activity. As shown in Fig. 5, in contrast to its effectiveness in inhibiting human placental aromatase activity, miconazole was a relatively weak inhibitor of cholesterol side chain cleavage activity. Clotrimazole and ketoconazole were found to be very effective inhibitors of cholesterol side chain cleavage activity and were much more potent than aminoglutethimide, a well-recognized inhibitor of this reaction.

Effect of miconazole on NADPH cytochrome c *(P-450) reductase activity*

To exclude the possibility that miconazole interacted not with cytochrome P-450 alone, but also with the other necessary component of the microsomal monooxygenase system, NADPH cytochrome *c* (P-450) reductase, we assayed this activity using the alternative electron acceptor, cytochrome *c*. Miconazole (10 μM), which is sufficient to inhibit the aromatase activity by over 90%, decreased placental microsomal reductase activity by only 7% of the control activity. Thus, the inhibition of aromatase activity by miconazole was apparently not mediated by an interaction of this compound with the NADPH cytochrome P-450 reductase component of the monooxygenase but, instead, due to its direct interaction with cytochrome P-450.

DISCUSSION

Miconazole and clotrimazole were shown to be effective inhibitors of estrogen biosynthesis in human placental microsomes. Under the assay conditions employed, miconazole was found to be nearly seventy times more effective an inhibitor than aminoglutethimide, a drug that is in current use for treatment of estrogen-dependent breast cancer [15].

The site of the inhibitory activity of miconazole appears to be the cytochrome P-450 component of the aromatase monooxygenase complex. The type II spectral perturbation observed upon the addition of miconazole to placental microsomal cytochrome P-450 is strongly suggestive of the formation of a nitrogenous ligand to the cytochrome P-450, most probably the interaction of an imidazole nitrogen of miconazole with the hemoprotein. This would be a mechanism analogous to that proposed for the inhibition of liver microsomal cytochromes P-450 by a variety of phenyl imidazole analogues [13]. The binding of miconazole to human placental cytochrome P-450 was reversible, as demonstrated by the ability to restore aromatase activity after dilution of the inhibitor. These results indicate that the mechanism of action of this inhibitor is different from that reported for various analogues of androstenedione which are reported to inactivate the enzyme irreversibly [12, 16, 17] and competitively [18].

A determination of the kinetic constants for the inhibition of aromatase activity by miconazole gave an apparent K_m value of 220 nM for androstenedione and an apparent K_i for miconazole inhibition of 55 nM. Thus, miconazole bound to the enzyme with approximately four times greater affinity than the substrate. The competitive kinetics of miconazole inhibition of aromatization of androstenedione is suggestive that these two compounds compete either for the same site or for two mutually exclusive sites on the enzyme. The marked ability of miconazole to effectively compete with androstenedione for the aromatase was further demonstrated spectrally by its ability to produce a type II spectral perturbation even in the presence of this substrate. In contrast to the inhibitory activity of miconazole on the aromatase, this compound was found to be only moderately effective as an inhibitor of the cholesterol side chain cleavage cytochrome P-450 of human placental mitochondria. The two other antimycotic agents tested, ketoconazole and clotrimazole, were found to be effective inhibitors of this activity.

The chemical features resulting in the selectivity of these compounds to inhibit different cytochromes P-450 are not yet clear. A comparison of the chemical structures of these antimycotic agents (see Fig. 1) affords no apparent simple structure–activity relationship. The only homologous structure other than the imidazole function is the presence of a chlorinated aromatic ring(s). All three imidazole compounds were found to produce a type II difference spectral change when added to liver microsomal cytochrome P-450 (data not shown). These results are suggestive of the presence of a nitrogenous ligand near the heme center. Presumably, this would come from an imidazole nitrogen. A determination of the possible role of other structural features acting to promote tight binding and specificity to inhibit different cytochromes P-450 will require the study of more closely related analogues of miconazole.

Since miconazole has been used as a systemic antifungal and antibacterial agent [19], the results of our study are suggestive that serious consideration be given to the evaluation of imidazole antimycotic agents, such as miconazole, as inhibitors of estrogen biosynthesis in situations that require suppression of estrogen formation.

Acknowledgements—We are grateful for the advice of Dr. R. W. Estabrook and the editorial assistance of Mrs. Betty Bushnell. This work was supported, in part, by grants from the National Institutes of Health, DHHS (CA-30253 and GM-16488). J. J. S. was supported, in part, by a grant-in-aid from the Chilton Foundation and a National Research Service Award (1-F32-HD-06596-01).

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