Evidence that products of the respiratory burst cause DNA damage is provided by the data of Table 1. The enzymes superoxide dismutase and catalase, which remove O_2^- and H_2O_2, respectively, had a pronounced but not complete inhibitory effect on PMA-induced DNA damage. Boiled enzymes did not protect the cells. High levels of enzyme were necessary to afford protection, as had been shown when cell damage was monitored by trypan blue staining (*4*). Although both O_2^- and H_2O_2 seem to be necessary for damage to occur (that is, protection is afforded by either superoxide dismutase or catalase), the mechanism of DNA damage has not been elucidated. Hydroxyl radicals are very reactive species and likely to be generated by O_2^- and H_2O_2, but OH· scavengers were ineffective in preventing damage. Similarly, the singlet oxygen scavengers diazabicyclooctane (DABCO) and tryptophan were ineffective. At high levels of dimethyl sulfoxide (DMSO) (0.5*M*), damage was decreased, but O_2^- production was also decreased, so that interpretation of the action of DMSO was uncertain (*7*). Thus, the actual sequence of events leading to DNA damage requires further investigation.

A number of different donors were examined to assess variability in the level of PMA-induced DNA damage in leukocytes. After cells were incubated for 40 minutes at 37°C with 10^{-8}*M* PMA, the measured amount of damage (expressed as induced DNA strand breaks per chromosome) was 148.7 ± 24.4 percent (average ± standard deviation) for 16 blood samples from 12 donors over a 2-month period. Variation between duplicate analyses of the same sample was on average only 4.3 percent, indicating that differences seen between individuals was considerably greater than experimental variability.

These results show that exposure of phagocytic cells to PMA can lead to extensive DNA damage through active oxygen species and also indicate that phagocytic cells may play a role in PMA-induced skin tumor promotion. According to this model, a tumor initiator such as 7,12-dimethylbenz[*a*]anthracene is responsible for a mutation in a critical gene of a skin cell. Subsequent repeated applications of PMA lead to inflammation, migration of phagocytic cells to the area, and then triggering of these cells to produce DNA-damaging oxygen radicals. A combination of mutational damage by the initiator and strand break damage by the promoter may be necessary for the eventual development of a malignant tumor. Benzoyl peroxide, recently shown to be a potent tumor promoter (*13*), can directly cause DNA strand break damage in human leukocytes (*7*).

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Norethisterone, a Major Ingredient of Contraceptive Pills, Is a Suicide Inhibitor of Estrogen Biosynthesis

Abstract. *Norethisterone (17α-ethynyl-19-nortestosterone) is an effective irreversible inhibitor of estrogen synthetase (aromatase), the enzyme responsible for the conversion of androgens to estrogens, even at a* 2×10^{-6} *molar concentration. This irreversible inactivation, which is directed toward the active site of aromatase and requires the cofactor-reduced nicotinamide adenine dinucleotide phosphate, is both time- and concentration-dependent. Ethisterone (17α-ethynyltestosterone), in contrast, is not a suicide inhibitor of aromatase even at concentrations of* 10^{-4} *molar.*

Norethisterone (NET) is widely used as the active ingredient in oral contraceptives (*1*). Even though the compound is taken by humans over prolonged periods, little information is available on the mechanism of its activity. In particular, there is no information on the irreversible inactivation of aromatase by NET. A high concentration (1 m*M*) of NET covalently modifies rat liver cytochrome P-450 (*2, 3*), but shows no competitive inhibitory action on microsomal aromatase at 780 μ*M* (*4*).

Development of an effective irreversible inhibitor of estrogen biosynthesis is the goal of many researchers. Such an inhibitor is sought for possible chemotherapeutic use in estrogen-dependent mammary cancers and also to aid in elucidating the mechanism of the aromatase reaction (*5–11*).

We have studied the effect of NET on the aromatase activity of human placental microsomes using the following procedure. A known amount of the inhibitory compound was incubated with lyophilized human placental microsomes (4 mg/ml) (*12*) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) (0.5 mg/ml) in 0.067*M* phosphate buffer (*p*H 7.49) in a total volume of 10 ml, the mixture being placed in a Dubunoff shaker at 37°C. At specific time intervals a 0.5-ml portion was assayed for aromatase activity. The activity was determined by means of a radiometric assay in which we used [1β-^{3}H,4-^{14}C]androstenedione as substrate and measured the ^3H_2O produced (*5, 12*). A portion of the mixture together with [1β-^{3}H,4-^{14}C]androstenedione (10.5 μ*M*, 1.33×10^7 disintegrations per minute per micromole for ^{3}H and 2.05×10^5 dis/min-μmole for ^{14}C) was incubated with 0.5 mg of NADPH in 0.067*M* phosphate buffer at a total volume of 2.0 ml for 10 minutes. The logarithmic percentage of aromatase activity was plotted against time for each run (Fig. 1) (*13*).

We found that NET caused a time- and dose-dependent irreversible inhibition of aromatase (Fig. 1B) at concentrations of

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2 μM to 50 μM. The stability of the aromatase preparation was measured under the incubation conditions with and without NADPH and with 2 μM androstenedione; the results are shown in Fig. 1A.

When we incubated 50 μM NET with various amounts of the natural substrate androstenedione (Fig. 1D) we found that this substrate hindered the NET inactivation of aromatase in a concentration-dependent manner, indicating that NET attacks at the active site of aromatization.

Mere addition of NET to microsomes, without NADPH, protected rather than inactivated aromatase, but the presence of NADPH caused rapid inactivation (Fig. 1C). This reveals that NET itself cannot inactivate the enzyme but that inhibition appears to be the result of a metabolic interaction of NET with aromatase. The data thus reveal that NET is a suicide inhibitor of aromatase.

Using the method of Kitz and Wilson (*14*) we calculated that the apparent dissociation constant for the initial reversible complex, K_i, was 1.7 μM and that the pseudo–first-order inactivation constant, K_{inact}, was 1.61×10^{-3} per second.

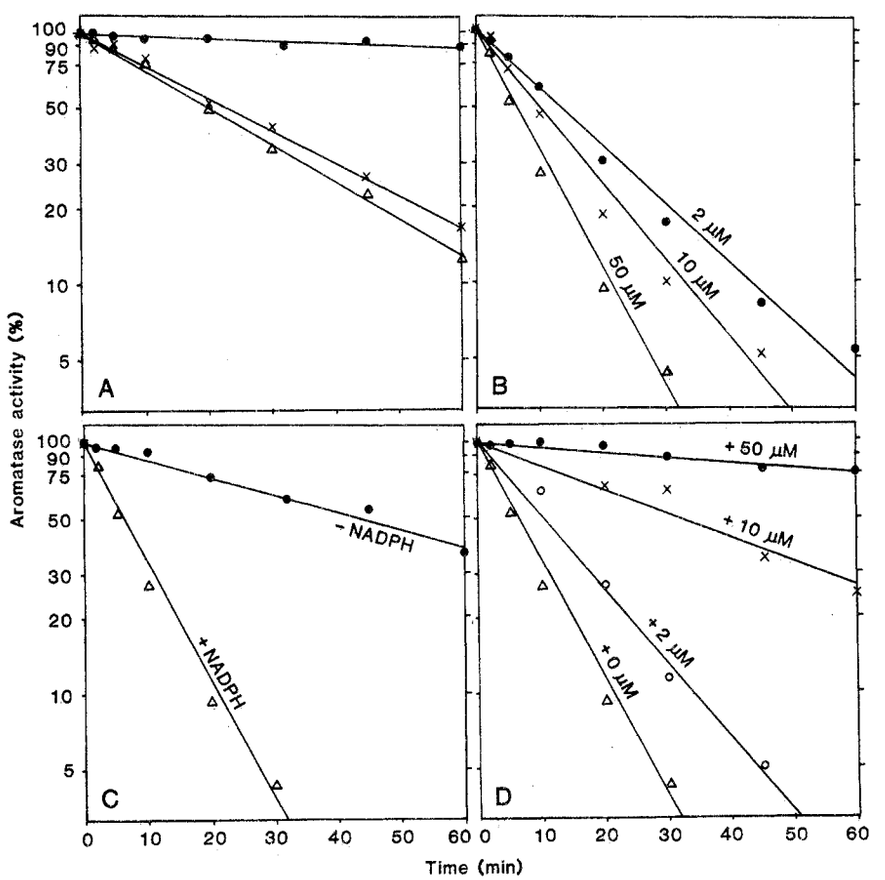
We also studied the effect of ethisterone on placental aromatase. Ethisterone differs from NET only in having the 19-angular methyl (10β-methyl) group instead of hydrogen. By using the same experimental procedure that we used for NET we found that ethisterone in the presence of NADPH neither protected nor inactivated aromatase, even at 50 μM and 100 μM concentrations (Fig. 2).

The effects of administering NET to humans have not been assessed, but NET (25 mg/kg) administered to rats reacts with the hepatic cytochrome P-450 to yield a covalently bound adduct (*2*, *3*). Since androgen aromatase is a molecular complex of a cytochrome P-450 and an NADPH cytochrome P-450 reductase (*15*), it is conceivable that this inactivation of aromatase by NET is due to an NADPH-dependent reaction of NET with aromatase P-450.

Covey *et al.* (*7*), Marcotte and Robinson (*8*), Perel *et al.* (*9*), and Johnston *et al.* (*10*) have synthesized various 19-substituted derivatives of androstenedione, including 19-ethynyl androstenedione, as potential therapeutic agents for estrogen-dependent breast cancer. The ready availability and effectiveness of NET make this compound also a candidate for clinical use.

The kinetic data for the inactivation of NET are comparable to the apparent K_i of 23 nM and 27μM and K_{inact} of 1.11 and 2.91×10^{-3} per second recently reported by Covey *et al.* (*7*) for 10β-propargyl-4-estrene-3,17-dione and 10β-[(1*S*)-1-hydroxy-2-propynyl]-4-estrene-3,17-dione, respectively. Marcotte and Robinson (*8*) also reported 10β-propargyl and 10β-difluoromethyl-4-estrene-3,17-dione to be NADPH-dependent irreversible inactivators of the aromatase in placental microsomes.

The reaction mechanism for the suicide inhibition of aromatase has not been elucidated. The addition of a methyl substituent at the 10β position of NET abolished its inhibitory activity. The presence of two axial methyl substituents at the β-side (10β and 13β) and an axial ethynyl group at the α-side (17α) of the steroid adds substantial bulk in the perpendicular direction to the steroid ring structure. Although the exact site of interaction is not known, this added bulkiness of ethisterone may have caused sufficient steric hindrance to prevent the suicide inhibition reaction. The postulated mechanism of stereospecific α-hydroxylation to the acetylenic group followed by further oxidation to a conjugated ketone proposed by Covey *et al.* (*7*) is not applicable to NET due to the lack of an oxidizable α-carbon to the acetylenic group. Whether or not NET forms a covalent bond directly to the heme por-



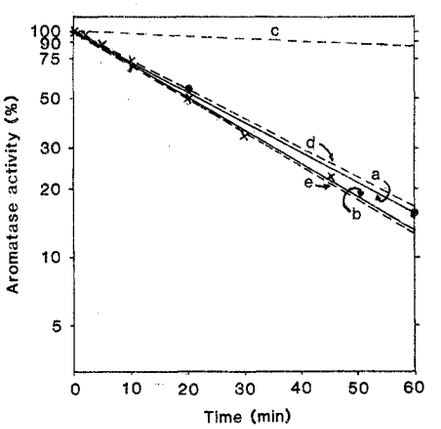


Fig. 1 (left). Suicide inhibition of aromatase by norethisterone (NET). (A) Stability of placental microsomes in 67 mM phosphate buffer at 37°C. Symbols: ●, With 2 μM androstenedione; △ and x, with and without NADPH, respectively. (B) Time and dose response of NET inactivation (2 μM to 50 μM NET with NADPH). (C) Cofactor dependence of NET inactivation (50 μM NET). (D) Change of NET inactivation (50 μM) on addition of androstenedione (0 μM to 50 μM). Fig. 2 (right). Effect of ethisterone on microsomal aromatase. *a*, 50 μM ethisterone (●); *b*, 100 μM ethisterone (x); *c*, *d*, and *e* are controls as presented in Fig. 1A (*c*, with 2 μM androstenedione; *d*, without NADPH; and *e*, with 600 μM NADPH).

tion of the aromatase, as might be suggested from studies by Ortiz de Montellano and co-workers (*2, 3*) on rat liver microsomal cytochrome P-450, has not been determined. Identification of the metabolite of NET suicide inhibition reaction of aromatase is in progress by use of ^{3}H-labeled NET.

These findings raise challenging questions on the molecular mechanism of the aromatase reaction and on the possible effects of NET on estrogen in humans taking contraceptive pills.

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Transmission of Oropouche Virus from Man to Hamster by the Midge *Culicoides paraensis*

Abstract. *Oropouche virus (arbovirus family Bunyaviridae, Simbu serological group) was experimentally transmitted from man to hamster by the bite of the midge* Culicoides paraensis. *Infection rates and transmission rates were determined after the midge had engorged on patients with viremia. The threshold titer necessary to enable infection or transmission by the midges was approximately 5.3 $\log_{10}$ of the median lethal dose of the virus in suckling mice per milliliter of blood. Transmission was achieved 6 to 12 days after* C. paraensis *had taken the infective blood meal. This represents conclusive evidence of transmission of an arbovirus of public health importance to man by a member of the Ceratopogonidae family.*

During the past two decades Oropouche (ORO) virus (arbovirus family Bunyaviridae, Simbu serological group) has been recognized as a major cause of human febrile illness in the Amazon region of Brazil. Between 1961 and 1980 numerous outbreaks occurred in urban centers of Pará State, in the eastern part of Amazonia (*1*). At least 165,000 persons were infected, including 130,000 in 1978–1980, when the greatest wave yet recorded affected 16 localities of this state, including the capital (*2, 3*). Outside of the Amazon region, human infection caused by ORO virus has been recognized only in Trinidad, where it was first isolated in 1955, but to date no epidemics have been recorded in that country (*4*).

Three types of clinical syndromes have been associated with ORO virus infection: (i) febrile illness, (ii) febrile illness with rash, and (iii) meningitis or meningismus. Although no fatalities have been attributed to the disease, many patients become severely ill, some to the point of prostration. Acute manifestations usually last one week or less, but many patients experience one or more episodes of recurrence of symptoms for a period of 1 or 2 weeks. Instances of meningitis associated with ORO virus infection were observed during the 1980 outbreak in Pará, and although no sequelae occurred among these patients, it is clear that meningitis is an aggravating component of ORO virus infection (*5*). A rash also is occasionally observed on the trunks, arms, and less commonly on the thighs (*3*).

Oropouche virus probably occurs in nature in two distinct cycles: a sylvatic cycle which is responsible for maintenance of the virus in nature, with primates, sloths, and possibly certain species of wild birds implicated as vertebrate hosts, with the sylvatic vector still unknown; and an urban cycle during which man may be infected and, once infected, probably serves as an amplifying host of the virus among hematophagous insects. Two insect species have been implicated as potential vectors in the urban cycle through epidemiological studies made during outbreaks: the ceratopogonid midge *Culicoides paraensis* and the mosquito *Culex p. quinquefasciatus* (*6, 7*). In laboratory transmission studies of both the suspect vectors the former was found to be the more efficient of the two, but hamsters were used in these experiments as the donor and recipient hosts (*8*). Viremia titers in experimentally infected hamsters are usually much higher than those in man; consequently, the question remained whether *C. paraensis* could become infected when feeding on the lower-titered viremia which is circulated when man is infected. In this report we describe the successful transmission of ORO virus from man to hamsters by the bite of *C. paraensis*. This observation represents the first definitive evidence of transmission of an arbovirus pathogenic to humans by a vector of the family Ceratopogonidae.

Suspected cases of ORO virus infection which occurred during the 1979–1980 Pará outbreak were chosen for the study. Most patients were selected during their first 2 days of illness when viremia titers are usually highest. Blood was collected from febrile patients, diluted 1:10 in phosphate-buffered saline (PBS) with 0.75 percent bovine albumin, and frozen at 70°C for subsequent virus titration. Viremia values were calculated by the method of Reed and Muench (*9*) after inoculation of serial tenfold blood dilutions intracerebrally into suckling mice. Virus identity was confirmed by complement-fixation (CF) tests with the use of mouse brains as antigen and ascitic fluid from hyperimmune mice (HMAF) prepared against the Belém ORO virus prototype strain (BeAn 19991).

All midges used in the transmission experiments were captured as they were attracted to humans at an agriculture research institute near Belém where banana and cacao trees are cultivated (*8*). The *Culicoides* were maintained at 22° to 25°C and 95 percent relative humidity and were provided with a 10 percent sucrose solution which was removed a few hours before they were allowed to feed on the patients.

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