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EFFECTS OF CLOMIPHENE *CIS* AND *TRANS* ISOMERS ON STEROL METABOLISM IN THE RAT*

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Abstract—Both of the geometric isomers of clomiphene cause an increase in the desmosterol content of tissues of rats receiving the compounds by gastric route for 10 days; these results indicate inhibition *in vivo* of sterol Δ^{24}-reductase. The *trans* isomer is approximately 10 times as potent as the *cis* isomer as an inhibitor of this enzyme. Both were less potent than triparanol. *Trans*-clomiphene reduced the total sterol content of plasma, adrenal and ovary more than did *cis*-clomiphene or triparanol (dose range, 1–10 mg/kg); it therefore has an effect on sterol metabolism in the rat additional to its effect on Δ^{24}-reductase. The desmosterol data demonstrate the importance of steric configuration of the triphenylethyl portion of the clomiphene molecule as a determinant of potency of Δ^{24}-reductase inhibition. A mechanism is postulated for inhibition of Δ^{24}-reductase by known inhibitors, involving interaction of the amino nitrogen of the inhibitor with an electrophilic group at the enzyme active site.

CLOMIPHENE is a compound which has been found useful for induction of ovulation in anovulatory women.[1–4] The chemical structure of clomiphene is shown in Fig. 1.

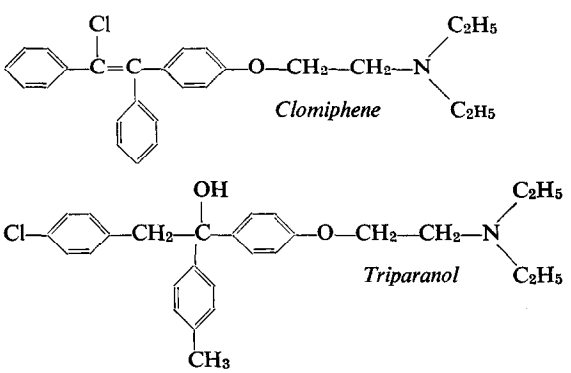


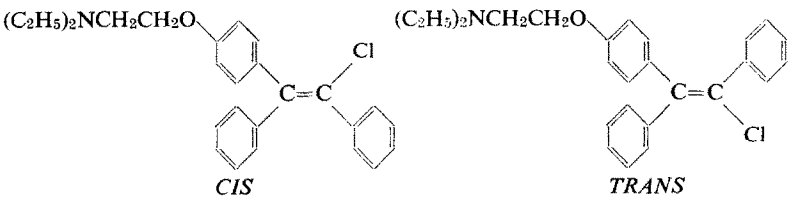
FIG. 1. Structures of clomiphene and triparanol.

It is a triphenylethylene, in which one of the phenyl groups is substituted in the *para*-position with a basic ether group, and the fourth hydrogen of the ethylene portion has been replaced by chlorine. For comparison, the structure of triparanol is also given in Fig. 1. This compound is a triphenylethanol, with the same type of basic ether substitution, and additional substitution in the remaining phenyl rings.

* This work was presented in part at the Third International Symposium on Drugs Affecting Lipid Metabolism, Milan, Italy (September, 1968).

Triparanol inhibits the biological reduction of the Δ^{24}-double bond that occurs in precursors of cholesterol and, in cholesterol-producing species, causes partial replacement of cholesterol by desmosterol (24-dehydrocholesterol).[5,6] The similarity of the structures of clomiphene and triparanol prompted us to determine the effects of clomiphene on sterol Δ^{24}-reductase in the rat *in vivo*, as reflected in the desmosterol content of various tissues. At the same time, total sterol (cholesterol plus desmosterol) contents of the same tissues were determined.

An important structural feature of clomiphene is its central double bond, which gives rise to geometric isomerism. Thus two forms of clomiphene exist, as shown in Fig. 2. The configurations shown are those reported by Richardson and Benson;[7]



CLOMIPHENE

FIG. 2. Configurations of clomiphene isomers.

in their notation, *cis* and *trans* refer to the relationship of the two unsubstituted phenyl rings in each structure.*

In addition to having different physical properties, *cis*- and *trans*-clomiphene have been shown to differ quantitatively in their abilities to facilitate PMS†-induced ovulation in immature rats and to increase uterine weight in immature mice.[8] It was deemed important to determine whether they also differ in their ability to inhibit Δ^{24}-reductase. The two isomers were therefore studied separately, and a marked difference in potency was found. In addition, *trans*-clomiphene was shown to have a lowering effect on the total sterol content of certain tissues, which could not have been due to its effect on sterol Δ^{24}-reductase.

METHODS

Sterol assays. Tissues were removed, weighed and frozen immediately after sacrifice of the animals, and stored frozen until analysis. Each tissue was pooled by dose group for analysis and hydrolyzed in 50% aqueous KOH for 45 min at 60°, followed by addition of absolute ethanol (5 vol./3 vol. of aqueous KOH) and heating for another 45 min at 60°. Five vol. of water were added, and the nonsaponifiable matter was extracted with petroleum ether (30–60°). The solvent was removed from an aliquot of the extract. All operations up to this point were carried out under nitrogen, in subdued light.

Trimethylsilyl ethers of the sterols in the residue were prepared by the method of Luukainen *et al.*[9] Gas chromatography (GLC) of the sterol ethers was carried out on an 8% ECNSS-S on Gas-Chrom P column, 6 ft × 4 mm, in a Barber–Colman model 10 instrument with argon ionization detector. Column temperature and pressure:

* In some literature references, cis-clomiphene is identified as isomer B, and trans-clomiphene as isomer A.

† PMS = serum gonadotropin of pregnant mares.

198°, 16·5 psi. Cholestane was internal standard (retention time, 7·2 min) and standards of pure cholesterol (retention time, 14·0 min) and desmosterol (retention time, 19·1 min) were employed. Results are expressed as milligrams of sterol per gram of tissue, wet weight.

Isolation and identification of desmosterol. Nonsaponifiable matter was isolated by the procedure described above from the livers of rats treated with *cis*-clomiphene or *trans*-clomiphene. Sterols were isolated from this material by digitonin precipitation according to Sperry and Webb.[10] The washed digitonides were cleaved with pyridine[11] and the free sterols extracted with diethyl ether. Sterol acetates were prepared as described by Galli and Grossi-Paoletti[12] and chromatographed on Silica gel G-15% $AgNO_3$ plates, 1 mm thick, with benzene–hexane (3:5) as the developing solvent. Sterol acetate areas were disclosed with iodine vapor. Areas having the same R_f value as desmosterol acetate (run on the same plate) were scraped off and eluted with $CHCl_3$ The materials thus isolated (65 mg from *trans*-clomiphene-treated livers, 6·1 mg from *cis*-clomiphene livers) were submitted to nuclear magnetic resonance (n.m.r.) and infrared (i.r.) spectroscopy. For both, comparison was made to authentic cholesterol, desmosterol ($\Delta^{5,24}$-cholestadien-3β-ol) and $\Delta^{5,25}$-cholestadien-3β-ol acetates. For n.m.r. sterol acetates were dissolved in $CHCl_3$, with trimethylsilane internal standard. The KBr pellet technique was used for i.r. spectroscopy.

EXPERIMENTAL

Identification of tissue sterol induced by treatment with clomiphene isomers. Pilot experiments showed that administration of either *cis*-clomiphene or *trans*-clomiphene to rats resulted in the appearance in the blood and tissues of a sterol having the retention time of desmosterol on GLC. In order to isolate this material for identification, two groups of twenty-five female, Charles River, Sprague–Dawley rats, average weight 140 g, were given respectively, 100 mg/kg/day of *cis*-clomiphene citrate or 30 mg/kg/day of *trans*-clomiphene citrate. Drugs were administered by gastric tube once daily for 10 days. Animals were placed on an *ad lib.* Purina Chow diet. Twenty-four of the rats receiving *trans*-clomiphene survived, but only six survived the 100 mg/kg dose of *cis*-clomiphene. These animals were sacrificed on the eleventh day, and the livers were removed for isolation and identification of the sterols (see Methods).

Determination of tissue sterol responses to cis-*clomiphene*, trans-*clomiphene and triparanol.* Female, Charles River, Sprague–Dawley rats, weighing 130–160 g, were placed on an *ad lib.* Purina Chow diet, and distributed into dose groups as shown in Table 1. Average starting weights for the groups were 145–149 g. Drugs were administered as suspensions or solutions in 0·25% aqueous Methocel, once a day, by gastric tube. Controls received vehicle only. On the eleventh day, 16–22 hr after the last dose, the rats were weighed and anesthetized with ether; blood was obtained by heart puncture, the animals were sacrificed, and organs and tissues removed immediately for sterol analysis by GLC (see Methods).

RESULTS

Identification of tissue sterol induced by treatment with clomiphene isomers. Treatment of rats for 10 days with doses of *cis*-clomiphene ranging from 10 to 100 mg/kg/day, or with *trans*-clomiphene, 1–30 mg/kg/day, resulted in the appearance of a single new tissue sterol peak on GLC, corresponding to the retention time of desmosterol. Thin-

TABLE 1. TREATMENT GROUPS

Treatment Drug*	Dose (mg/kg)	No. rats	Av. body wt. gain (g)
Control (vehicle)		6	31
Trans-clomiphene citrate	1	6	7
	3	6	3
	10	6	13
Cis-clomiphene citrate	1	5†	8
	3	5†	20
	10	6	12
	30	6	19
Triparanol‡	1	6	21
	3	6	32
	10	6	36

* Administration in 0·25% aqueous Methocel once daily for 10 days by gastric tube.
† One animal received the wrong dose on day 4; they were eliminated from the study.
‡ Triparanol free base.

TABLE 2. SPECIFIC SPECTRAL CHARACTERISTICS USEFUL IN IDENTIFICATION OF DESMOSTEROL (AS ACETATE)

Spectrum	Group	Cholesterol acetate	Desmosterol acetate	$\Delta^{5,25}$-Cholestadien-3-β-ol acetate
Nuclear magnetic resonance	C-26 and C-27 vinyl	0·87* d	1·62, 1·68 s	1·70 s
		5·37 d (C-6)	5·42 m (C-6)	5·18 m (C-6)
			5·13 m (C-24)	4·69 m (C-26)
	C-18	0·70 s	0·68 s	0·69 s
	C-19	1·03 s	1·00 s	1·03 s
	C-21	0·95 d	1·02 d	1·01 d
Infrared	gem-dimethyl (C-26, C-27)	1375 cm^{-1}-unsymmetrical doublet	1375 cm^{-1}-symmetrical doublet	1375 cm^{-1}-unsymmetrical doublet
	C-26			890 cm^{1} terminal methylene peak
		3100–2100 cm^{1} region differs in relative intensities of peaks		

* Chemical shifts in ppm from internal trimethylsilane; s = singlet, d = doublet, m = multiplet. (Value represents centre of gravity of multiplet.)

layer chromatography (TLC) on Silica gel G-$AgNO_3$ of the acetates of the sterols isolated from livers of rats treated with the highest doses of the clomiphene isomers also showed only two sterols to be present, corresponding to cholesterol and desmosterol acetate standards. The apparent desmosterol acetate areas were isolated and subjected to n.m.r. and i.r. spectroscopy; both spectra were identical to corresponding spectra of authentic desmosterol acetate and different from spectra of cholesterol acetate and $\Delta^{5,25}$-cholestadien-3β-ol acetate. This was true for material isolated from livers of animals treated with both clomiphene isomers. Specific spectral identification points are given in Table 2.

The sterol induced in rat tissues by treatment with both clomiphene isomers is thus established as desmosterol by four criteria: GLC, TLC, n.m.r. and i.r.

Effects of cis-*clomiphene,* trans-*clomiphene and triparanol on tissue sterols.* Results of GLC assays of various tissues for desmosterol are presented in Fig. 3, A. In addition to the tissues represented in this figure, skin and ocular lens were also assayed. No

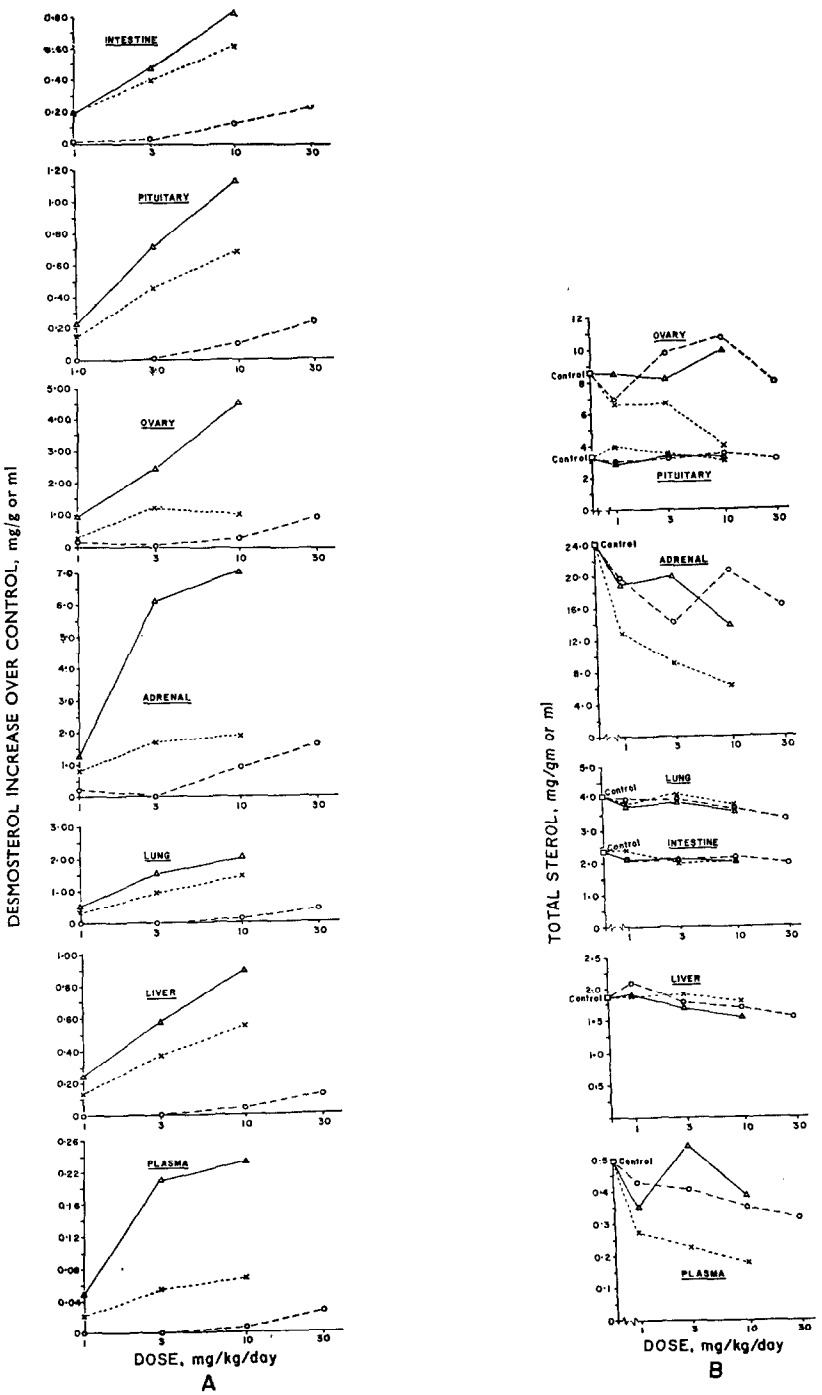


FIG. 3. Effects of *cis*-clomiphene, *trans*-clomiphene and triparanol on rat tissue sterols. (A) Increase in tissue desmosterol over control. ○ — — ○, *cis*-clomiphene; × - - - - - ×, *trans*-clomiphene; △———△, triparanol. (B) Tissue total sterols. Symbols as in A; control values are indicated on the ordinate. Ten-day oral administration.

desmosterol could be detected in any of the lenses from animals treated with the clomiphene isomers, and only a trace amount was found at the highest dose of triparanol. It seems likely that 10 days was not sufficient time for a detectable increase of desmosterol in this tissue, in which such an increase would be expected to occur by the gradual deposition of old cell residues occurring with the continuous replacement of lens epithelium. The changes in sterol pattern in rat skin due to Δ^{24}-reductase inhibition are very complex, and cannot be determined quantitatively by GLC alone.[12,13] Qualitatively, changes similar to those described by Clayton *et al.*[13] and by Horlick and Avigan[14] were seen in skin extracts from rats treated with triparanol and *trans*-clomiphene, but could not be seen with certainty in skin extracts from *cis*-clomiphene-treated rats. It seems likely that they may have occurred to a much lesser degree, consistent with results in other tissues with *cis*-clomiphene.

The data in Fig. 3 show that in all tissues examined, the order of desmosterol-increasing potency was: triparanol > *trans*-clomiphene > *cis*-clomiphene. The first two compounds produced an effect in most tissues at the 1 mg/kg dose, whereas *cis*-clomiphene was active only at 10 and 30 mg/kg. In other experiments, not reported here, the absolute desmosterol responses to the two isomers differed from the present results, but the relative responses were always the same, i.e. *trans* > *cis*. The same order of activity occurs when the compounds are administered subcutaneously in olive oil vehicle.

Two different patterns of response to *trans*-clomiphene can be distinguished. In liver, lung, small intestine and pituitary, the absolute increase of desmosterol due to *trans*-clomiphene is more than half that due to triparanol, while in plasma, adrenal

TABLE 3. DESMOSTEROL CONTENT OF TISSUES OF RATS TREATED WITH *TRANS*-CLOMIPHENE

Dose (mg/kg)	Desmosterol content of tissue (% of total sterols)						
	Plasma	Adrenal	Ovary	Liver	Intestine	Lung	Pituitary
1·0	11	6·2	4·6	7·0	9·3	8·1	6·0
3·0	27	19	18	19	22	24	16
10	41	29	25	31	31	39	25

and ovary it is less than half (with the exception of adrenal at the 1 mg/kg dose level). The second desmosterol response pattern is coupled with a marked reduction of total sterols (cholesterol plus desmosterol) in the same tissues (Fig. 3, B). In other words, in plasma, adrenal and ovary, not only is the desmosterol response to *trans*-clomiphene reduced in comparison to that of other tissues, but cholesterol is reduced more as well. The relationship between these effects is seen more clearly in Table 3, in which the desmosterol response to *trans*-clomiphene is expressed in terms of per cent of total sterols. In these terms, the desmosterol response of plasma, adrenal and ovary cannot be distinguished from responses of other tissues to *trans*-clomiphene.

DISCUSSION

The data show that both *cis*- and *trans*-clomiphene can inhibit sterol Δ^{24}-reductase in the intact rat, and thus cause an increase in desmosterol in the tissues. It has previously been observed by us* and others[15] that the basic ether side chain is a requirement for inhibition of this enzyme *in vivo* by compounds related to triparanol. The present data show that steric configuration, as well as the composition, of the triphenylethyl portion of the molecule is an important determinant of potency of inhibition *in vivo*; *cis*-clomiphene is less than one-tenth as active as *trans*-clomiphene. Hughes *et al.*[15] have shown that the *trans* configuration was more active than the *cis* in a series of alkyl cyanostilbene basic ethers, utilizing Liebermann–Burchard colorimetric analysis of rat plasma sterols. This method of analysis would reflect either desmosterol replacement of cholesterol or total sterol reduction or both. Their results are therefore consistent with our findings, although not as definitive.

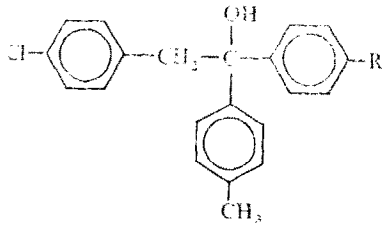
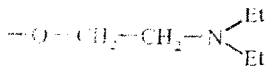
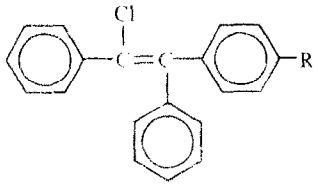
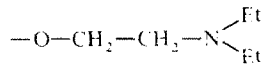
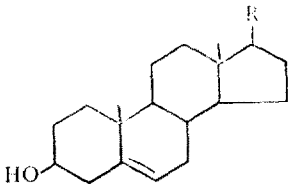
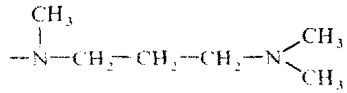
It is obvious from Fig. 3 that considerable replacement of cholesterol by desmosterol can occur in certain tissues (liver, lung, intestine, pituitary) with only moderate reduction of total tissue sterols, and that the total sterol responses to the three agents used were very similar in these tissues. In contrast, the responses of plasma, adrenal and ovary to *trans*-clomiphene were markedly different from the responses of the same tissues to *cis*-clomiphene and triparanol. *Trans*-clomiphene resulted in unexpectedly low desmosterol levels in these tissues, and in an increased reduction of cholesterol; the ratio of desmosterol to cholesterol did not differ, therefore, from the ratio in other tissues. Evidently, *trans*-clomiphene has an effect on sterol distribution or metabolism in the rat additional to its effect on Δ^{24}-reductase. This effect is either much smaller or nonexistent with *cis*-clomiphene and triparanol, in the dose ranges studied.

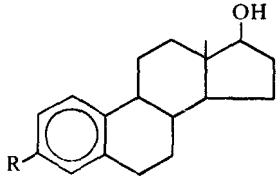
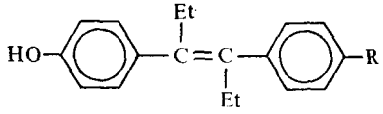
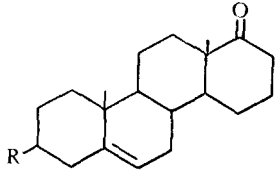
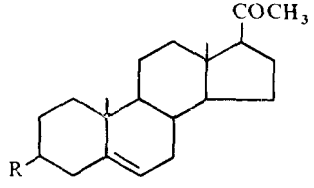
Landon and Greenberg[16] and Morris and Chaikoff[17] have provided evidence, based on kinetics of cholesterol specific activity in adrenal and plasma, that adrenal cholesterol is derived primarily from the blood plasma in the rat. Similar published evidence has not been found for ovary, but this organ, like the adrenal, is capable of depleting stored cholesterol under appropriate pituitary hormonal stimulation[18] and, also like the adrenal, functions as a producer of steroid hormones. It seems likely, therefore, that the marked effects of *trans*-clomiphene on sterols of the adrenal and ovary are secondary to an effect on plasma sterols. What the primary effect may be cannot be deduced from available data, but it appears to apply to cholesterol and desmosterol similarly. The data do not indicate a redistribution of plasma sterol to any of the tissues examined, including liver.

This study and others,[15] * have shown that compounds that are potent inhibitors of sterol Δ^{24}-reductase are composed of a steroid or pseudosteroid (hereafter included under the term "steroid") nucleus and a side chain containing a basic function, attached to a terminal ring of the steroid structure. It seems reasonable to assume that the steroid portion of the inhibitor occupies the site on the enzyme normally occupied by the nucleus of the Δ^{24}-sterol substrate, e.g. desmosterol. In the potent diazacholesterol inhibitors,[19,20] the nucleus of the inhibitor is identical to that of the substrate, i.e. androst-5-ene-3β-ol. The side chain must, then, correspond to the isooctenyl side chain of the Δ^{24}-sterol. Table 4 shows the nuclei and side chains of representative inhibitors. In every case except 20,25-diazacholesterol, the nitrogen atom of the terminal amino group is separated from the steroid nucleus by two carbon atoms and an oxygen

* T. Kariya and T. R. Blohm, unpublished results.

TABLE 4. STEROID* NUCLEI AND BASIC SIDE CHAINS OF REPRESENTATIVE INHIBITORS OF STEROL Δ^{24}-REDUCTASE

Inhibitor	Steroid moiety*	Side chain (R)	Reference no.
Triparanol			(5, 6)
Clomiphene			This paper
20, 25-Diazacholesterol			(19, 20)

Estradiol basic ether derivative		$-O-CH_2-CH_2-N(Et)_2$	(15)
Stilbestrol basic ether derivative		$-O-CH_2-CH_2-N(Et)_2$	(15)
3β-(2-Diethylaminoethoxy) androst-5-ene-17-one hydrochloride (U-18, 666 A)		$-O-CH_2-CH_2-N(Et)_2$	(21)
3β-(2-Dimethylaminoethoxy)-pregn-5-ene-20-one		$-O-CH_2-CH_2-N(CH_3)_2$	(22)

* Includes "pseudosteroid" groups, as in stilbestrol, triparanol, clomiphene.

atom. Nucleus-to-nitrogen distance (measured in Dreiding stereomodels[23]) is 4·68Å. The distance between C-17 and C-24 of desmosterol is 4·84 Å, measured extended in the plane of the steroid ring. (A distance of 5·12 Å is obtained for the most fully extended conformation of the side chain, but because of the β-configuration at C-17, this conformation is nonplanar for the molecule as a whole.) The inhibitor side chains thus correspond closely to the main branch of the isooctenyl side chain of Δ^{24}-sterols, with the amino nitrogen of the inhibitor corresponding to C-24 of the substrate. In 20,25-diazacholesterol, the terminal nitrogen corresponds more closely to C-25 of the substrate (6·00 Å vs. 6·04 Å). Since the C-24, C-25 double bond of the substrate is the operative site for the enzyme, the amino group of the inhibitors would appear to be the group that interacts with the active site of the enzyme.

Enzymatic reduction of Δ^{24} requires NADPH as coenzyme.[24] A preponderance of evidence indicates that transfer of hydrogen from NADPH to substrate involves transfer of H^- from C-4 of nicotinamide ring, accompanied or followed by capture of H^+ from the environment.[25] One function of the enzyme in this reaction must be to create a sufficient electronic deficit around one of the doubly bonded C-atoms to permit a bond-forming attack by H^- or its equivalent. An electrophilic group at the enzyme active site would be required for this function.* It is postulated that inhibition of the enzyme involves interaction between such an electrophilic active site and the unbonded electrons of the amino nitrogen of the inhibitor. Such an interaction would be much stronger than that between the electrophilic site and Δ^{24}, and would account for the high potency of Δ^{24}-reductase inhibitors.

* Since submission of this paper, a citation has appeared, work by D. Steinberg, J. Avigan, R. Dexter and H. Fales, cited by D. Steinberg and J. Avigan, in *Methods in Enzymology*, Vol. 15, *Steroids and Terpenoids* (Ed. R. B. CLAYTON), p. 521, Academic Press, New York (1969) indicating that deuterium from D_2O is incorporated exclusively into the 25-position during enzymatic reduction of Δ^{24}. This would indicate that the site of electrophilic attack by the enzyme postulated here is C-24.

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REFERENCES

1. D. E. HOLTKAMP, J. G. GRESLIN, C. A. ROOT and L. J. LERNER, *Proc. Soc. exp. Biol. Med.* **105**, 197 (1960).
2. R. B. GREENBLATT, W. E. BARFIELD, E. C. JUNGCK and A. W. RAY, *J. Am. med. Ass.* **178**, 101 (1961).
3. R. W. KISTNER, *Am. J. Obstet. Gynec.* **92**, 380 (1965).
4. D. E. HOLTKAMP, R. E. STAPLES, J. G. GRESLIN and R. H. DAVIS, *Proc. Fifth World Congr. Fertility and Sterility, Excerpta Med. Int. Congr.*, series 133, p. 68 (1967).
5. T. R. BLOHM and R. D. MACKENZIE, *Archs Biochem. Biophys.* **85**, 245 (1959).
6. D. STEINBERG and J. AVIGAN, *J. biol. Chem.* **235**, 3123 (1960).
7. A. RICHARDSON, JR. and H. D. BENSON, *153rd National Meeting Am. Chem. Soc.*, abstract M-4 (1967).
8. L. W. SELF, D. E. HOLTKAMP and W. L. KUHN, *Fedn Proc.* **26**, 534 (1967).
9. T. LUUKAINEN, W. J. A. VANDENHEUVEL, E. O. A. HAAHTI and E. C. HORNING, *Biochim. biophys. Acta* **52**, 599 (1961).
10. W. M. SPERRY and M. WEBB, *J. biol. Chem.* **187**, 97 (1950).
11. R. SCHOENHEIMER and H. DAM, *Hoppe-Seyler's Z. physiol. Chem.* **215**, 59 (1933).
12. G. GALLI and E. GROSSI-PAOLETTI, *Lipids* **2**, 72 (1967).

13. R. B. CLAYTON, A. N. NELSON and I. D. FRANTZ, JR., *J. Lipid Res.* **4**, 166 (1963).
14. L. HORLICK and J. AVIGAN, *J. Lipid Res.* **4**, 160 (1963).
15. G. M. K. HUGHES, P. F. MOORE and R. B. STEBBINS, *J. med. Chem.* **7**, 511 (1964).
16. E. J. LANDON and D. M. GREENBERG, *J. biol. Chem.* **209**, 493 (1954).
17. M. D. MORRIS and I. L. CHAIKOFF, *J. biol. Chem.* **234**, 1095 (1959).
18. E. T. BELL, S. MUKERJI and J. A. LORAINE, *J. Endocr.* **28**, 321 (1964).
19. M. J. THOMPSON, J. DUPONT and W. E. ROBBINS, *Steroids* **2**, 99 (1963).
20. R. E. RANNEY and E. G. DASKALAKIS, *Proc. Soc. exp. Biol. Med.* **116**, 999 (1964).
21. W. A. PHILLIPS and J. AVIGAN, *Proc. Soc. exp. Biol. Med.* **112**, 233 (1963).
22. E. W. CANTRALL, R. LITTELL, S. M. STOLOR, W. P. CEKLENIAK, H. J. ALBERS, S. GORDON and S. BERNSTEIN, *Steroids* **1**, 173 (1963).
23. U. S. Patent no. 2,974,425.
24. J. AVIGAN and D. STEINBERG, *J. biol. Chem.* **236**, 2898 (1961).
25. H. SUND, H. DIEKMANN and K. WALLENFELS, *Adv. Enzymol.* **26**, 115 (1964).