The Relationship between Structure and Activity in a Novel Series of Serum Cholesterol-Lowering Agents[1]

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In vivo and *in vitro* effects on cholesterol metabolism are presented for a series of 145 compounds which are structural analogs of the potent, orally active, cholesterol-lowering agent, *trans*-1,4-bis(2-chlorobenzylaminomethyl)cyclohexane dihydrochloride. On the basis of these results it has been possible to define the structural requirements for cholesterol-lowering activity. These are symmarized in terms of the following features: two, basic, secondary nitrogens separated by a distance of 5.59–6.30 A, the internitrogen area being occupied by a rigid nonaromatic system; and the nitrogens bearing groups of benzylic or cycloaliphatic character, these groups being subject to specific limitations with respect to size, flexibility, and nature and position of substitution. The mechanism of action of several of the more potent compounds has been studied and they are shown to be inhibitors of the enzymatic conversion of 7-dehydrocholesterol to cholesterol.

Previous reports from these laboratories have described the synthesis[3] and biological properties[1b,4-10] of *trans*-**1**,4-bis(2-chlorobenzylaminomethyl)cyclohexane dihydrochloride,[11] a potent cholesterol-lowering agent which acts by inhibiting the biosynthesis of cholesterol through blocking the enzymatic conversion of 7-dehydrocholesterol to cholesterol[12,13] and which has been used, along with Triparanol to elucidate the final stages in the pathway of cholesterol biosynthesis.[12,13] This compound represents a novel type of cholesterol-lowering agent, both with regard to its structure and to its mechanism of action, the inhibition of 7-dehydrocholesterol Δ^7-reductase.[12,13] We have synthesized and examined the pharmacological properties of a series of related structures in the search for a compound possessing the most favorable biological profile, and in order to define a possible relationship between molecular architecture and biological response. The details of these studies are reported herewith.

(1) (a) Part XX of a series entitled "Agents Affecting Lipid Metabolism." (b) Part XIX: P. Hill, *Biochem. J.*, in press.

(2) Deceased July 8, 1965.

(3) L. G. Humber, *J. Med. Chem.*, **7**, 826 (1964).

(4) C. I. Chappel, J. Dubuc, D. Dvornik, M. Givner, L. G. Humber, M. Kraml, K. Voith, and R. Gaudry, *Nature*, **201**, 497 (1964).

(5) A. V. Marton and C. I. Chappel, *Experientia*, **20**, 517 (1964).

(6) A. V. Marton, E. Greselin, M. Givner, and K. Voith, *Endocrinology*, **75**, 469 (1964).

(7) M. Givner, M. Kraml, D. Dvornik, and R. Gaudry, *Nature*, **203**, 317 (1964).

(8) M. L. Givner and D. Dvornik, *Biochem. Pharmacol.*, **14**, 611 (1965).

(9) M. L. Givner and D. Dvornik, *Proc. Soc. Exptl. Biol. Med.*, **117**, 3 (1964).

(10) (a) P. Hill and D. Dvornik, *Circulation*, **30** (Suppl. III), 14 (1964); (b) P. Cushman and J. G. Hilton, *Federation Proc.*, **24**, 449 (1965); (c) L. Horlick, *Circulation*, **30** (Suppl. III), 16 (1964); (d) P. Hill and D. Dvornik, *Arch. Biochem. Biophys.*, in press.

(11) This compound has been assigned the code number AY-9944.

(12) (a) D. Dvornik, M. Kraml, J. Dubuc, M. L. Givner, and R. Gaudry, *J. Am. Chem. Soc.*, **85**, 3309 (1963); (b) M. Kraml, J. F. Bagli, and D. Dvornik, *Biochem. Biophys. Res. Commun.*, **15**, 455 (1964).

(13) D. Dvornik, M. Kraml, and J. F. Bagli, *J. Am. Chem. Soc.* **86**, 2739 (1964).

Materials and Methods

The syntheses of the compounds investigated in this study have been described previously.[3,14-16]

Effect on Serum Sterol Levels.—Twenty-one male hooded rats (Long-Evans, weighing 90–100 g) were used for each compound. The animals were kept on Purina food and divided into groups of seven, one control group and two groups treated at different dose levels. Both food and water were given *ad lib.* Prior to treatment with the test compound, blood samples were collected for determination of serum sterol levels. The test compound was administered orally for 7 consecutive days, then, 3 hr after the last treatment and after 4 hr of fasting, the rats were killed, blood was collected by heart puncture, and serum sterol levels were determined. During the postmortem examination, organ weights were recorded. Changes were observed only in the adrenals and typical results are shown in Table I. The dose levels of the test compounds were 10 and 75 μmoles/kg of body weight. The dose level and toxicity values are expressed in μmoles/kg in order to facilitate comparisons of compounds within this series with molecular weights varying from 217 to 820.

Determination of Sterol Levels.—It has been demonstrated that *trans*-1,4-bis(2-chlorobenzylaminomethyl)cyclohexane dihydrochloride and several of the compounds to be discussed in this report are inhibitors of the conversion of 7-dehydrocholesterol to cholesterol,[12,13] and, as a result, cause the accumulation of 7-dehydrocholesterol. Thus, two types of sterol determinations were employed. "Total sterols" were determined using a semiautomatic method with the Technicon Autoanalyzer.[17] With this method 7-dehydrocholesterol gives only 40% of the color yield of cholesterol. As a result "% serum sterol decrease" in Tables I–IV gives a value which is lower than the true cholesterol reduction, but which is higher than the actual total sterol reduction. For some of the compounds, a differential sterol determination was employed. Thus, 7-dehydrocholesterol and cholesterol were determined, either by the "slow- and fast-acting sterol" method of Moore and Baumann[18a] or by a combination of a specific ultraviolet assay for 7-dehydrocholesterol[18b] and the semiautomated method (*vide supra*). Details of the calcula-

(14) L. G. Humber, *J. Med. Chem.*, **8**, 401 (1965).

(15) L. G. Humber, G. Myers, L. Hawkins, C. Schmidt, and M. Boulerice, *Can. J. Chem.*, **42**, 2852 (1964).

(16) L. G. Humber, *J. Med. Chem.*, **9**, 441 (1966).

(17) J. B. Levine and B. Zak, *Clin. Chim. Acta*, **101**, 381 (1964).

(18)(a) P. R. Moore and C. A. Baumann, *J. Biol. Chem.*, **195**, 615 (1952); (b) E. I. Mercer and J. Glover, *Biochem. J.*, **80**, 552 (1961).

tions involved have been presented previously,[8] and the results are given in Table V.

Determination of Acute Toxicities.—The LD_{50} values were determined in albino mice. The compounds were administered orally or occasionally intraperitoneally (see Tables I–IV) at three or more dose levels. Deaths at the end of 24 hr were used to estimate the LD_{50} by the method of Miller and Tainter.[19]

Effect on Hepatic Cholesterogenesis *in Vitro*.—The effects of the test compounds on the incorporation of mevalonate-2-^{14}C into cholesterol by rat liver homogenates were estimated as previously described,[20] and the results are presented in Tables I–IV.

Site of Inhibition.—Selected compounds were tested for their ability to block the enzyme 7-dehydrocholesterol Δ^7-reductase. The Δ^7-reductase activity was assayed by the procedure of the Kandutsch[21] which consists in measuring the disappearance of 7-dehydrocholesterol in the presence of liver enzymes and suitable cofactors. Details of the method have been presented previously,[22] and the results are shown in Table V.

Results and Discussion

We have recently described the events which have led from our interest in mevalonic acid analogs to the development of *trans*-1,4-bis(2-chlorobenzylaminomethyl)cyclohexane dihydrochloride.[22] In that study we had used almost exclusively as the parameter for comparison of the various compounds the inhibition of incorporation of mevalonate-2-^{14}C into cholesterol by rat liver homogenates. This technique had proved invaluable for determining the intrinsic capacity of a compound to interfere with hepatic cholesterogenesis *in vitro*, especially for compounds which had no detectable effect on serum sterol levels in normal laboratory animals. However, such compounds, *e.g.*, N,N′-dibenzylethylenediamine,[22] were shown to be active in the intact animal by studying their effects on the hypercholesteremia which accompanies the nephrotic syndrome induced by the aminonucleoside of puromycin.[23a] This technique has subsequently been developed into a general screening method for cholesterol-lowering agents which act by a variety of mechanisms.[23b] We considered it desirable, however, to study the effects of the various compounds on sterol metabolism in normal animals where gross pathologic and metabolic disorders were absent, and while this was not possible with slightly active compounds, it was realized with the potent compounds that have since become available. Thus, the prototype of the compounds to be discussed in this paper, 1,4-bis(benzylaminomethyl)cyclohexane,[22] causes a 32% lowering of sterol levels at a dose level of 10 μmoles/kg orally in the normal rat, and we decided to accept as candidates for possible further study only those compounds which were significantly active at a dose level of 75 μmoles/kg or less. Thus, in this work, we have not relied exclusively on *in vitro* enzyme inhibition, but have screened compounds initially in the intact animal using the two dose levels mentioned above.

The subsequent discussion of the relationship between structure and activity is based predominately on results obtained in the intact animal. There are included, however, a number of compounds, which have been tested only for their effects on cholesterol biosynthesis *in vitro*, and which show no activity at 10^{-6} *M* or, which show activity only at higher concentrations. Such compounds are regarded as being inactive since, of the compounds which have been studied by both the *in vivo* and *in vitro* methods (see Tables I–IV), significant reductions of serum sterol levels are obtained only when there is *in vitro* activity at a concentration of 10^{-6} *M*.

Compounds of Table I.—It has been demonstrated previously[22] that replacing the ethylenediamine group of N,N′-dibenzylethylenediamine by the 1,4-bis(aminomethyl)cyclohexane moiety resulted in a 100-fold increase in the capacity of the resulting compound to inhibit hepatic cholesterogenesis *in vitro*. This finding has prompted us to investigate the effects of further molecular modifications, and initially, we have studied the effects of introducing various substituents into the aromatic rings of 1,4-bis(benzylaminomethyl)cyclohexane, as well as the effect of the configuration of the carbon atoms bearing the substituents of the cyclohexane ring. The results are shown in Table I.

The effect of the configurations of carbons 1 and 4 of the cyclohexane ring is illustrated by examination of the three pairs of *cis* and *trans* isomers studied (**1** and **2**, **3** and **4**, and **10** and **11**). There is no significant difference in either the *in vivo* or *in vitro* results between the *cis* and *trans* isomers, although there is such a difference in the acute toxicities, the *cis* isomers being considerably more toxic than the *trans*.

The effect of configuration on duration of action has been studied in one *cis–trans* pair, **3** and **4**. This has been done by determining, *in vitro*, the capacity of livers from animals, pretreated with the test compounds, to inhibit the incorporation of mevalonate-2-^{14}C into cholesterol. It is found that the *trans* isomer (**4**) causes a 55% inhibition of hepatic cholesterogenesis 48 hr after administration of a single oral dose of 10 μmoles/kg, while the *cis* isomer (**3**) does not cause any inhibition after this period. The longer duration of action of the *trans* isomer may be attributable to a reduced rate of metabolism for this compound.

We have next examined the effect of the position of the substituents on the aromatic rings. Generally, we find that enhancement of activity with respect to the unsubstituted prototype is obtained only when single *ortho* substituents of unique character are introduced. When these substituents are, respectively, in the *ortho*, *meta*, and *para* positions, one observes a gradual decrease in activity both *in vivo* and *in vitro*. This decrease is demonstrated for *ortho*, *meta*, and *para* isomers with chlorine (**4–6**), bromine (**11–13**), fluorine (**14–16**), and methyl (**17** and **18**). Several compounds, polysubstituted in each aromatic ring, have been studied, and with one exception (**8**) these compounds are either inactive or possess a low order of activity (**7**, **9**, **19–21**, and **30–33**).

Having found that substitution in the *ortho* position enhances activity more than *meta* or *para* substitution, a series of compounds was compared which contains ten different groupings situated in the *ortho* position (**1-4**, **10**, **11**, **14**, **17**, **22–26**, and **34**). In this series, activity cannot be correlated with the physicochemical

(19) L. C. Miller and M. L. Tainter, *Proc. Soc. Exptl. Biol. Med.*, **57**, 261 (1944).

(20) L. G. Humber, M. Kraml, and J. Dubuc, *Biochem. Pharmacol.*, **11**, 755 (1962).

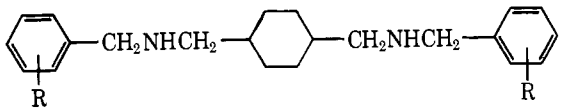
(21) A. A. Kandutsch, *J. Biol. Chem.*, **237**, 358 (1962).

(22) M. Kraml, L. G. Humber, J. Dubuc, and R. Gaudry, *J. Med. Chem.*, **7**, 500 (1964).

(23) (a) S. Frenk, I. Antonowicz, J. M. Craig, and J. Metcoff, *Proc. Soc. Exptl. Biol. Med.*, **89**, 424 (1955); (b) A. V. Marton, C. I. Chappel, K. Voith, and L. G. Humber, unpublished observations.

TABLE I

SUBSTITUTED 1,4-BIS(BENZYLAMINOMETHYL)CYCLOHEXANES[a]



			In vivo results				
			10 μmoles/kg		75 μmoles/kg		
No.	R	LD_{50} (mice), μmoles/kg[b]	% sterol decrease in serum	% adrenal wt increase	% sterol decrease in serum	% adrenal wt increase	*In vitro* results % inhib (final concn, *M*)
1	H[c]	—	24	0	63	53	—
2	H[d]	—	32	25	45	47	45 (10^{-6})
3	2-Chloro[c]	753	58	18	—	—	76 (10^{-6})
4	2-Chloro[d]	1166	62	34	74	56	83 (10^{-6})
5	3-Chloro	1556	11	0	—	—	—
6	4-Chloro	702[e]	—	—	—	—	30 (10^{-5})
7	2,6-Dichloro	2047	0	0	17	0	—
8	2,4-Dichloro	789[e]	—	—	—	—	75 (10^{-6})
9	3,4-Dichloro	233[e]	—	—	—	—	90 (10^{-4})
10	2-Bromo[c]	897	44	21	71	61	—
11	2-Bromo[d]	1720	57	23	74	91	—
12	3-Bromo	—	19	0	29	0	—
13	4-Bromo	634	0	0	16	0	—
14	2-Fluoro	1190	56	28	60	—	—
15	3-Fluoro	—	30	25	58	47	—
16	4-Fluoro	—	12	0	42	12	—
17	2-Methyl	213[e]	—	—	—	—	84 (10^{-6})
18	4-Methyl	1830	0	0	0	0	10 (10^{-5})
19	2,4,6-Trimethyl	1976	0	0	0	0	61 (10^{-5})
20	2-Chloro-5-methyl	>2840	0	0	10	0	—
21	2-Chloro-6-methyl	>1785	11	0	48	19	—
22	2-Methoxy	109[e]	—	—	—	—	58 (10^{-5})
23	2-Hydroxy[f]	793	11	0	38	0	—
24	2-Nitro	925	45	29	65	66	—
25	2-Amino[g]	—	0	0	17	10	—
26	2-Methylthio	225	14	0	71	40	—
27	4-Isopropyl	—	0	0	0	0	11 (10^{-5})
28	4-Acetamido	—	12	13	10	12	—
29	4-Dimethylamino[h]	294	—	—	—	—	76 (10^{-4})
30	2,3-Dimethoxy	1455	0	0	0	0	—
31	3,4-Dimethoxy	—	0	0	0	0	0 (10^{-5})
32	3,4,5-Trimethoxy	—	0	0	0	0	—
33	3,4-Dibenzyloxy	—	0	0	0	0	—
34	2-Trifluoromethyl[d]	900	65	82	82	71	87 (10^{-6})

[a] All compounds were administered as dihydrochloride salts except where indicated otherwise; all compounds are mixtures of *cis*- and *trans*-1,4 isomers except where indicated. [b] All acute toxicities were determined by oral administration except where indicated. [c] A *cis*-1,4 isomer. [d] A *trans*-1,4 isomer. [e] This acute toxicity was determined by intraperitoneal injection. [f] Administered as a diacetate salt. [g] Administered as a diacid maleate salt. [h] Administered as a tetrahydrochloride salt.

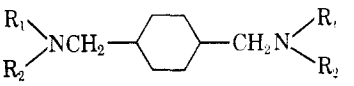
properties of the groups. For example, *ortho* substituents with widely different electronic characters yield active compounds: halogen and methylthio (+R, −I), methyl (+R, +I), nitro (−R, −I), and trifluoromethyl (−I). Similarly, *ortho* substituents of different bulk (hydrogen, fluorine, and bromine with van der Waals radii of 1.2, 1.4, and 2.0 A, respectively) and with widely separated group dipole moments (nitro, chlorine, and methyl: −3.9, −1.5, and +0.4 D., respectively) also yield active compounds.[24]

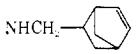
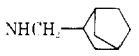
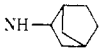
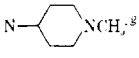
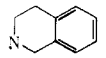
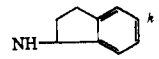
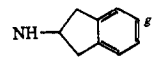
Interestingly, the disubstituted benzyl derivatives **7** (2,6-dichloro) and **21** (2-chloro-6-methyl) possess only weak activity. In these compounds, each *ortho* position bears a substituent which, when present in the similar position of a monosubstituted benzyl derivative, had resulted in highly active compounds. This suggests that when only one of the *ortho* positions is occupied, the compound is capable of assuming a conformation which is forbidden when both *ortho* positions are occupied. A conformation which suggests itself is one in which the nitrogen is essentially coplanar with the aromatic ring; coplanarity is not possible in a di-*ortho* disubstituted derivative because of the crowding between the nitrogen and one of the substituents.

In our experimental protocol, we have routinely examined organ weights after 7 days of treatment with the test compound. We find that when total serum sterol levels are depressed to 65% of normal or more, there is a concomitant increase in adrenal weight and these values are presented for the compounds in Table I. One of these compounds, *trans*-1,4-bis(2-chlorobenzylaminomethyl)cyclohexane dihydrochloride (**4**), has been studied more extensively with regard to its

(24) The p*k* values of selected compounds in Table I have been determined and no correlation is found between basicity and cholesterol-lowering activity: M. Givner, unpublished observations.

TABLE II: N,N'-DI(ARALKYL AND NONAROMATIC) DERIVATIVES OF 1,4-BIS(AMINOMETHYL)CYCLOHEXANE[a]



No.	$-N<^{R_1}_{R_2}$	1,4 configuration	LD_{50} (mice), μmoles/kg[b]	*In vivo* results: % sterol decrease in serum, 10 μmoles/kg	75 μmoles/kg
35	NH_2	*cis/trans*	>9200	0	0
36	$NHCH_3$	*cis/trans*	>5770	0	0
37	NHC_2H_5	*cis/trans*	>5170	0	0
38	$NH(CH_2)_3CH_3$	*trans*	3210	0	27
39	$NHCH_2CH(CH_3)_2$	*cis/trans*	2870	37	67
40	$NH(CH_2)_2CH(CH_3)_2$	*cis/trans*	2400	38	73
41	$NH(CH_2)_5CH_3$	*trans*	1092	0	0
42	$NHCH_2CH{=}C(CH_3)(CH_2)_2CH{=}C(CH_3)_2$[c]	*cis/trans*	2340	0	10
43	$NH(CH_2)_2CH(CH_3)(CH_2)_3CH(CH_3)_2$	*cis/trans*	2100	27	27
44	$NHCH_2CH(CH_2)_2CH{=}CHCH_2$	*cis/trans*	1615	47	66
45	$NHCH_2$-cyclohexyl[d]	*trans*	1422	65	83
46	$NHCH_2$-cyclohexyl	*cis*	501	72	61
47	$NHCH_2$-cyclobutyl	*trans*	2565	28	69
48	$NHCH_2$-cyclopentyl	*cis/trans*	1450	52	78
49		*trans*	2106	33	66
50		*trans*	2320	19	66
51	$NHCH_2C{=}CH(CH_2)_3CH_2$[e]	*trans*	1554	0	70
52	$NH(CH_2)_2$-cyclohexyl	*trans*	3200	0	0
53	$NH(CH_2)_3$-cyclohexyl	*cis/trans*	>3240	0	11
54	$NHCH_2CH(CH_2)_4O$	*trans*	2920	0	39
55	$NHC(CH_3)_3$	*trans*	1710	0	0
56	$NHC(CH_3)_2CH_2C(CH_3)_3$	*trans*	627	10	21
57	NH-cyclopentyl	*trans*	2140	13	60
58	NH-cyclohexyl[f]	*trans*	1347	75	69
59	NH-cycloheptyl	*trans*	360	73	—
60	NH-cyclooctyl[g]	*trans*	476	23	66
61		*trans*	620	65	—
62	$NHCH(CH_2)_3CH(CH_3)CH_2$[e]	*trans*	1210	76	56
63	$NHCH(CH_2)_2CH(CH_3)CH_2CH_2$	*trans*	858	69	82
64		*trans*	1930	0	0
65	$NHCH(CH_2)_4CH(OH)$[g,h]	*trans*	>3540	0	24
66	$NHCH(CH_2)_4CH(OH)$[g,i]	*trans*	>3540	0	15
67	Pyrrolidino	*trans*	>4340	0	0
68	Piperidino	*trans*	>3980	0	0
69	Hexamethylenimino	*trans*	2340	0	49
70	$N(CH_3)CH_2C_6H_5$	*trans*	1297	18	52
71	$N<^{(CH_2)_3CH_3}_{CH_2\text{-}2\text{-}ClC_6H_4}$	*trans*	>3460	18	2
72		*trans*	1208	0	51
73	$N<^{COOC_2H_5}_{CH_2\text{-}2\text{-}ClC_6H_4}$	*trans*	>3740	0	12
74		*trans*	>2680	72	79
75		*trans*	>1792	0	12
76	$NH(CH_2)_2C_6H_5$[j]	*cis/trans*	425[m]	—	—
77	$NHCH_2CH(CH_3)C_6H_5$	*cis/trans*	1650	0	16
78	$NHCH_2$-1-naphthyl[n]	*cis/trans*	2723	0	—
79	$NHCH_2$-2-naphthyl[o]	*cis/trans*	2620	0	0
80	$NHCH_2$-2-furyl	*cis/trans*	3355	0	10
81	$NHCH_2$-2-thienyl	*cis/trans*	2087	0	36
82	$NHCH_2$-2-pyridyl	*cis/trans*	>2560	0	—
83	$NHCH_2$-4-pyridyl	*cis/trans*	—	0	0

[a] All basic compounds were administered as dihydrochloride salts except where indicated to the contrary. [b] All acute toxicities were determined orally except where indicated. [c] This compound is a mixture of the 6- and 7-octenyl derivatives. [d] *In vitro* 63% at 10^{-6} *M*. [e] Administered as the diacetate salt. [f] *In vitro* 94% at 10^{-6} *M*. [g] Administered as the free base. [h] A low-melting, ether-soluble isomer. [i] A high-melting, ether-insoluble isomer. [j] Administered as the dihydrobromide salt. [k] *In vitro* 71% at 10^{-6} *M*. [l] *In vitro* 63 at 10^{-5} *M*. [m] This acute toxicity was determined intraperitoneally. [n] *In vitro* 8% at 10^{-5} *M*. [o] *In vitro* 18% at 10^{-5} *M*.

effects on adrenal function and morphology.[6,7,9,10b,25] Whether these adrenal changes are due to a decrease in serum or adrenal sterol levels,[6,25] inhibition of adrenal cholesterogenesis,[9] or inhibition of adrenal steroidogenesis[7,10b] remains unanswered.

Compounds of Table II.—The results of studies on a series of derivatives wherein the central 1,4-bis(aminomethyl)cyclohexane moiety is retained and the nitrogen substituents include nonaromatic as well as aromatic moieties other than benzyl, are presented in Table II. 1,4-Bis(aminomethyl)cyclohexane itself (**35**) is inactive, and, as the chain length of the nitrogen substituents is increased from methyl (**36**) to ethyl (**37**) to *n*-butyl (**38**), slight activity appears at the higher dose level. Increasing the chain length of the nitrogen substituents to *n*-heptyl (**41**) or dimethyloctyl (**43**) does not yield more potent compounds. Branching of the chain in the α position to the nitrogen as in the *t*-butyl (**55**) and *t*-octyl (**56**) derivatives yields inactive compounds in contrast to β or γ branching (the isobutyl and isopentyl derivatives **39** and **40**, respectively) which gives compounds of a high order of activity.

We have next examined the effects of a series of alicyclic substituents attached to each nitrogen and find that these substituents, whether attached to the nitrogens directly (**57–60**, **62**, and **63**) or through one intervening methylene group (**45–48**), yield compounds of a high order of activity which is retained when the alicyclic group is bridged (**50** and **61**) or when it contains a double bond (**44**, **49**, and **51**). A highly specific structural requirement for activity is dramatically illustrated with the cyclohexylmethyl derivative (**46**) whose high activity is completely lost by inserting one or two extra methylene groups to yield the β-cyclohexylethyl and γ-cyclohexylpropyl analogs (**52** and **53**, respectively). Similarly, the β-phenethyl derivatives (**76** and **77**) are inactive. We suggest that the specific structural requirement operative here is a necessity for an unhindered nitrogen available for binding which may not be met in the cyclohexylethyl and -propyl derivatives where, because of the extra degrees of freedom of rotation introduced, conformations with the cyclohexyl ring folded back over the nitrogen may be predominant. The relationship between acute toxicity and 1,4 configuration that had been noted earlier in Table I also holds in nonaromatic derivatives. Thus, the *cis*-cyclohexylmethyl compound (**46**) is about three times as toxic as the *trans* isomer **45**. We note also, an unexpected relationship between toxicity and ring size in the group of 1,4-*trans*-cycloalkylaminomethyl derivatives **57–60**. As the ring size increases from the comparatively rigid cyclopentyl and cyclohexyl systems to cycloheptyl and cyclooctyl, there is observed about a fourfold increase in the toxicity of the flexible seven- and eight-membered rings.

The introduction of polar groupings into the nitrogen substituents uniformly reduces activity, as demonstrated for the tetrahydropyranyl (**54**), piperidino (**64**), and the hydroxy-substituted cyclohexyl derivatives **65** and **66**. Similarly, replacing the benzene ring of **1** (Table I) by other aromatic or by heteroaromatic systems (**78–83**) almost completely abolishes activity.

(25) A. V. Marton, M. Givner, K. Voith, and C. Chappel, manuscript in preparation.

The effect of rendering the nitrogens tertiary is illustrated in the series of compounds, **67–72**, and it is seen that this structural change causes a reduction in the activity of the resultant compounds. Compound **73**, a N,N′-dicarbethoxy derivative of **4** shows virtually no activity, a finding consistent with previous observations on nonbasic compounds in this series.[22] A dramatic difference is seen between the 1-indanyl (**74**) and the 2-indanyl (**75**) derivatives; the former is one of the most potent compounds in our series (25% lowering of total sterol levels at 3 μmoles/kg orally), but the latter is virtually inactive. This high degree of specificity is probably related to the benzylamine structure of **74** in contrast to the β-phenethylamine structure of **75**.

Compounds of Table III.—We have noted in a previous publication,[22] the dramatic effect on cholesterol biosynthesis of inserting the 1,4-bis(aminomethyl)-cyclohexane moiety between two benzyl groups. We were interested in pursuing this finding further, and in Table III we describe a series of analogs of **4** wherein the diamine moiety is replaced by others of unique shape, bulk, electron density, and internitrogen distance. In this series of compounds the central diamine groups bear substituents on the nitrogens of types which have been shown to yield highly active compounds when attached to the nitrogens of 1,4-bis(aminomethyl)cyclohexane itself (*cf.* Table II). Table III also gives the internitrogen distances of the various diamines as measured on Dreiding models, the most elongated conformations being chosen for comparison with compounds where conformational changes affect the internitrogen distance. Compound **4**, containing the *trans*-1,4-bis(aminomethyl)cyclohexane moiety, has a N–N distance of 7.42 A and in the homologous alkylene series of N,N′-di(2-chlorobenzyl) derivatives (**84–93**), with N–N distances varying between 3.7 and 13.8 A, we see that activity rapidly drops on either side of the range from *ca.* 6–9 A. In an attempt to define more precisely the optimum internitrogen distance for effective enzyme inhibition, we have studied the relationship between these parameters in a series of compounds containing cyclic diamine "nuclei." Such compounds, if considerably more rigid in the internitrogen area than the acyclic derivatives, should be of more value in this study. Thus, compounds **98** and **99** containing the *trans*-1,4-bis-(2-aminoethyl)cyclohexane moieties (N–N = 9.65 A) are virtually inactive, and compounds with somewhat shorter internitrogen distances retain activity, *e.g.*, the spiro[3.3]heptane derivative **112** (N–N = 8.43 A) and the tricyclodecane derivative **116** (N–N = 8.80 A). At the other end of the range are found highly active compounds with the 1,4-diaminobicyclo[2.2.2]octane nucleus (**117**, N–N = 5.59 A) and the *trans*-1,4-diaminocyclohexane derivatives **101–103** and **105** having internitrogen distances of 5.59 A. Compound **104**, however, the *cis* isomer of **105**, having an N–N distance of only 4.17 A, is virtually inactive.[26] The loss of activity in this compound is not due to the nature of the 1,4 configuration, since, as noted previously, the *cis* and *trans* isomers **3** and **4** are equipotent, the N–N

(26) Compounds **104** and **105** are, unambiguously, *cis* and *trans* isomers, respectively. Compounds **101–103** were synthesized by methods where mixtures of isomers would be expected.[14] In view of their high activity, however, they are undoubtedly the *trans* isomers.

TABLE III
N,N′-DISUBSTITUTED DIAMINES[a]

$$R_1R_2{>}NAN{<}^{R_1}_{R_2}$$

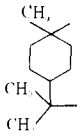
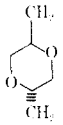
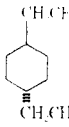
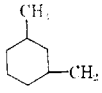
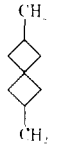
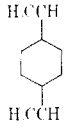
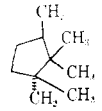
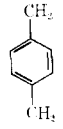
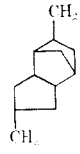
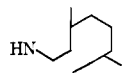
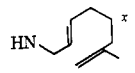
No.	A	$-N<^{R_1}_{R_2}$	N–N′ distance, A	LD_{50} (mice), μmoles/kg	*In vivo* results—% sterol decrease in serum: 10 μmoles/kg	75 μmoles/kg
84	$(CH_2)_2$	$NHCH_2$-2-ClC_6H_4	3.74	1695	0	0
85	$(CH_2)_3$		4.84	1634	0	0
86	$(CH_2)_4$		6.01	911	12	46
87	$(CH_2)_5$		7.11	779	46	78
88	$(CH_2)_6$		8.20	433	23	70
89	$(CH_2)_7$		9.25	1160	0	32
90	$(CH_2)_8$		10.56	1125	0	0
91	$(CH_2)_9$		11.60	2275	0	0
92	$(CH_2)_{10}$		12.78	2625	0	0
93	$(CH_2)_{11}$		13.80	4075	0	0
94	$CH(CH_3)(CH_2)_4CH(CH_3)_2$		8.20	750	15	46
95		$NHCH_2C_6H_5$[c]	*d*	>2422	10	23
96		$NHCH_2$-2-ClC_6H_4	*d*	>2790	13	22
97		$NHCH_2$-2-ClC_6H_4	7.42	—	0	27
98		NH-cyclohexyl[e]	9.65	—	10	25
99		$NHCH_2$-2-ClC_6H_4	9.65	>2840	0	0
100		NH-cyclohexyl[e,g]	—	2250	0	0
101		$NHCH_2$-cyclohexyl[e,f]	—	585	58	52
102		$NH(CH_2)_2$-cyclohexyl[e,f]	—	770	25	76
103		$NH(CH_2)_2$-2-ClC_6H_4[e,f]	—	487	42	79
104		$NHCH_2$-2-ClC_6H_4[h]	4.17 (*cis*)	1260	0	27
105		$NHCH_2$-2-ClC_6H_4[e,i,j]	5.59 (*trans*)	930	52	76
106		NH-cyclohexyl[e,k]	6.60	1180	81	53
107		$NHCH_2$-cyclohexyl[e,]	6.60	1215	73	76
108		$NH(CH_2)_2$-cyclohexyl[e]	6.60	1150	0	16
109		$NHCH_2$-2-ClC_6H_4[m]	6.60	1250	72	82
110		NH-(*dl*-1-indanyl)[n]	6.60	2016	59	73
111		$NHCH_2$-2-ClC_6H_4[e]	*o*	880	28	64
112		$NHCH_2$-2-ClC_6H_4	8.43	1240	14	45
113		$NHCH_2$-2-ClC_6H_4[f]	7.42	1420	12	48
114		$NHCH_2$-2-ClC_6H_4[p]	6.7	711	67	—
115		$NHCH_2C_6H_5$	7.4	—	0	0
116		NH-cyclohexyl	8.80	882	69	85

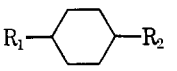
TABLE III (*Continued*)

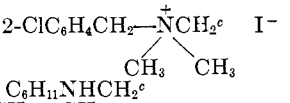
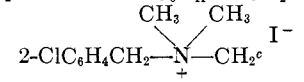
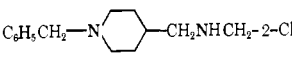
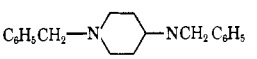
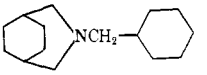
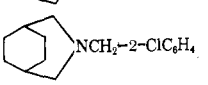
No.	A	$-N<^{R_1}_{R_2}$	N–N[b] distance, A	LD_{50} (mice), μmoles/kg	*In vivo* results: % sterol decrease in serum, 10 μmoles/kg	75 μmoles/kg
117		$NHCH_2$-2-ClC_6H_4[q]	5.59	1510	70	80
118		$NHCH_2$-2-ClC_6H_4[r]	6.75	1726	75	72
119		NH-cyclohexyl[s]	6.75	860	68	68
120		$NHCH_2$-2-$CF_3C_6H_4$[t]	6.75	2322	67	70
121		$NHCH_2$-cyclohexyl[u]	6.75	1500	79	64
122		NH-(*dl*-1-indanyl)[v]	6.75	1590	66	78
123		$C_9H_{10}N$[w,x]	6.75	156	34	—
124	$H_3CCH(CH_2)_4HCCH_3$		8.20	1800	0	—
125			8.20	—	10	11

[a] All compounds were administered as dihydrochloride salts except where indicated. [b] Measured on Dreiding models. [c] Administered as a diacid maleate salt. [d] A mixture of *cis* and *trans* isomers. The N–N distance (*cis*) is 5.73 A and N–N distance (*trans*) is 6.53 A. [e] Administered as a diacetate salt. [f] A mixture of *cis* and *trans* isomers. [g] *In vitro* 100% at 10^{-3} *M*. [h] A pure *cis* isomer; *in vitro* 0% at 10^{-6} *M*. [i] A pure *trans* isomer. [j] *In vitro* 97% at 10^{-6} *M*. [k] *In vitro* 84% at 10^{-6} *M*. [l] *In vitro* 60% at 10^{-6} *M*. [m] *In vitro* 96% at 10^{-6} *M*. [n] *In vitro* 70% at 10^{-6} *M*. [o] A mixture of *cis* and *trans* isomers. The N–N distance (*cis*) is 6.4 A and N–N distance (*trans*) is 7.1 A. [p] *In vitro* 87% at 10^{-6} *M*. [q] *In vitro* 89% at 10^{-6} *M*. [r] *In vitro* 93% at 10^{-6} *M*. [s] *In vitro* 100% at 10^{-6} *M*. [t] *In vitro* 100% at 10^{-6} *M*. [u] *In vitro* 93% at 10^{-6} *M*. [v] *In vitro* 84% at 10^{-6} *M*. [w] $C_9H_{10}N$ ≡ 1,2,3,4-tetrahydroisoquinolino. [x] Administered as the free base.

TABLE IV

MISCELLANEOUS COMPOUNDS[a]



No.	R_1	R_2	LD_{50} (mice), μmoles/kg	*In vivo* results: % sterol decrease in serum, 10 μmoles/kg	75 μmoles/kg
126	$C_6H_5CH_2NHCH_2$	$C_6H_5CH_2OCH_2$[b]	1530	0	14
127	$C_6H_5CH_2NHCH_2$	$C_6H_5CH_2SCH_2$[b]	602	28	0
128	$C_6H_5CH_2NHCH_2$	$C_6H_5CH_2SO_2CH_2$[b]	—	0	0
129	$C_6H_5CH_2NHCH_2$	CH_2OH[b]	—	0	0
130	2-$ClC_6H_4CH_2NHCH_2$	$C_6H_5CH_2SCH_2$[b]	1870	0	0
131	2-$ClC_6H_4CH_2NHCH_2$	$C_6H_{11}CH_2NHCH_2$[c]	801	67	—
132	2-$ClC_6H_4CH_2NHCH_2$	$C_6H_{11}NHCH_2$[c]	444	72	—
133	2-$ClC_6H_4CH_2NHCH_2$	CH_2Cl[b]	—	0	0
134	2-$ClC_6H_4CH_2NHCH_2$	CH_2NH_2[c]	1400	0	16
135	2-$ClC_6H_4CH_2NHCH_2$	H	3280	0	0
136	2-$ClC_6H_4CH_2NHCH_2$		1660	0	0
137	$C_6H_{11}CH_2NHCH_2$	$C_6H_{11}NHCH_2$[c]	1140	68	77
138			—	0	0
139		2-$ClC_6H_4CH_2NHCO$[c]	3340	0	0
140		$C_6H_5CONHCH_2$[b]	—	0	—
141		2-$ClC_6H_4CH{=}NCH_2$[c]	5800	0	0
142			1518	28	32
143			1160	0	0
144			4460	0	0
145			4780	0	0

[a] All basic compounds were administered as dihydrochloride salts except where indicated. All acute toxicities were determined orally. [b] A mixture of *cis*- and *trans*-1,4 isomers. [c] A *trans*-1,4 isomer.

distances of both isomers (7.05 and 7.42 A for *cis* and *trans*, respectively) being within the range where activity is predicted. The inability of the *cis*-**1**,4-diaminocyclohexane isomer (**105**) to lower serum sterol levels *in vivo* or to inhibit hepatic cholesterogenesis *in vitro* is probably due to its internitrogen distance being less than that minimum which is compatible with effective enzyme inhibition.

TABLE V

In Vitro AND *in Vivo* DATA ON SITE OF INHIBITION OF SELECTED COMPOUNDS[a]

No.	Structure	*In vitro* results: % inhib of incorp of 2-^{14}C-MVA → Chol (100 γ M)	*In vitro* results: % inhib of 7-dehydrocholesterol Δ^7-reductase (10 γ M)	*In vivo* results[b]: Serum sterol components mg % 7-Dehydrocholesterol[c]	Cholesterol[c]
4	2-ClC$_6$H$_4$CH$_2$NHCH$_2$-C$_6$H$_4$-CH$_2$NHCH$_2$-2-ClC$_6$H$_4$	90	98	14	17
104	2-ClC$_6$H$_4$CH$_2$NH-C$_6$H$_4$-NHCH$_2$-2-ClC$_6$H$_4$	—	—	9[d]	61[d]
105	2-ClC$_6$H$_4$CH$_2$NH-C$_6$H$_4$-NHCH$_2$-2-ClC$_6$H$_4$	97	100	7	22
132	C$_6$H$_{11}$NHCH$_2$-C$_6$H$_4$-CH$_2$NHCH$_2$-2-ClC$_6$H$_4$	—	—	6	13
109	2-ClC$_6$H$_4$CH$_2$NHCH$_2$-C$_6$H$_4$-CH$_2$NHCH$_2$-2-ClC$_6$H$_4$	97	100	7	22
117	2-ClC$_6$H$_4$CH$_2$NH-C$_6$H$_4$-NHCH$_2$-2-ClC$_6$H$_4$	89	—	15	13
118	2-ClC$_6$H$_4$CH$_2$NHCH$_2$-C$_6$H$_4$-CH$_2$NHCH$_2$-2-ClC$_6$H$_4$	93	94	8	20
119	C$_6$H$_{11}$NHCH$_2$-C$_6$H$_4$-CH$_2$NHC$_6$H$_{11}$	—	—	15	25
120	2-CF$_3$C$_6$H$_4$CH$_2$NHCH$_2$-C$_6$H$_4$-CH$_2$NHCH$_2$-2-CF$_3$C$_6$H$_4$	—	—	21	27
123	RCH$_2$-C$_6$H$_4$-CH$_2$R[e]	—	—	14	57

[a] All compounds were used as dihydrochloride salts except **105** and **123** which were used as the diacetate and the free base, respectively. [b] The dose was 10 μmoles/kg per day for 7 days. [c] In the control groups no 7-dehydrocholesterol is detectable and the cholesterol level is 80–100 mg %. [d] This compound is inactive at the 10-μmole dose level; these results were obtained using 75 μmoles/kg. [e] R = tetrahydroisoquinolino.

We have also studied compounds with intermediate N–N distances (see Table III). Worthy of mention are the 1,4-bis(aminomethyl)bicyclo[2.2.2]octane derivatives, **118–123** (N–N = 6.75 A), all of which are highly active. Compounds with this rigid nucleus are incapable of assuming conformations wherein the two nitrogens are separated by less than 6.3 A. We have suggested previously[22] that the two nitrogens of the inhibitor interact with complementary sites on an enzyme surface *which are separated by a unique distance*. If this hypothesis were valid, the apparently anomalous situation would arise whereby, on the one hand, compounds having a *maximum* internitrogen distance of 5.59 A (**117** and **105**) and, on the other, compounds with a *minimum* internitrogen distance of 6.3 A (**118–123**) are both of high activity. These two groups of compounds lower sterol levels by the same mechanism, the inhibition of 7-dehydrocholesterol Δ^7-reductase. Thus, all of the capacity of **105** and **118** to inhibit the incorporation of mevalonate-2-^{14}C into cholesterol is accounted for by their action on the enzyme converting 7-dehydrocholesterol to cholesterol (see Table V). In the serum of rats treated with these compounds, cholesterol levels are reduced and considerable amounts of 7-dehydrocholesterol are found. 7-Dehydrocholesterol normally cannot be detected in rat serum,[8] and thus, we interpret the presence of this component in the serum of animals treated with various compounds (see Table V) to indicate that they indeed act by a common mechanism, the inhibition of 7-dehydrocholesterol Δ^7-reductase. We are thus forced to abandon our previous hypothesis (*vide supra*) in favor of one which pictures the enzyme as bearing on its surface a pair of anionic sites which are involved with binding the inhibitor, where the distance between these sites is *not* invariable. This situation would arise if the active sites are situated on the surface of a polypeptide chain which is capable of undergoing changes in its secondary or tertiary structure, within a narrow range, in order to accommodate the interactions between enzyme and inhibitor.[27] A variety of other "nuclei" have been studied and the results are given in Table III. Notably, substitution of the *trans*-1,4-bis(aminomethyl)-2,5-dioxane moiety (**97**) for the *trans*-1,4-bis(aminomethyl)cyclohexane group (**4**, Table I) leads to almost complete loss of activity, and introduction of the electron-rich benzene ring (**115**) in place of the saturated cyclohexyl ring has a similar effect. The cyclohexene derivatives (**106–110**), however, are among the most potent compounds that we have studied. Thus, there are factors other than internitrogen distance which determine activity and we have suggested previously[22] that van der Waals attractive forces between enzyme and inhibitor might play a major role.

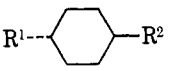
Compounds of Table IV.—In Table IV are shown the results obtained from a study of the effects of a series of miscellaneous structural changes. Replacement of one of the nitrogens of 1,4-bis(benzylaminomethyl)-cyclohexane (**1**, Table I) by oxygen, sulfur, or the sulfone group (**126**, **127**, and **128**, respectively) destroys the activity of the resulting compounds; a similar result is obtained when a benzyl- or a 2-chlorobenzyl-aminomethyl group is replaced by hydrogen (**135**), hydroxymethyl (**129**), chloromethyl (**133**), or aminomethyl (**134**) groups. Mono- and bisquaternary derivatives (**136** and **138**), nonbasic derivatives (**139**-

(27) For a discussion of these types of interactions see J. L. Webb, "Enzyme and Metabolic Inhibitors," Academic Press Inc., New York, N. Y., 1963, Chapter 6, and references therein.

141), and the tertiary amines (**142**–**145**) are all characterized by a lack of high activity. Table IV contains three compounds of exceptionally high activity (**131**, **132**, and **137**). These unsymmetrical 1,4-*trans*-disubstituted cyclohexane derivatives were prepared utilizing substituents which, when present in both the **1** and the 4 positions of the cyclohexane ring, had given rise to highly active compounds (**4**, **45**, and **58**). While the degree of sterol lowering produced at the **10**-μmole/kg dose level is virtually the maximum obtainable, so that the potencies of these compounds cannot be compared with those of their symmetrical parents, we nevertheless note a marked change in their toxicities. Thus, **131** and **132**, each of which possesses one aromatic and one nonaromatic side chain, are considerably more toxic than one would expect from the toxicities of their symmetrical parents (see Table VI), so toxic

TABLE VI

TOXICITIES OF SOME SYMMETRICAL AND NONSYMMETRICAL 1,4-*trans*-DISUBSTITUTED CYCLOHEXANE DERIVATIVES



No.	R^1	R^2	LD_{50}, μmoles/kg (oral)
4	$2\text{-}ClC_6H_4CH_2NHCH_2$		1166
45	$C_6H_{11}CH_2NH$		1422
58	$C_6H_{11}NHCH_2$		1347
131	$2\text{-}ClC_6H_4CH_2NHCH_2$	$C_6H_{11}CH_2NHCH_2$	801
132	$2\text{-}ClC_6H_4CH_2NHCH_2$	$C_6H_{11}NHCH_2$	444

in fact, that at the 75-μmole/kg dose level, none of the animals survived 7 days of treatment. This increased toxicity might reflect a greater affinity of these compounds for the enzyme surface, or, a reduced rate of metabolic detoxification.

General Discussion.—From the compounds that have been studied we have obtained a number of highly active cholesterol-lowering agents, some of which are being studied more extensively. We have been able to define many of the structural requirements for effective inhibition of cholesterol biosynthesis in this series of compounds. A previous study of a number of compounds of fairly homogeneous structural type[22] had suggested what such requirements might be, and this study allows us to define them more precisely in terms of the following features: two basic secondary nitrogens separated by a distance of from 5.59 to 6.30 A, the internitrogen area being occupied by a rigid, nonaromatic system; and the nitrogens having substituents of benzylic or cycloaliphatic character, cycloaliphatic substituents being subject to specific limitations with respect to bulk, flexibility, and linear dimensions and benzylic substituents being subject to specific limitation with respect to position and type of aromatic substitution.

This series of cholesterol-synthesis inhibitors bears no structural relationship to the enzyme substrate, 7-dehydrocholesterol. This suggests that these compounds do not compete with 7-dehydrocholesterol for a specific binding site, as is likely the case in the inhibition of 7-dehydrocholesterol Δ^7-reductase by 7-azacoprostanol,[28] or in the inhibition of 24-dehydrocholesterol Δ^{24}-reductase by 22,25-diazacholestanol.[29,30] One of the compounds reported herein, *trans*-**1**,4-bis(2-chlorobenzylaminomethyl)cyclohexane has been shown by Givner, *et al.*,[7] to be a potent inhibitor of adrenal steroid **11**β-hydroxylase, a key enzyme in adrenal steroidogenesis which is known to be reduced triphosphopyridine nucleotide (TPNH) dependent.[31] The enzyme 7-dehydrocholesterol Δ^7-reductase also requires TPNH as coenzyme[21,32] and it is possible that the active compounds described in this paper, as well as some other recently reported inhibitors of the enzyme,[33] interfere with the proper utilization of pyridine nucleotide coenzymes in sterol biosynthetic processes. The compounds, however, would not be direct competitors of these coenzymes since it has been shown that *trans*-**1**,4-bis(2-chlorobenzylaminomethyl)cyclohexane dihydrochloride (**4**) has no inhibitory effect on either the lactic or glucose 6-phosphate dehydrogenases.[34]

Whatever the ultimate mechanism of inhibition may be, it is clear from the diversity of structural types which result in the accumulation of 7-dehydrocholesterol, that either the enzyme itself exerts a very low degree of specificity or that there are several independent sites at which inhibition will result in interference with 7-dehydrocholesterol reduction. Further work will be necessary to elucidate the mechanism of action of these compounds.

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(28) D. Dvornik and M. Kraml, unpublished observations.

(29) D. Dvornik and M. Kraml, *Proc. Soc. Exptl. Biol. Med.*, **112**, 1012 (1963).

(30) While this manuscript was in preparation, R. Niemiro and R. Fumagilli [*Biochim. Biophys. Acta*, **98**, 624 (1965)] have shown that *trans*-1,4-bis(2-chlorobenzylaminomethyl)cyclohexane dihydrochloride and Triparanol are noncompetitive inhibitors of the Δ^7- and Δ^{24}-reductases, respectively, and that 20,25-diazacholesterol, which is an inhibitor of both the Δ^7- and Δ^{24}-reductases, is a competitive inhibitor with respect to the Δ^7-reductase.

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