Influence of 4,4′-(Isopropylidenedithio)bis(2,6-di-*t*-butylphenol) (DH-581) on Experimental Atherosclerosis in Rabbits[1] (35461)

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Barnhart *et al.* (1, 2) have reported that 4,4′-(isopropylidenedithio)bis(2,6-di-*t*-butylphenol) (DH-581) (Fig. 1) exerts a hypocholesteremic effect in monkeys, rats, and mice. This compound has also been shown to reduce serum lipids in man (3). We have found that DH-581, when fed to rats at a level of 0.3%, causes significant reductions in serum cholesterol and triglyceride levels, but also leads to an increase in liver size (4). Experiments in which we studied the influence of two dosage levels of DH-581 on cholesterol-induced atherosclerosis in rabbits are the subject of this report.

Methods. Male, Dutch-belted rabbits (1800–2100 g) were used throughout. The rabbits were fed a diet of rabbit chow augmented with 2% cholesterol suspended in 6% corn oil. The diets of the test animals were further augmented with 0.3 or 1.0% DH-581.

At the end of the feeding period rabbits were bled, weighed, and killed. The livers were weighed and samples were taken for cholesterol determination. Aortas were graded for atheromata on a 0–4 scale (5) which has been shown to correlate with aortic cholesterol content (6). The aortic arch and thoracic aorta were graded separately. Serum and liver cholesterol levels were determined by the method of Mann (7); the liver cholesterol determinations were carried out on 1-g portions of liver that had been saponified in 15% alc. KOH. Triglycerides were assayed by the Van Handel and Zilversmit procedure (8). Phospholipids were determined by the method of Fiske and Subbarow (9).

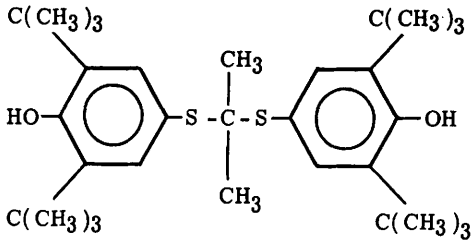

FIG. 1. 4,4′-(Isopropylidenedithio)bis(2,6-di-*t*-butylphenol).

Aortas were freed of adventitia, pooled by group and extracted with chloroform–methanol, 2:1, for 5 hr to extract all the aortic lipid (10). Free and total cholesterol of the aortas was determined by the Sperry–Webb procedure (11).

Gas–liquid chromatography of fatty acid methyl esters was carried out with a column containing 15% ethylene glycol succinate on 100–120 mesh gas chrom P. An Argon ionization detector was used. The column was standardized for retention times and quantitated at 160° and 20 psi of argon gas, using purified standards.

We are indebted to Dr. M. P. Dacquisto of the Dow Chemical Co., Zionsville, Indiana for generously supplying the DH-581 (Probucol; Biphenabid) used in these experiments.

Results. In the first three experiments (Table I) we fed DH-581 at a 0.3% level to rabbits who were simultaneously being fed rabbit chow plus 2% cholesterol suspended in 6% corn oil. In the first experiment we maintained a large group of rabbits on the control (cholesterol-corn oil) diet and autopsied a group that was comparable to the DH-581-fed group in average serum cholesterol

[1] Supported by Grant (HE-03299) and Research Career Award (4-K6-HE-734) from the National Heart and Lung Institute and a grant-in-aid from the Human Health Research and Development Labs, Dow Chemical Co., Zionsville, Indiana.

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TABLE I. Influence of DH-581 (0.3%) on Experimental Atherosclerosis in Rabbits Fed 2% Cholesterol and 6% Corn Oil.

Expt.:	1		2		3	
	DH-581	Control	DH-581	Control	DH-581	Control
No.[d]	9/10	9/10	10/10	10/10	7/11	8/11
Wt gain (g)	239	84	239	212	187	240
Liver wt (g)	124.9	106.3	135.9	129.8	143.0	138.6
Serum lipids (mg/100 ml)						
Cholesterol	2606 ± 600[e]	2608 ± 560	2062 ± 200	2258 ± 436	1943 ± 170	1441 ± 177
Triglyceride	271 ± 33	198 ± 31	361 ± 48	246 ± 44	348 ± 58[a]	167 ± 23
Phospholipid	107 ± 14	109 ± 12	71 ± 8	75 ± 9	164 ± 11	124 ± 16
Liver lipids (g/100 g)						
Cholesterol	1.24 ± 0.09	1.14 ± 0.20	1.88 ± 0.08	1.71 ± 0.09	3.31 ± 0.25[b]	4.33 ± 0.36
Triglyceride	1.05 ± 0.14	1.11 ± 0.08	0.80 ± 0.10	1.07 ± 0.09	1.56 ± 0.05[c]	2.34 ± 0.03
Phospholipid	0.33 ± 0.10	0.29 ± 0.13	0.30 ± 0.12	0.28 ± 0.10	0.27 ± 0.10	0.27 ± 0.10
Atheromata						
Arch	1.2	1.3	1.6	2.2	1.7	1.3
Thoracic	0.9	0.9	1.0	1.5	1.1	1.0

[a] $p < .02$; [b] $p < .05$; [c] $p < .01$.
[d] Survival ratio.
[e] Standard error.

level and starting weight. The results indicate that there were few differences between the groups, except for a slight elevation in serum triglyceride levels in the DH-581 group. In the next two experiments the severity of the observed atheromata varied directly with serum cholesterol levels. In Expt. 3, the DH-581 group had higher serum cholesterol, triglyceride, and phospholipid levels and more severe atheromata; nevertheless, this group exhibited significantly lower liver cholesterol and triglyceride levels. The averages over all three experiments of serum cholesterol and triglyceride levels were 2218 and 322 mg/100 ml, respectively, for the DH-581-fed group, as compared to 2133 and 207 mg/100 ml for the controls. Liver cholesterol levels were 2.04 and 2.30 g/100 g for the DH-581 and control groups, respectively. The distribution of atheromata for the three experiments are shown in Table II. The average atheroma grade of the DH-581-fed rabbits was about 14% lower than that of the controls.

We repeated the feeding experiments using a higher level (1%) of DH-581 in the atherogenic diet. The results of these experiments (Expts. 4–6) are shown in Table III. At the 1% level, DH-581 was consistently hypocholesteremic. Liver cholesterol and triglyceride levels were also lower than the controls in all three experiments, but serum triglyceride levels were variable. Atheromata were consistently less severe in the rabbits fed 1% DH-581. The average serum cholesterol and triglyceride levels were 1283 and 224 mg/100 ml for the test rabbits, compared to 1889 and 190 mg/100 ml in the controls. The liver cholesterol levels were 4.01 g/100 g in the DH-581 rabbits and 5.56 g/100 g in rabbits fed cholesterol and corn oil without drug supplement.

A summary of the distribution of atheromata is given in Table IV. Rabbits fed 1% DH-581 exhibited significantly less severe atheromata in the aortic arch ($p < .05$), as well as in the thoracic aorta ($p < .01$).

The pooled aortas of the rabbits in Expts. 4–6 were extracted with chloroform–methanol, 2:1, and a sample of the dried chloroform

TABLE II. Distribution of Atheromata in Rabbits Fed 2% Cholesterol and 6% Corn Oil With and Without 0.3% DH-581 (summary of 3 expts.).

Grade	DH-581 (26/31)[a]		Control (27/31)	
	Arch	Thoracic	Arch	Thoracic
4.0	—	—	—	—
3.5	1	—	1	—
3.0	—	—	2	1
2.5	3	1	3	2
2.0	6	1	6	2
1.5	3	3	4	5
1.0	9	13	6	7
0.5	4	8	5	10
0.0	—	—	—	—
	1.49 ± 0.15[b]	1.00 ± 0.09	1.61 ± 0.17	1.17 ± 0.14

[a] Survival ratio.
[b] Standard error.

extract was subjected to thin-layer chromatography on Silica gel G to separate the cholesteryl esters. The developing solvent was petroleum ether–ethyl ether–glacial acetic acid, 75:24:1. The cholesteryl esters were subjected to transesterification using BF_3-methanol and the fatty acid methyl esters were separated by gas-liquid chromatography. The results, including data obtained from aortas of normal rabbits, are shown in Table V.

Discussion. When administered as 0.3% of the diet to cholesterol-fed rabbits, DH-581 has no effect upon serum cholesterol levels and is somewhat hypertriglyceridemic. Over-

TABLE III. Influence of DH-581 (1%) on Experimental Atherosclerosis in Rabbits Fed 2% Cholesterol and 6% Corn Oil.

Expt.:	4		5		6	
	DH-581	Control	DH-581	Control	DH-581	Control
No.[e]	10/10	10/10	10/10	10/10	8/10	9/10
Wt gain (g)	69	254	356	110	278	337
Liver wt (g)	141.3	141.4	154.2	125.0	131.9	130.3
Serum lipids (mg/100 ml)						
Cholesterol	1137 ± 161[f][a]	1889 ± 112	1690 ± 190	2069 ± 223	957 ± 99[c]	1689 ± 250
Triglyceride	99 ± 13	94 ± 16	390 ± 56	248 ± 49	174 ± 18[d]	232 ± 17
Phospholipid	—	—	154 ± 5	147 ± 8	147 ± 7	145 ± 5
Liver lipids (g/100 g)						
Cholesterol	1.94 ± 0.32[b]	4.57 ± 0.38	5.54 ± 0.38	6.65 ± 0.64	4.67 ± 0.40	5.44 ± 0.54
Triglyceride	0.45 ± .04[a]	0.75 ± .08	0.88 ± .05	1.03 ± 0.24	0.60 ± .07	0.80 ± 0.14
Phospholipid	—	—	0.33 ± .03	0.34 ± .02	0.32 ± .01	0.29 ± .02
Atheromata						
Arch	1.2	1.8	2.0	2.4	1.5	2.0
Thoracic	0.7	1.4	1.1	1.6	1.0	1.2

[a] $p < .01$; [b] $p < .001$; [c] $p < .02$; [d] $p < .05$.
[e] Survival ratio.
[f] Standard error.

TABLE IV. Distribution of Atheromata in Rabbits Fed 2% Cholesterol and 6% Corn Oil With and Without 1.0% DH-581 (summary of 3 expts.).

	DH-581 (28/30)[c]		Control (29/30)	
Grade	Arch	Thoracic	Arch	Thoracic
4.0	—	—	2	—
3.5	2	—	2	—
3.0	—	—	3	—
2.5	4	1	3	3
2.0	4	1	7	4
1.5	7	4	6	8
1.0	6	9	4	11
0.5	5	13	2	3
0.0	—	—	—	—
	1.57 ± 0.16[d]	0.93 ± 0.10	2.05 ± 0.18[a]	1.38 ± 0.11[b]

[a] $p < .05$; [b] $p < .01$.
[c] Survival ratio.
[d] Standard error.

all, DH-581 at this dosage effects an 11% reduction in liver cholesterol levels. When fed at the 0.3% level to rats, this compound did not significantly affect liver cholesterol or triglyceride levels (4). It did, however, affect liver size in rats (4), which alerted us to the possibility that a hepatomegalic effect might also be observed in cholesterol-fed rabbits. Such was not the case: in the three experiments in which DH-581 was fed at the 0.3% level, liver size (liver wt/100 g of body wt) of the treated rabbits was only slightly higher than that of the controls. The exact values in the three experiments were 5.31 vs 4.97, 6.15 vs 6.08, and 6.21 vs 6.18 for DH-581-fed and control rabbits, respectively. When DH-581 was administered as 1% of the diet, the liver (% body wt) values were 5.47 vs 5.11, 6.62 vs 5.99, and 6.15 vs 5.84 for the DH-581 and control groups for each of the three experiments. The average difference in these values was about 4% in the first series of experiments and 8% in the second. When rats were fed 0.3% DH-581 for 21 days, their liver size (% body wt) increased by 26% (4); when the drug was fed at the 0.25% level for 14 days, the increase was about 13% (2).

When rabbits were fed 1% DH-581 with the atherogenic diet, there were significant reductions in serum and liver cholesterol levels and the difference between test and control serum triglyceride levels was smaller. At this level the drug significantly reduced atheromata. The magnitude of this effect on cholesterol-induced lesions in rabbits compares favorably with results obtained using other hypocholesteremic agents (6, 12, 13).

The free/ester cholesterol ratio (FC/EC) in the aortas was drastically reduced after 8

TABLE V. Aortic Cholesteryl Ester Fatty Acids (% composition).

	Expt.: 4		5		6		Normal
Fatty acid	DH-581	CO	DH-581	CO	DH-581	CO	aorta
14:0	—	—	2.0	3.4	3.0	4.2	6.6
16:0	20.4	23.9	16.6	17.4	17.3	22.4	27.2
16:1	11.8	7.5	9.3	11.6	8.0	10.1	15.2
18:0	9.8	10.2	6.7	7.1	8.1	11.7	14.1
18:1	32.2	37.3	34.4	37.1	34.4	33.7	22.9
18:2	21.7	18.9	28.0	18.6	25.0	16.7	14.1
18:3	4.0	2.3	3.0	4.8	3.8	1.5	—

weeks of the atherogenic regimen. The FC/EC ratio in normal aortas was 20. In the three experiments in which 1% DH-581 was added to the diet, the FC/EC ratios for test and control rabbit aortas were: 1.82 and 1.11; 0.50 and 0.71; and 2.13 and 1.73. Other investigators had reported changes in FC/EC ratio from a normal level of about 10 to 0.40–0.60 in aortas of rabbits fed cholesterol (14–16) and we have shown a drop in ratio from 20 to 2 in rabbits fed a cholesterol-free, semisynthetic atherogenic regimen (17). In most cases, the group of aortas with more severe average atherosclerosis show a lower FC/EC ratio, but this is not uniformly true, as seen from our Expt. 5 and from the experiments of Swell *et al.* (15).

The ratio of cholesteryl oleate to linoleate in the human atherosclerotic plaque is much higher than that observed in the serum (18). Normal human aortas also show an increasing level of cholesteryl oleate with aging (19). Table V shows that in each experiment the oleate/linoleate ratio was higher in the group showing the more severe atherosclerosis. Even in the normal rabbit aortas this ratio is quite high, but despite the linoleate-rich diet (corn oil) the average increase of cholesteryl oleate relative to cholesteryl linoleate was higher in the CO groups than it was in the DH-581 groups. St. Clair (20) has demonstrated increased oleic acid synthesis in atherosclerotic pigeon aortas; thus the higher oleate/linoleate ratio may be related to stimulation of fatty acid synthesis due to increased cholesterol deposition and to severity of atherosclerosis.

The mechanism of action of DH-581 cannot be attributed to an anorectic effect, since rabbits fed 0.3% of the drug gained 30–40% more weight than did the controls, whereas the average weight gain of rabbits fed 1% DH-581 was equivalent to that of the controls. Barnhart *et al.* (2) considered the possibility that DH-581 inhibits cholesterol synthesis. Colmore *et al.* (21) have confirmed the hypocholesteremic action of DH-581 in man and could detect neither desmosterol nor 7-dehydrocholesterol in the sera of treated patients. Thus, if DH-581 inhibits cholesterol synthesis it must be at one of the early steps. We have found that suitably fortified liver mitochondrial preparations from rats (6/group) fed 0.3% DH-581 for 3 weeks oxidized less cholesterol-26-^{14}C to $^{14}CO_2$ (7.6%) than did control preparations (11.3%). These differences were significant ($p<.01$). Thus the hypocholesteremic effect is not due to increased cholesterol degradation.

No data relative to the influence of DH-581 on cholesterol absorption are currently available.

Summary. The influence of orally administered 4,4′-(isopropylidenedithio)bis(2,6-di-*t*-butylphenol) (DH-581) on the development of cholesterol-induced atherosclerosis in rabbits has been investigated. When fed at a level of 0.3% to rabbits who are also being given a diet augmented with 2% cholesterol and 6% corn oil, DH-581 does not affect the course of the cholesterol-induced atherosclerosis. At this dietary level, however, DH-581 did cause reductions in liver triglycerides. When administered at a level of 1% of the atherogenic diet, DH-581 significantly lowers the severity of atheromata in the arch ($p<.05$) and thoracic aorta ($p<.01$) of rabbits.

These rabbits also showed consistently lower serum cholesterol and liver cholesterol and triglyceride levels. Serum triglyceride levels were variable.

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Received Nov. 17, 1970. P.S.E.B.M., 1971, Vol. 136.