logical sections, and Mrs. Bernie Powell and Miss Judy Chesner for technical assistance.

1. Bernhard, W., Tournier, P., Ann. de l'Inst. Pasteur, 1964, v107, 447.
2. Millonig, G., Proc. 5th Internat. Congress for Electron Microscopy, Academic Press, 1962, v2, 8.
3. Reynolds, E. S., J. Cell. Biol., 1963, v17, 208.
4. Fortner, J. G., Cancer Res., 1961, v21, 1491.
5. Weinberger, M. A., Banfield, W. G., J. Nat. Cancer Inst., 1965, v34, 459.
6. Trentin, J. J., Van Hoosier, G. L., Jr., Shields, J., Stephens, K., Stenback, W. A., Parker, J. C., Nat. Cancer Inst. Monograph 20, 1966, p147.
7. Stansly, P. G., Prog. Exp. Tumor Res., F. Homburger, ed., Hafner Publ. Co. Inc., N. Y., 1965, v7.

Received May 9, 1966. P.S.E.B.M., 1966, v122.

Effect of the Cholesterol Biosynthesis Inhibitor AY-9944 on Serum Lipoproteins of Rats, Pigs and Dogs. (31366)

P. HILL AND D. DVORNIK (Introduced by W. T. Rockhold)
(With the assistance of Patricia Ford)
Department of Biochemistry, Ayerst Research Laboratories, Montreal, Canada

The agent *trans*-1,4-*bis*(2-chlorobenzylaminomethyl) cyclohexane dihydrochloride (AY-9944)(1) inhibits the biosynthesis of cholesterol by interfering with the enzymatic transformation of 7-dehydrocholesterol to cholesterol(2,3). Given orally to laboratory animals, *e.g.*, in rats, AY-9944 significantly lowers serum cholesterol and—in spite of accumulation of the intermediate 7-dehydrocholesterol biosynthesized instead of cholesterol—also causes reduced total sterol levels in the serum(4). In view of its potency, it was of interest to investigate the distribution of sterols and phospholipids in the serum lipoproteins of rats, pigs and dogs treated with AY-9944(5).

Methods. Albino or hooded rats, weighing approximately 120 g, were given orally 10 μmoles (4.6 mg)/kg/day of AY-9944 for 7 days; to facilitate analysis the sera from 5 rats were pooled. Young, 4-5-week-old male pigs received *per os* 5, 10 and 50 μmoles (2.3, 4.6 and 23.2 mg)/kg/day of AY-9944 for 12 weeks; individual blood samples were taken after 20 and 104 days. In another study, young pigs of the same age were given orally 25 μmoles (11.6 mg)/kg/day of AY-9944 for 28 days. Treatment was stopped for 10 days and was then repeated for 18 days. Individual blood samples were taken on days 14, 28, 38 and 56. Young beagle hounds were administered orally either 25 (11.6 mg) or 50 μmoles (23.2 mg)/kg/day of AY-9944; a third group received daily 50 μmoles/kg of the agent and the dose was increased every month by 10 μmoles/kg/day until a final dose of 110 μmoles/kg/day was reached. AY-9944 was given for 5 days per week and treatment was continued for a total of 104 weeks. In a second study in dogs, 5-8-year-old beagle hounds were given orally 30 μmoles (13.9 mg)/kg/day of AY-9944 for 28 weeks.

The serum lipoproteins were separated by preparative ultracentrifugation(6) into fractions of very low density (VLD; $d<1.019$), low density (LD; *d*, 1.019-1.063) and high density (HD; *d*, 1.063-1.21) respectively. Cholesterol and 7-dehydrocholesterol were determined as reported earlier(7) and the phospholipid levels by the semi-automated procedure of Kraml(8).

Results. Rats (Table I). Oral administration of 10 μmoles/kg/day of AY-9944 caused a fall in serum cholesterol which was partly compensated by 7-dehydrocholesterol. The effect was greater in hooded rats, notably in females, than in albinos. Cholesterol was reduced in all lipoprotein fractions. 7-Dehydrocholesterol predominated in HD lipoproteins and was not detected in the LD fraction. In spite of different sterol levels in male hooded and albino rats, their 7-dehydrocholesterol:cholesterol ratios in the VLD and HD lipoproteins were virtually identical (Table II).

Pigs. As presented in Table III, administra-

TABLE I. Effect of AY-9944 (10 μmoles (4.6 mg)/kg/Day *per os* for 7 Days) on Sterol and Phospholipid Distribution in Serum Lipoproteins of Hooded and Albino Rats (Expressed in mg/100 ml Serum).

Serum lipoprotein fraction	Lipid constituent*	Hooded rats — Control ♂	Hooded rats — Control ♀	Hooded rats — Treated ♂	Hooded rats — Treated ♀	Albino rats, male — Control	Albino rats, male — Treated
$d<1.019$ (VLD)	Cholesterol	34	28	11.5	4	33	24
	7-Dehydrocholesterol	0.0	0.0	4.0	3.0	0.0	7.5
	Phospholipids	32	19	16	11	41.5	39
d, 1.019-1.063 (LD)	Cholesterol	8	6	1	2	10.5	2.5
	7-Dehydrocholesterol	0.0	0.0	0.0	0.0	0.0	0.0
	Phospholipids	15	13	11	9	27.5	22
d, 1.063-1.21 (HD)	Cholesterol	52	54	16	10	37	12.5
	7-Dehydrocholesterol	0.0	0.0	11.5	8.0	0.0	9.0
	Phospholipids	58	49	58.5	44	24	27
Total serum	Cholesterol	94	88	28.5 (−70%)†	16 (−82%)	80.5	39 (−51.5%)
	7-Dehydrocholesterol	0.0	0.0	15.5 (+16.5%)	11 (+12.5%)	0.0	16.5 (+20.5%)
	Phospholipids	105	77	85.5 (−18.5%)	64 (−17%)	93.0	88

* Each value is the average of two serum samples, each pooled from a group of five rats.
† Percent of concentration in control serum.

tion of AY-9944 *per os* produced in the serum very low levels of cholesterol together with a certain amount of 7-dehydrocholesterol. The concentrations of both were dose-dependent: increasing doses of AY-9944 caused greater reduction in cholesterol content and enhanced accumulation of 7-dehydrocholesterol. The dose-dependent lowering of serum phospholipids detected after 20 days was no longer observed after 83 days of AY-9944 administration. Cholesterol was reduced in all lipoproteins and all contained 7-dehydrocholesterol. The dependence of the effect on the dose and duration of treatment with AY-9944 is also reflected in the 7-dehydrocholesterol: cholesterol ratio (Table IV).

After 20 days of AY-9944 the phospholipids were reduced in all lipoprotein fractions; their slight increase observed after 83 days of treatment with 10 and 50 μmoles/kg/day of AY-9944 was accompanied by a redistribution resulting in accumulation of phospholipids in the HD lipoproteins.

TABLE II. 7-Dehydrocholesterol : Cholesterol Ratio in Serum Lipoprotein Fractions of Rats Treated with AY-9944 (10 μmoles/kg/Day, *p.o.* for 7 Days).

Serum lipoprotein fraction	7-Dehydrocholesterol/cholesterol — Hooded rats ♀	Hooded rats ♂	Albino rats ♂
$d<1.019$ (VLD)	.75	.35	.31
d, 1.019-1.063 (LD)	—	—	—
d, 1.063-1.21 (HD)	.80	.72	.74

The fall in serum cholesterol and phospholipid levels together with the accumulation of 7-dehydrocholesterol as caused by AY-9944 (25 μmoles/kg/day) given *per os* for 28 days, was reversed virtually to control values following a recovery period of 10 days (Table V). Repeated administration of AY-9944 led again to a typical response.

Dogs. As in rats and pigs, AY-9944 given orally to young beagles, depressed the serum cholesterol content and caused appearance of 7-dehydrocholesterol. No such elevation was observed on prolonged administration of the higher doses. The fall in cholesterol appeared to be greatest in the VLD fraction, whereas 7-dehydrocholesterol occured mainly in the HD lipoprotein fraction (Table VI).

In old dogs a daily dose of 13.9 mg/kg of AY-9944 given for 28 weeks produced a considerably lower proportion of 7-dehydrocholesterol in serum sterols (about 1/10) than was detected in young animals (approximately 1/5 of the total sterol content) treated for a similar period of time (25 weeks of 11.6 mg/kg/day of AY-9944) (Table VII).

Discussion. In all 3 species studied, oral administration of AY-9944 caused greatly reduced serum sterol levels. The fall in

TABLE III. Effect of Chronic Administration *per os* of AY-9944 on Distribution of Sterols and Phospholipids in Serum Lipoproteins of Young Growing Male Pigs (Expressed in mg/100 ml Serum).

Serum lipoprotein fraction	Lipid constituent	20 day treatment				83 day treatment			
			AY-9944, μmole/kg/day*				AY-9944, μmole/kg/day		
		Control	5	10	50	Control	5	10	50
$d<1.019$ (VLD)	Cholesterol	58.3	30.9	21.3	16.0	45.7	20.8	12.6	3.9
	7-Dehydrocholesterol	0.0	5.3	5.3	14.3	0.0	7.0	11.6	7.0
	Phospholipids	41.7	21.6	17.6	13.2	33.1	30.3	28.5	17.2
d, 1.019-1.063 (LD)	Cholesterol	15.7	24.0	13.5	11.3	15.5	18.2	9.0	5.6
	7-Dehydrocholesterol	0.0	2.4	3.1	9.7	0.0	4.7	5.5	13.6
	Phospholipids	17.5	13.2	10.8	11.2	16.9	17.7	18.1	24.6
d, 1.063-1.210 (HD)	Cholesterol	44.0	19.6	23.5	8.4	58.7	25.2	28.3	6.5
	7-Dehydrocholesterol	0.0	3.6	5.8	9.3	0.0	9.4	21.0	27.0
	Phospholipids	78.2	51.2	41.0	37.6	73.0	56.0	81.4	89.2
	Cholesterol	118.0	74.5 (−37%)†	58.3 (−50.5%)	35.7 (−70%)	119.9	64.2 (−46.5%)	49.9 (−58%)	16.0 (−87%)
Total serum	7-Dehydrocholesterol	0.0	11.3 (+10%)	14.2 (+12%)	33.3 (+28%)	0.0	21.1 (+18%)	38.1 (+32%)	47.6 (+40%)
	Phospholipids	137.4	86.0 (−37.5%)	69.4 (−49.5%)	62.0 (−55%)	123.0	104.0 (−15.5%)	129.0 (+5%)	131.0 (+6.5%)

* 2.3 mg, 4.64 mg and 23.2 mg/kg/day respectively.

† Percent of cholesterol concentration in control serum.

TABLE IV. 7-Dehydrocholesterol : Cholesterol Ratios in Serum Lipoprotein Fractions of Young Growing Male Pigs Following Chronic Oral Administration of AY-9944.

Serum lipoprotein fraction	7-Dehydrocholesterol/cholesterol ratios following AY-9944, *per os*, in μmole/kg					
	20 day treatment			83 day treatment		
	5	10	50	5	10	50
d<1.019 (VLD)	.17	.25	.89	.33	.92	1.79
d, 1.019-1.063 (LD)	.10	.23	.86	.26	.61	2.43
d, 1.063-1.21 (HD)	.18	.25	1.10	.37	.74	4.15

cholesterol content was only partly counterbalanced by the appearance of 7-dehydrocholesterol, the intermediate which is biosynthesized instead of cholesterol in laboratory animals treated with AY-9944(2). The content and especially the composition of serum sterols depended on dose and duration of treatment. In pigs, higher doses produced lower cholesterol and higher 7-dehydrocholesterol levels, a phenomenon enhanced further by prolonged treatment. A similar tendency was noted in dogs.

Following a rest period of 10 days, in the serum of pigs treated with AY-9944, with a trace of 7-dehydrocholesterol left, cholesterol rose to control level while phospholipids approached that of controls. A similar observation was made earlier in rats(9).

In spite of considerable differences between different species in the content of serum lipids and their distribution between lipoprotein fractions(10), AY-9944 caused in rats, pigs and dogs a fall of cholesterol in all lipoprotein fractions. However, the distribution of 7-dehydrocholesterol between lipoprotein fractions varied considerably from species to species: in rats and dogs the bulk of 7-dehydrocholesterol was usually associated with the HD lipoprotein fraction; in pigs, this preference was observed only after prolonged treatment with higher doses of AY-9944; no 7-dehydrocholesterol was detected in rat serum LD lipoproteins.

In respect to serum phospholipids, the slight reduction observed in hooded rats treated with AY-9944 occurred mainly in their VLD lipoprotein fraction. In pigs the effect

TABLE V. Effect on Pig Serum Sterols and Phospholipids of Oral Administration of AY-9944 (25 μmoles, *i.e.*, 11.6 mg/kg/Day) Followed by a Recovery Period and Repeated Treatment (Expressed as mg/100 ml Serum).

Serum lipid constituent	AY-9944, 25 μmoles/kg/day *per os*										AY-9944, 25 μmoles/kg/day					
	After 14 days				After 28 days				10-day recovery				After 18 days			
	Control		Treated		Control		Treated		Control		Treated		Control		Treated	
	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
Cholesterol	64	67	22 (−70%)*	40 (−45%)	105	110	18 (−83%)	13 (−88%)	106	110	109	74	112	114	19 (−83%)	33 (−69%)
7-Dehydrocholesterol	0.0	0.0	12 (+16.5%)	21 (+29%)	0.0	0.0	20 (+19%)	27 (+25%)	0.0	0.0	1.0	1.0	0.0	0.0	25 (+22%)	29 (+26%)
Phospholipids	98	58	67 (−14%)	62 (−20%)	129	137	64 (−52%)	61 (−54%)	117	140	117	109	120	129	86 (−31%)	109 (−12.5%)

* % of mean value from controls.

depended on duration of treatment: after 20 days the reduction was dose-dependent and was detected in all 3 lipoprotein fractions; prolonged treatment with higher doses of AY-9944 caused phospholipid levels to reverse to virtually normal values.

Recently we have discussed the importance of age of laboratory animals in studies of cholesterol biosynthesis inhibitors(7). In accordance, the effect of AY-9944 on serum lipoproteins was also studied in old dogs. In spite of considerable variation in the lipoprotein lipid content from animal to animal (11), AY-9944 caused in all dogs the appearance of 7-dehydrocholesterol in concentrations similar to those found in young animals. The considerably smaller proportion of 7-dehydrocholesterol in the serum total sterols, on the other hand, may indicate decreased sterol catabolism in old animals.

TABLE VI. Effect of Prolonged Oral Administration of AY-9944 on Sterol Distribution in Serum Lipoproteins of Young Beagles (Expressed as mg/100 ml Serum).

AY-9944 treatment			Serum lipoprotein fraction							
			d <1.019 (VLD)		*d*, 1.019-1.063 (LD)		*d*, 1.063-1.21 (HD)		Total serum	
μmole/kg /day	Duration (wk)	Sex	Δ^5*	$\Delta^{5,7}$†	Δ^5	$\Delta^{5,7}$	Δ^5	$\Delta^{5,7}$	Δ^5	$\Delta^{5,7}$
25	25	♂	57	5	14.5	3	41	19.5	112.5	27.5
(11.6 mg)		♀	24	6	12	3	99	26	135	35
	104	♂	8	7	13	4	78	63	99	74
		♂	13	18	11	5	102	37	126	60
		♀	7	3	14	1	46	62	67	68
		♀	10	5	10	0.0	84	57	104	62
50	25	♂	46	13	14	3	65	12	125	28
(23.2 mg)		♂	24	16.5	19	11	99	41	142	68.5
		♀	8	9	10	9	62	50	80	68
	104	♂	10	4	7	2	43	44	60	50
		♀	8	2	10	0.0	22	39	40	41
110‡	30	♂	14	0.0	56	0.0	113	50	183	106
(51.0 mg)		♂	8.5	0.0	30	6	17	24	55.5	30
		♀	24	2.5	15	4	82.5	38	121.5	44.5
Control		♂	132	0.0	36	0.0	140	0.0	308	0.0
		♀	139	0.0	59.5	0.0	171	0.0	369.5	0.0

* Cholesterol.
† 7-Dehydrocholesterol.
‡ Dogs administered 50 μmole/kg/day AY-9944 with increments of 10 μmole/kg/day each month to a terminal dose of 110 μmole/kg/day.

TABLE VII. Effect of AY-9944 (30 μmoles, *i.e.*, 13.9 mg/kg/Day Given Orally for 28 Weeks) on Sterol and Phospholipid Distribution in Serum Lipoproteins of Old Dogs (Expressed as mg/100 ml Serum).

Serum lipoprotein fraction	Lipid constituent	Control			AY-9944 treated			
		♂	♀	♀	♂	♂	♀	♀
d, <1.019 (VLD)	Cholesterol	163	117	71	19	14	54	31
	7-Dehydrocholesterol	0.0	0.0	0.0	9.0	4.0	21.0	2.0
	Phospholipids	98	64	100	40	37	92	39
d, 1.019-1.063 (LD)	Cholesterol	55	51	70	28	23	56	40
	7-Dehydrocholesterol	0.0	0.0	0.0	0.0	3.0	0.0	0.0
	Phospholipids	183	140	160	77	54	79	86
d, 1.063-1.21 (HD)	Cholesterol	70	44	153	146	81	158	147
	7-Dehydrocholesterol	0.0	0.0	0.0	15.5	12.0	9.0	14.0
	Phospholipids	260	187	156	269	179	287	230
Total serum	Cholesterol	288	212	294	193	118	268	218
	7-Dehydrocholesterol	0.0	0.0	0.0	24.5	19	30	16
	Phospholipids	541	391	416	386	270	358	355

Regarding 7-dehydrocholesterol, readily detectable in animals treated with AY-9944, the level in the serum reflects the rate of its biosynthesis(12) and degradation as well as its transportability by serum lipoproteins. Concerning the rate of biosynthesis, any dose of AY-9944 higher than the minimal dose which completely blocks cholesterol formation from 7-dehydrocholesterol should finally cause a similar, maximal concentration of serum 7-dehydrocholesterol. Actually, in pigs, higher doses and prolonged administration of AY-9944 resulted in progressively higher serum 7-dehydrocholesterol levels. Assuming the rate of catabolism of 7-dehydrocholesterol was not affected, this observation suggests enhanced biosynthesis of 7-dehydrocholesterol in pigs treated with AY-9944 as compared to that of cholesterol in untreated controls. The increase may indicate failure of the biosynthesized 7-dehydrocholesterol to activate the negative feedback mechanism of homeostatic control of cholesterogenesis normally triggered off by elevated levels of cholesterol(13).

The striking elevation of serum 7-dehydrocholesterol following prolonged administration of the highest dose to pigs may be indicative of secondary effects of AY-9944 on serum lipoproteins.

Summary. AY-9944, an orally active inhibitor of cholesterol biosynthesis, caused in rats, pigs and dogs greatly reduced serum cholesterol levels, only partly compensated by 7-dehydrocholesterol formed instead of cholesterol by the action of AY-9944. The distribution of 7-dehydrocholesterol between serum lipoprotein fractions differed from cholesterol and varied from species to species. Levels of 7-dehydrocholesterol and their distribution may reflect the rate of sterol biosynthesis.

We acknowledge with gratitude the collaboration of Dr. E. Greselin who supervised all experiments with animals and we thank Mr. J. Dubuc and his staff for the analyses of lipids.

1. Humber, L. G., J. Med. Chem., 1964, v7, 826.
2. Dvornik, D., Kraml, M., Dubuc, J., Givner, M., Gaudry, R., J. Am. Chem. Soc., 1963, v85, 3309.
3. Kraml, M., Bagli, J. F., Dvornik, D., Biochem. Biophys. Res. Comm., 1964, v15, 455.
4. Chappel, C. I., Dvornik, D., Hill, P., Kraml, M., Gaudry, R., Circulation, 1963, v28, 651.
5. Hill, P., Dvornik, D., ibid., 1964, v30, Suppl. III, 14.
6. Hillyard, L. A., Entenman, L., Feinberg, H., Chaikoff, I. L., J. Biol. Chem., 1955, v214, 79.
7. Givner, M. L., Dvornik, D., Biochem. Pharmacol., 1965, v14, 611.
8. Kraml, M., Clin. Chim. Acta, 1966, v13, 442.
9. Chappel, C. I., Dubuc, J., Dvornik, D., Givner, M., Humber, L., Kraml, M., Voith, K., Gaudry, R., Nature, 1964, v201, 497.
10. Pezold, F. A., Lipide und Lipoproteine in Blutplasma, Springer Verlag, Berlin, 1961, 156, and references cited therein.
11. Lewis, L. A., Green, A. A., Page, I. H., Am. J. Physiol., 1952, v171, 391.
12. Dvornik, D., Givner, M. L., Rochefort, J. G., Circulation, 1965, v32, Suppl. II, 10.
13. Siperstein, M. D., Fagan, V. M., J. Biol. Chem., 1966, v241, 602 and references cited therein.

Received May 12, 1966. P.S.E.B.M., 1966. v122.

Regeneration of Insulin from its Inactive Reduction Product in the Presence of Pancreatic Islets. (31367)

OTHON B. KOTOULAS* AND LILLIAN RECANT
Departments of Medicine, Preventive Medicine and Pathology, Washington University School of Medicine, St. Louis, Mo.

Tomizawa and Halsey(1) isolated an insulin degrading enzyme from beef liver. Tomizawa(2) and Katzen and Stetten(3) demonstrated that this liver enzyme catalyzes the reductive cleavage of the interchain disulfide bonds of insulin by virtue of H^+ transfer from reduced glutathione (GSH) and they proposed the name glutathione-insulin transhydrogenase for the enzyme.

Subsequently, Katzen, Tietze and Stetten

* Trainee (NIAMID) in Nutrition Grant 5T1 AM5341.