Effect of Hypocholesteremic Agents on Protozoa.* (27125)

S. Aaronson, Barbara Bensky, M. Shifrine and Herman Baker
Haskins Laboratories, New York, School of Veterinary Medicine, University of California, Davis, and Seton Hall College of Medicine and Dentistry, Jersey City, N. J.

Compounds that interfere with sterol synthesis in mammals have been described recently: triparanol† (MER-29, 1-[4(diethylaminoethoxy)-phenyl]-1-*p*-tolyl) - (*p*-chlorphenyl)-ethanol) prevented the reduction of desmosterol to cholesterol in both the rat and man(1), and benzmalecene† (*N*-methyl-2,3-di-*p*-chlorophenylpropyl)-maleamic acid) inhibited *in vitro* conversion of mevalonic acid to cholesterol(2). Triparanol lowered serum cholesterol in rat and man(3) as did benzmalecene(4). We tested the effect of a group of hypocholesteremic compounds on the multiplication of several protozoa; sterol-synthesizing and non-sterol-synthesizing protozoa were included(5) to compare inhibition of growth with inhibition of cholesterol synthesis as seen in mammals. This comparison would make protozoa useful for screening hypocholesteremic agents.

Procedure. We used the phytoflagellates, *Ochromonas danica, O. malhamensis,* and *Euglena gracilis* var. Z, and the ciliate *Tetrahymena pyriformis,* var. 1, mating-type II. The phytoflagellates were grown in the light at 26°C in 5 ml of nutrient media (6) in 10-ml micro-Fernbach flasks, covered with plastic caps; *Tetrahymena* was grown in the dark at 26°C in 5 ml of nutrient medium(7) in 25-ml micro-Fernbach flasks. All chemicals were obtained from commercial sources. Purified fatty acids were purchased from Applied Science Laboratories, Inc., State College, Pa.

Results. Besides triparanol and benzmalecene, 2 other compounds reported to lower serum cholesterol were tried: nicotinuric acid (8) and 3-pyridylacetic acid(9). Neither nicotinuric acid nor 3-pyridylacetic acid has proven useful in lowering serum cholesterol in humans. The latter 2 compounds had no effect on multiplication while the former 2 compounds, especially triparanol, inhibited the 4 protozoa at relatively low concentration (Table I). Detailed results are presented for *O. danica. O. malhamensis* responded to these anti-sterols as did *O. danica;* the effect of hypocholesteremic agents on *Euglena* and *Tetrahymena* was not studied in detail.

The effects of sterols, sterol precursors, and impure fatty acids and fatty-acid mixtures on triparanol inhibition are presented in Table II. Farnesol, squalene, cholesterol, and ergosterol prevented inhibition by 0.3 mg % of triparanol as well but no better than did the impure fatty acids, *e.g.,* palmitic, stearic or oleic acid, while complex lipid mixtures such as the Tweens or TEM-4 (a diacetyl tartaric ester of tallow monoglycerides) prevented the inhibition of 10 times more inhibitor. Comparison of the effectiveness of pure fatty acid in preventing triparanol inhibition is shown in Tables III and IV. The only effective compounds were the saturated 12-carbon acid, lauric acid, and the unsaturated 18-carbon acids: oleic, linoleic, and linolenic acids. On a molar basis, oleic and linolenic acids best prevented inhibition by triparanol. The low activity of linoleic may be attributed to the low activity of esters in this system: methyl oleate was much less effective than oleic acid. We suspect that these phytoflagellates lack an esterase, although our evidence is indirect; they do not hydrolyze acetylcholine, propionylcholine or butyrylcholine.

Discussion. In mammals, benzmalecene inhibits cholesterol synthesis at the mevalonic acid stage(2), and triparanol inhibits at the reduction of desmosterol to cholesterol(1). Low concentrations of these agents inhibited multiplication in both sterol-synthesizing protozoa and in the non-sterol-synthesizing pro-

* Supported by grants from Nat. Inst. Health, U.S.P.H.S. and Nat. Vitamin Foundation.

† We thank the following for generous samples of compounds: Dr. F. J. Murray, Wm. S. Merrell, Cincinnati, Ohio for triparanol; and Dr. E. M. Schultz of Merck, Sharp and Dohme Research Labs., West Point, Pa.

TABLE I. Effect of Hypocholesteremic Agents on Multiplication of Protozoa.

Compound	Conc. (mg %) giving complete inhibition of multiplication			
	O. danica	*O. malhamensis*	*E. gracilis*	*Tetrahymena*
Triparanol	.3–.6	.1	2.0	.3
Benzmalecene	3.0	3.0	20.0	.3
Nicotinuric acid	*		*	*
3-Pyridylacetic acid	*		*	*

* No inhibition at 50.0 mg %.

tozoan, *Tetrahymena*. In *Ochromonas* species this inhibition was prevented by the sterols, cholesterol and ergosterol, as well as by the sterol precursors, farnesol and squalene. This suggests a partial inhibition of sterol synthesis at mevalonic acid. The main site of inhibition was the synthesis of unsaturated fatty acids; either oleate or linolenate alone was more effective than the sterols in preventing inhibition by benzmalecene or triparanol. Combination of oleate with sterols or their precursors was no more effective against the inhibitors than oleate alone. The interrelationship between sterols, fatty acids, and especially unsaturated fatty acids in reversing of the inhibition of protozoa induced by the hypocholesteremic agents remains unclear. However, a nutritional requirement for both a sterol and unsaturated fatty acid has been reported for several protozoa: *Trichomonas gallinae, T. gallinarum*(10), *T. foetus*(11), *Hypotrichomonas acosta*(12), *Peranema trichophorum*(13) and *Tetrahymena corlissi* Th-X(14).

Since the protozoa described here were in-

TABLE II. Effect of Lipids on Inhibition of *O. danica* by Triparanol.

Compound	Highest conc. tested (mg %)	Conc. of triparanol (mg %) .3	1.0	3.0
Sterol precursors				
Mevalonic acid lactone; geraniol	10	—	—	—
Farnesol; squalene	10	+	—	—
Sterols				
Cholesterol; ergosterol	10	+	—	—
Lanosterol; β-sitosterol; stigmasterol	10	—	—	—
Fatty acids (impure)				
Tween 20; Tween 80	100	+	+	—
Tween 40; Tween 60; TEM T-4	100	+	+	+
Palmitic acid; stearic acid; oleic acid	100	+	—	—
Above 3 + cholesterol each at	1	+	—	—

\+ = prevents inhibition.

TABLE III. Effect of Pure Fatty Acids on Inhibition of *O. danica* by Triparanol.

Fatty acid*	Conc. of triparanol (mg %) .6	1.0	3.0
Acetic; propionic; butyric; valeric; caproic; caprylic; capric; myrstic; palmitic; stearic; Mix No. 1; Mix No. 2; Mix No. 3	—	—	—
Lauric	+	+	—
Oleic	+	+	+
Methyl oleate; methyl linoleate; linolenic	+	—	—

* Fatty acids used in a concentration of 10.0 mg % except for 2-6 carbon acids which were used to 100 mg %.

Mixtures of odd and even numbered fatty acids; No. 1 = mixture of 6-11 carbons, No. 2 = mixture of 11-15 carbons, No. 3 = 15-19 carbons.

\+ = prevents inhibition; — = no effect.

TABLE IV. Ability of Fatty Acids to Prevent Inhibition of *O. danica* by Triparanol.

Compound	Conc. (mM)	Optical density Conc. of triparanol (mM) .0069†	.023†	.069
0		2.03	0	0
Lauric acid	.035	2.68	0	0
" "	.375	2.69	2.54	0
" "	.750	2.75	2.76	0
Oleic acid	.0035	2.15	0	0
" "	.035	2.81	0	0
" "	.105	2.88	2.81	0
" "	.350	2.93	2.85	2.73
Methyl linoleate	.0034	2.60	0	0
" "	.034	—	0	0
" "	.34	2.72	0	0
Linolenic acid*	.036	2.84	0	0
" "	.108	2.90	3.02	0

* .36 mM toxic.

† .0069 mM = .3 mg %.
.023 mM = 1.0 mg %.

hibited by hypocholesteremic agents found to be active in humans they may be useful for screening compounds inhibiting sterol or fatty acid synthesis.

Summary. The hypocholesteremic agents, triparanol and benzmalecene, inhibit the multiplication of the sterol-synthesizing phytoflagellates, *Ochromonas danica*, *O. malhamensis*, *Euglena gracilis*, and the non-sterol-synthesizing ciliate, *Tetrahymena pyriformis*. The inhibition of multiplication of the *Ochromonas* species may be prevented by cholesterol, ergosterol, farnesol, squalene, lauric acid, oleic acid, linoleic acid, and linolenic acid. Oleic acid and linolenic acid were most effective.

1. Steinberg, D., Avigan, J., *J. Biol. Chem.*, 1960, v235, 3127.

2. Huff, J. W., Gilfillan, J. L., PROC. SOC. EXP. BIOL. AND MED., 1960, v103, 41.

3. Avigan, J., Steinberg, D., Vromanz, H. E., Thompson, M. J., Mosettig, E., *J. Biol. Chem.*, 1960, v235, 3123.

4. Page, I. H., Schneckloth, R. E., *Circulation*, 1959, v20, 1075.

5. Aaronson, S., Baker, H., *J. Protozool.*, 1961, v8, 274.

6. Aaronson, S., Scher, S., *ibid.*, 1960, v7, 156.

7. Stokstad, E. L. R., Seaman, G. R., Davis, R. J., Hutner, S. H., in *Methods of Biochemical Analysis*, 1956, v3, 23, Interscience Publishers, Inc., New York.

8. Miller, O. N., Hamilton, J. G., Goldsmith, G. A., *Am. J. Clin. Nutrit.*, 1960, v8, 480.

9. Guggenheim, K., Diamant, E. J., *Biochem. J.*, 1958, v69, 56.

10. Shorb, M. S., Lund, P. G., *J. Protozool.*, 1959, v6, 122.

11. Sanders, M., *ibid.*, 1957, v4, 118.

12. Lee, J. J., Pierce, S., *ibid.*, 1960, v7, 402.

13. Storm, J., Hutner, S. H., *Ann. N. Y. Acad. Sci.*, 1953, v56, 901.

14. Holz, G. G., Jr., Wagner, B., Erwin, J., Britt, J. J., Blech, K., *Comp. Biochem. Physiol.*, 1961, v2, 202.

Received September 19, 1961. P.S.E.B.M., 1962, v109.

Permeability of Ehrlich Cells to Uracil, Thymine and Fluorouracil.* (27126)

JOHN A. JACQUEZ‡ (Introduced by C. C. Stock)

Division of Experimental Chemotherapy, Sloan-Kettering Institute for Cancer Research, and the Sloan-Kettering Division, Cornell University Graduate School of Medical Sciences, New York

Ehrlich ascites cells(1) and human erythrocytes(2) are highly permeable to adenine. Little is known about the permeabilities of cells to the pyrimidines although the work of Bosch *et al.*(3) and Heidelberger *et al.*(4) suggests that uracil and fluorouracil readily enter Ehrlich ascites cells. This paper reports our studies on the permeability of Ehrlich ascites cells to uracil, thymine and fluorouracil.

Methods and materials. The Ehrlich ascites used was a hypotetraploid line carried in Swiss albino mice by weekly intraperitoneal injections of 0.1 ml of ascites. For experiments, 6-day ascites was collected into 50 ml Krebs-Ringer bicarbonate "KRB", containing 0.2 mg heparin. Obviously bloody ascites was discarded. Faintly pink ascitic fluid containing small numbers of erythrocytes was accepted for use. The resulting suspension was diluted with an equal volume of KRB and centrifuged to collect the cells. After one wash, the cells were resuspended in KRB. Osmotic hemolysis(1) was not used.

Details of the technics utilized have been described(5,6). Experiments were conducted at 5°C and 39°C. Heinicke reaction vessels were used. Three ml of cell suspension was placed in one arm and 3 ml of a solution of pyrimidine in KRB in the other arm. Glucose was not added to the medium because it was found that uracil and uridine were metabolized at a considerably higher rate than

* This work was supported by grants from Am. Cancer Soc. and by CCNSC contract, Nat. Cancer Inst., P.H.S.

‡ Sloan-Kettering Institute, Rye, New York.