3. McCollum's diet I, slightly modified, expressed on the basis of dry weight, includes whole wheat, 70 percent; casein, 16 percent; whole milk powder, 11 percent; iodized NaCl, 0.75 percent; $CaCO_3$, 1.7 percent; and a vitamin concentrate in an amount to give 1.5 USP units of vitamin D and 7.5 units of vitamin A per gram of diet.
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8. I thank S. Dobbs for histologic and animal husbandry work, C. Reather for photomicrography, and S. C. Marks, Jr., for the acid phosphatase histochemical analyses. Supported by grant AM 12491 from the National Institute of Arthritis and Metabolic Diseases.

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Hepatic Peroxisome Proliferation: Induction by Two Novel Compounds Structurally Unrelated to Clofibrate

Abstract. *Two hypolipidemic compounds [4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio]acetic acid, and 2-chloro-5-(3,5-dimethylpiperidinosulfonyl)benzoic acid (tibric acid) greatly increased the number of peroxisomes (microbodies) in liver cells of rats and mice. This augmented peroxisome population was accompanied by significant elevation of liver catalase activity. These two hypolipidemic peroxisome proliferators are structurally different from ethyl* α-p-*chlorophenoxyisobutyrate (clofibrate) and other hypolipidemic, aryloxyisobutyrate derivatives which cause hepatic peroxisome proliferation. Induction of peroxisome proliferation by these structurally unrelated hypolipidemic compounds suggests a possible relation between hepatic peroxisome proliferation and hypolipidemia.*

Peroxisomes (microbodies), cytoplasmic constituents characterized morphologically by a single limiting membrane and a finely granular or homogeneous matrix, have recently been recognized as ubiquitous structures in animal and plant cells (*1*). In liver and kidney cells these organelles possess catalase and several H_2O_2-generating oxidases (*2, 3*), but their precise functional significance remains unknown. Nearly 10 years ago, de Duve and Baudhuin (*2*) suggested that hepatic and renal peroxisomes may participate in gluconeogenesis, while Allen and Beard (*4*) proposed that these organelles, by degrading H_2O_2, protect cells from random peroxidation. Although no satisfactory evidence supports these hypotheses, protection against heavy concentrations of H_2O_2 may be one of the principal properties of these catalase-containing organelles present in almost every cell type (*5*). Based on the close morphologic association of peroxisomes and lipid droplets in liver cells, Novikoff and Shin (*6*) postulated that peroxisomes might play a role in lipid metabolism. The observation that a potent hypolipidemic drug, clofibrate (ethyl-α-*p*-chlorophenoxyisobutyrate, CPIB), induced peroxisome proliferation in liver cells of rats and mice (*7, 8*) also suggested a relation between hepatic peroxisome proliferation and hypolipidemia. However, additional studies (*9*) favored the hypolipidemia and peroxisome proliferation of clofibrate to be independent properties.

Recently, a number of potent hypolipidemic analogs of clofibrate were shown to induce profound proliferation of peroxisomes in liver cells (*10, 11*). Whether this indicates a relationship or accidentally related properties of structurally related compounds could not be decided (*11*). Up to now, all known peroxisome proliferators possessed hypolipidemic properties; and all are closely related to clofibrate (Fig. 1, compounds **1** to **5**). We now report that [4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio]acetic acid (Wy-14,643) and 2-chloro-5-(3,5-dimethylpiperidinosulfonyl)benzoic acid (tibric acid; CP-18,524) (Fig. 1, compounds **6** and **7**), two new hypolipidemic compounds with chemical structure distinctly different from clofibrate or its analogs, also produce marked proliferation of hepatic peroxisomes in rats and mice. The stimulation of hepatic peroxisome proliferation by these structurally unrelated hypolipidemic compounds suggests that the peroxisome proliferative and hypolipidemic responses are interrelated.

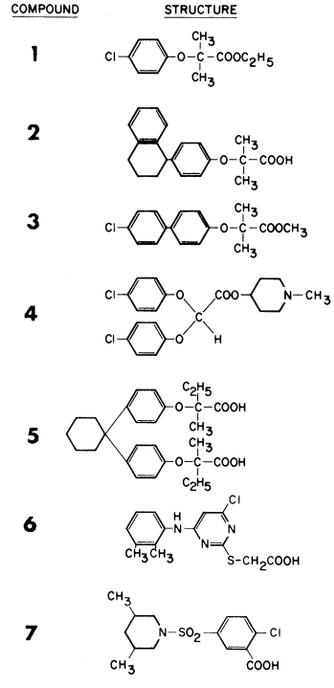

Fig. 1. Chemical structures of peroxisome proliferators. Compounds **1** to **5** are aryloxyisobutyrate derivatives: **1**, ethyl-α-*p*-chlorophenoxyisobutyrate (clofibrate; CPIB); **2**, 2-methyl-2-[*p*-(1,2,3,4 - tetrahydro - 1 - naphthyl)phenoxy]propionic acid (nafenopin); **3**, methyl-2-[4-(*p*-chlorophenyl)phenoxy]2-methyl propionate (methyl clofenapate); **4**, 1-methyl-4-piperidylbis(*p*-chlorophenoxy) acetate (SaH 42-348); **5**, 1,1-bis[4′-(1′′-carboxy-1′′-methylpropoxy) - phenyl]cyclohexane (S-8527). Compound **6**, [4-chloro-6-(2,3,-xylidino)-2-pyrimidinylthio]acetic acid (Wy-14,643) and compound **7**, 2-chloro-5-(3,5-dimethylpiperidinosulfonyl)benzoic acid (tibric acid; CP-18,524), are not clofibrate analogs, but are as effective as aryloxyisobutyrate derivatives (compounds **1** to **5**) in inducing peroxisome proliferation and hypolipidemia.

Inbred male F-344 rats (Simonson Laboratories Inc., Gilroy, California), 125 to 150 g, and male Swiss Webster mice (supplied by a local dealer), 20 to 25 g, were housed in individual cages. The drugs, Wy-14,643 and tibric acid, were added to ground Purina laboratory chow at a level of 0.125 percent or 0.25 percent (by weight), which was always available to the animals. Liver biopsies were obtained from animals treated with these compounds for 1 to 4 weeks; after fixation for 2 hours in 2 percent OsO_4 buffered with *S*-collidine to *p*H 7.4, they were processed for electron microscopic examination (*11*). Portions of liver fixed in neutral buffered formalin were processed for light microscopy. For cytochemical localization of peroxisome catalase, samples of liver were fixed in 2.5 percent glutaraldehyde buffered with 0.1*M* sodium cacodylate, *p*H 7.4, for 4 hours. After fixation, the tissues were washed overnight in the cacodylate buffer. Sections (40 μm thick), prepared with a tissue chopper, were incubated for 30 to 45 minutes at 37°C in alkaline 3,3′-diaminobenzidine (DAB) medium (*12*). After incubation, the tissue was postfixed with OsO_4 and processed for electron microscopy.

Both compounds produced a significant ($P < .001$) increase in liver weights of rats and mice at 0.125 percent as well as 0.25 percent dose levels after 1 to 2 weeks of treatment (Table 1). Examination of these livers by light microscopy revealed enlarged hepatocytes containing abundant acidophilic granular cytoplasm. By electron microscopy, the liver cells showed a profound increase in the number of peroxisome profiles together with proliferation of smooth endoplasmic reticulum (Fig. 2). These organelles were numerous at 2 weeks and displayed considerable vari-

Table 1. Effect of administration of [4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio]acetic acid (Wy-14,643) and 2-chloro-5-(3,5-dimethylpiperidinosulfonyl)benzoic acid (tibric acid) on liver weight (grams per 100 grams of body weight and liver catalase activity (units per milligram of protein) in male F-344 rats and Swiss Webster Mice. The drugs were mixed in ground Purina chow at the doses (by weight) shown, and fed for 2 weeks. The values are expressed as mean ± standard error. The numbers in parentheses represent the number of animals used

Drug	Dose (%)	F-344 male rats		Swiss Webster male mice	
		Liver weight	Liver catalase	Liver weight	Liver catalase
Control		3.7 ± 0.41 (6)	38 ± 3.9 (6)	6.20 ± 0.13 (6)	33 ± 1.8 (6)
Wy-14,643	0.125	7.6 ± 0.11* (5)	81 ± 2.1* (5)	15.4 ± 0.49* (5)	78.3 ± 1.3* (5)
Wy-14,643	0.25	9.1 ± 0.79* (5)	78 ± 4.2* (5)	15.9 ± 0.13* (5)	77 ± 2.4* (5)
Tibric acid	0.125	7.7 ± 0.36* (4)	78 ± 3.4* (4)	14.1 ± 0.28* (5)	79 ± 3.6* (5)
Tibric acid	0.25	8.04 ± 0.38* (5)	84 ± 5.4* (5)	13.1 ± 0.55* (8)	89 ± 2.0* (8)

*Significantly different from control ($P < .001$).

ation in size and shape. The peroxisomes proliferated to the same extent in rats and mice treated with these two hypolipidemic drugs. Both drugs appeared equally potent at 0.125 percent dose level and produced no additional increase in peroxisome population at the higher level (0.25 percent). Treatment for 3 and 4 weeks did not result in an additional increase in peroxisome population although the proliferative effect was sustained. The electron-opaque reaction product was found in all these peroxisome profiles after incubation of the tissue in a standard DAB cytochemical medium for localization of catalase (*12*), a marker enzyme for peroxisomes. This staining reaction was abolished when sections were incubated in DAB medium containing 0.02*M* 3-amino-1,2,4-triazole, an inhibitor of the peroxidatic activity of catalase in peroxisomes (*12*). These cytochemical studies further confirmed peroxisome proliferation.

In order to establish whether hepatic peroxisome proliferation induced by these two new hypolipidemic drugs is accompanied by an elevation of catalase activity, livers of rats and mice fed these compounds for 2 weeks were perfused via the portal vein with cold saline, and 5 percent liver homogenates were prepared for the determination of catalase activity by the spectrophotometric method of Lück (*13*). Total protein was measured by the method of Lowry *et al.* (*14*). Both Wy-14,643 and tibric acid produced a significant ($P < .001$) increase in hepatic catalase activity in rats and mice (Table 1). The specific activity of liver catalase in the treated animals was slightly more than doubled when compared to appropriate controls. These findings correlate well with the hepatic peroxisome proliferation observed by electron microscopic and cytochemical studies.

Our studies establish that Wy-14,643 and tibric acid are capable of inducing peroxisome proliferation in liver cells of rats and mice and that this effect is sustained as long as the drugs are administered. Clofibrate and its analogs as well as the two new compounds reported here are capable of lowering serum cholesterol and triglyceride levels to a significant extent (*15–17*). It is also evident from these and other studies that these compounds also possess the hepatic peroxisome proliferative property. In view of the earlier studies of Caravaca *et al.* (*18*) which demonstrated a hypocholesterolemic effect in rabbits and humans treated with bovine hepatic catalase, Svoboda and Azarnoff (*8*), initially suggested that the hypolipidemic effect of clofibrate might be mediated by the endogenous elevation of liver catalase resulting from increased microbody population. In subsequent studies, however, it was concluded that the hepatic peroxisome proliferation induced by clofibrate is independent of its hypolipidemic effect (*9*). This was, in part, based on the finding that thyroidectomy abolished the hypolipidemic effect of this drug (*19, 20*) but failed to prevent the peroxisome proliferative effect of clofibrate (*9*). Accordingly, these two effects of clofibrate appeared to be dissociable. However, the data of Best and Duncan (*20*), on rats made hypothyroid by thyroidectomy and given clofibrate, do not substantiate the contention of Platt and Thorp (*19*) that thyroidectomy abolished the hypolipidemic effect of clofibrate. Best and Duncan (*20*) actually observed significant reduction in serum cholesterol in the thyroidectomized animals treated with clofibrate. Since this drug caused peroxisome proliferation also in thyroidectomized rats (*9*), the conclusion that these two effects are independent appears untenable.

Our observations that Wy-14,643 and tibric acid produced hepatic peroxisome proliferation and enhanced catalase activity indicate that the hypolipidemic and peroxisome proliferative effects, at least, are two common properties of some potent hypolipidemic drugs. Studies of the relation between structure and activity of clofibrate analogs suggested that the compounds exhibiting peroxisome proliferative activity are *p*-substituted phenoxyacetates (*21*). Compound Wy-14,643, a pyrimidine derivative, is several times more potent than clofibrate in reducing serum cholesterol (*16*). Likewise, tibric acid, a piperidinosulfonyl benzoic acid derivative, also possesses significant hypolipidemic activity (*17*). Since these two compounds are structurally different from one another and from clofibrate and its hypolipidemic analogs, it is reasonable to assume that peroxisome proliferation is closely related to

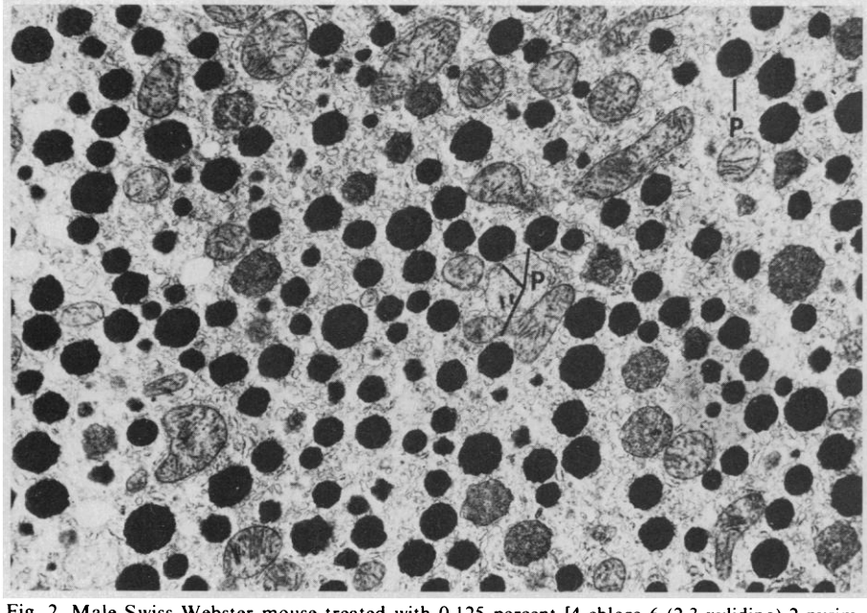

Fig. 2. Male Swiss Webster mouse treated with 0.125 percent [4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio]acetic acid (Wy-14,643) in the diet for 2 weeks. Marked increase in peroxisome profiles (*P*) in liver cell cytoplasm is evident. There is considerable variation in size and shape of these organelles. The tissue was fixed in OsO_4 and processed for electron microscopy; the sections were stained with lead hydroxide (×9500).

the hypolipidemic property of these drugs and not to independent actions. In addition, to our knowledge, all known peroxisome proliferators have hypolipidemic properties including acetylsalicylic acid, which produces a minimal to moderate increase in peroxisome profiles (*22*). The mechanism by which these hypolipidemic drugs produce peroxisome proliferation in the liver cells and their role in lipid metabolism are not understood. The frequent association of hepatic peroxisome proliferation with drug-induced hypolipidemia suggests that either peroxisome catalase or some other peroxisomal enzyme may be responsible for the hypocholesterolemic and hypotriglyceridemic effects (*11, 23*). However, only if a nonhypolipidemic peroxisome proliferator is found can these two effects be considered unrelated. Until such a compound is identified, it is reasonable to direct future studies towards clarification of the role of peroxisomes in lipid metabolism.

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1 April 1975

Circadian Periodicity in *Neurospora*: Alteration by Inhibitors of Cyclic AMP Phosphodiesterase

Abstract. *Three methyl xanthine inhibitors of adenosine 3',5'-monophosphate phosphodiesterase—theophylline, aminophylline, and caffeine—lengthen the period of the circadian conidiation rhythm of* Neurospora. *The effects are seen in wild-type strains and in three mutant strains with genetically altered period lengths. These results suggest the possible involvement of adenosine 3',5'-monophosphate in the control of circadian rhythmicity.*

Recent interest in the molecular basis of circadian rhythmicity has focused on the possible role of membranes in this phenomenon. Much of the impetus for this thinking has come from experiments in which substances likely to affect membrane structure or function alter circadian periodicity. For example, ethyl alcohol and D_2O cause phase shifts or changes of period length in the leaf movement rhythm of *Phaseolus* (*1*), in the activity rhythm of the isopod *Excirolana* (*2*), and the phototaxis rhythm of *Euglena* (*3*). In addition, pulses of K^+ cause phase shifts in the spontaneous rhythmic firing of the optic nerve of *Aplysia* (*4*), and valinomycin, which alters membrane transport of K^+, causes phase shifts in *Phaseolus* (*5*) and *Gonyaulax* (*6*). Much of this type of information has been incorporated in the ion-flux model of the circadian clock proposed by Njus *et al.* (*7*).

Adenosine 3',5'-monophosphate (cyclic AMP) is ubiquitous in both higher and lower organisms and plays a variety of regulatory roles in these systems (*8*). Adenylate cyclase and cyclic AMP phosphodiesterase, the enzymes that control endogenous cyclic AMP levels, are often mem-

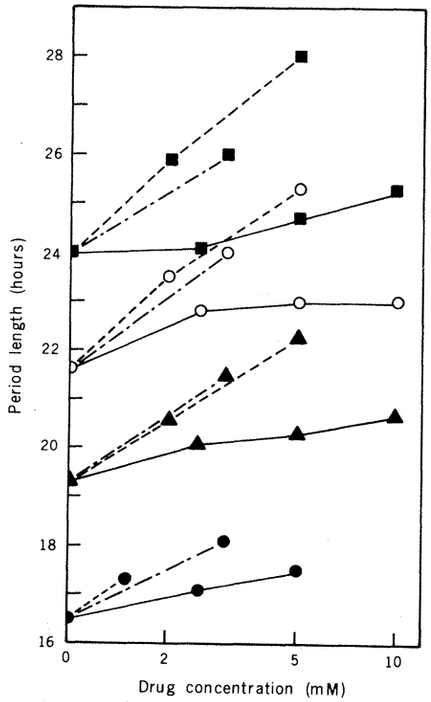

Fig. 1. Lengthening the period of the circadian rhythm of conidiation in *Neurospora crassa*. Each line represents one strain tested with one drug at the indicated concentrations. Symbols used to denote the various strains are as follows: ○, *frq*$^+$; ●, *frq-1*; ▲, *frq-2*; and ■, *frq-3*. Symbols used to denote different drugs are as follows: ———, theophylline; ------, aminophylline; and — — —, caffeine. Each point represents the mean of 12 replicate growth tubes. In all but three cases, the standard deviations ranged from 0.2 to 0.6. In the other three cases they ranged from 0.7 to 0.9.