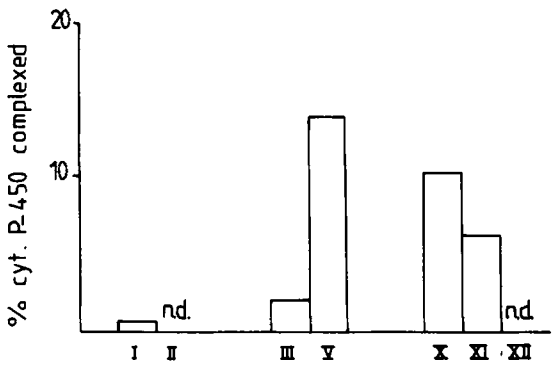

Figure 4—*Effect of substitution in the aromatic part of the substrates on metabolic intermediate-complexing activity, indicated as percentage of cytochrome* P_{450} *complexed (obtained from Table I).*

ligand responsible for such complex formation is an *N* oxidized primary amine, probably a nitroso compound or a nitroxide radical (11). Presumably the main mechanism leading to the ultimate metabolic intermediate ligand is *N*-oxidation of the secondary amines, since *N*-hydroxytofenacine (XV) leads to a large extent to complex formation. *N*-Oxidation of the *N*-hydroxy-*N*-alkylamine may lead to an unstable *N*-hydroxy-*N*-alkylamine *N*-oxide, which after rearrangement is readily *N*-dealkylated. The *N*-dealkylated *N*-hydroxylamine is rather unstable and may give the nitroxide radical. Direct *N*-oxidation of the primary amine does not have an important contribution to metabolic intermediate complex formation inasmuch as I and II do not produce a metabolic intermediate complex to any large degree.

Interpretations of data become more difficult if the effect of substitution at the aromatic portion of the molecule comes into play (Fig. 4). Substitution on one phenyl group greatly affects complexation. Moreover, the substituent on the amine function influences the effect of substitution on the aromatic part in an unpredictable manner. Clearly more compounds are needed to unravel the effect on metabolic intermediate complexation if substitution occurs in different parts of the molecule simultaneously.

In conclusion, evaluation of the relationship between structure and biological activity may be helpful in development of compounds which will show the desired pharmacological effect without metabolic intermediate complexation. One may also design compounds with prominent complexing activity with specific cytochrome P_{450} subforms, which may be used for pharmacological or toxicological purposes.

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Hypolipidemic Activity of 3-*N*-(1′,8′-Naphthalimido)propionic Acid in Rodents

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Abstract □ 3-*N*-(1′,8′-Naphthalimido)propionic acid was synthesized and shown to effectively lower both serum cholesterol and triglyceride levels in rats and mice. In hyperlipidemic mice, serum lipid levels were lowered significantly, approaching normal levels of cholesterol and below normal levels of triglyceride. The serum lipid levels were reduced due to accelerated clearance of cholesterol *via* the biliary route as well as a lowering of available acetyl CoA in the cytoplasm for liver *de novo* cholesterol and triglyceride syntheses. The liver regulatory enzyme of fatty acid and triglyceride syntheses were depressed by drug treatment. Significant reduction of liver lipids as well as the lipid content of the lipoprotein fractions were observed. The agent possessed a safe therapeutic index when used as a hypolipidemic agent.

Keyphrases □ 3-*N*-(1′,8′-Naphthalimido)propionic acid—hypolipidemic activity in rodents, cholesterol and triglycerides reduction □ Antihyperlipidemic agents—3-*N*-(1′,8′-naphthalimido)propionic acid, activity in rodents, cholesterol and triglycerides reduction

N-Substituted cyclic imides have been shown to be potent hypolipidemic agents in rodents at the low dose of 20 mg/kg/d. Substitutions of four carbon atoms or an oxygen atom for one of the carbons provided the most active agent of the phthalimide, saccharin, and the 1′,8′-naphthalimide series (1). These agents effectively lowered both serum cholesterol and tri-

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Table I—Effects of 3-*N*-(1′,8′-Naphthalimido)propionic Acid on Serum Cholesterol and Triglyceride Levels of Male CF_1 Mice and Male Holtzman Rats

Compound	Rats: Dose, mg/kg/d	Rats: Serum Cholesterol Day 9	Rats: Serum Cholesterol Day 14	Rats: Serum Triglyceride Day 14	Mice: Dose, mg/kg/d	Mice: Serum Cholesterol Day 9	Mice: Serum Cholesterol Day 16	Mice: Serum Triglyceride Day 14
3-*N*-(1′,8′-Naphthalimido)propionic Acid	10	81 ± 6[b]	65 ± 7[b]	69 ± 4[b]	10	91 ± 7	78 ± 5[b]	88 ± 6
	20	78 ± 7[b]	57 ± 5[b]	66 ± 6[b]	20	92 ± 6	68 ± 6[b]	58 ± 5[b]
					40	87 ± 8	64 ± 5[b]	70 ± 4[b]
					60	81 ± 6[c]	74 ± 7[b]	72 ± 6[b]
Control (1% carboxymethylcellulose)		100 ± 5[c]	100 ± 6[d]	100 ± 6[e]		100 ± 9[f]	100 ± 7[g]	100 ± 8[h]

[a] Expressed as percent of control (mean ± *SD*); $n = 6$. [b] $p \leq 0.001$. [c] 118 mg%. [d] 122 mg%. [e] 137 mg%. [f] 73 mg%. [g] 78 mg%. [h] 110 mg%.

Table II—Effect of 3-*N*-(1′,8′-Naphthalimido)propionic Acid on Weights of Major Organs from Male Holtzman Rats after Dosing Intraperitoneally[a]

Organ	Control (1% Carboxymethylcellulose): Organ Weight, g	Control (1% Carboxymethylcellulose): Percent of Body Weight	3-*N*-(1′,8′-Naphthalimido)propionic Acid: Organ Weight, g	3-*N*-(1′,8′-Naphthalimido)propionic Acid: Percent of Body Weight
Liver	15.2	3.46	16.2	3.99
Lung	2.1	0.48	2.5	0.63
Heart	1.5	0.34	1.2	0.29
Stomach	3.0	0.68	2.3	0.58
Kidney	3.1	0.71	2.5	0.61
Spleen	1.1	0.25	1.0	0.25
Brain	1.6	0.36	1.9	0.47
Large intestine	4.8	1.09	3.6	0.89
Small intestine	10.3	2.35	10.43	2.58
Percent increase in animal weights during experiment		31		33

[a] For 14 d at 20 mg/kg/d; $n = 6$.

glyceride levels. They were active in both normal and hyperlipidemic mice. In the latter case, the agents lowered blood lipids to maintain normal ranges of cholesterol and triglycerides. The LD_{50} values of the drugs appeared to be high, *i.e.*, >2 g/kg, indicating a safe therapeutic index. No other deleterious side effects were noted, and the effects on lipid metabolism were totally reversible. The cyclic imides have been observed to reduce regulatory enzymes of lipid synthesis and to accelerate lipid excretion *via* the bile. The agents significantly lowered cholesterol and triglyceride content of the lipoprotein fractions of blood in rats. The *N*-propionic acid substitution of the cyclic ring provided one of the more active derivatives of the phthalimide (2) and saccharin (3) series in the hypolipidemic screen. Preliminary testing indicated the same findings for the propionic acid derivative of 1′,8′-naphthalimide. Thus, an in-depth study of the metabolic effects of 3-*N*-(1′,8′-naphthalimido)propionic acid was undertaken in rodents, and those results are reported.

EXPERIMENTAL SECTION

Source of Compounds—3-*N*-(1′,8′-Naphthalimido)propionic acid was prepared as follows. 1′,8′-Naphthalic anhydride (19.8 g, 0.1 mol) and 8.9 g (0.1 mol) of β-alanine were refluxed in 300 mL of dimethylformamide for 1.25 h. The resulting solution was cooled, and the solvent was removed *in vacuo* to yield a pink solid residue which was a single spot by TLC analysis. The solid was washed with ethanol to remove the pink color, yielding 24 g (89%) of an off-white solid, mp 225–243°C. Recrystallization of an analytical sample from ethyl acetate gave 3-*N*-(1′,8′-naphthalimido)propionic acid, mp 231–233°C. ^{1}H-NMR (Me_2SO-d_6): δ 7.62–8.47 (m, 6, ArH), 4.26 (t, 2, $N-CH_2$), and 2.60 ppm (t, 2, $CH_2-C=O$).

Anal.—Calc. for $C_{15}H_{11}NO_4$: C, 66.91; H, 4.12. Found: C, 66.96; H, 4.11.

Antihyperlipidemic Screens in Normal Rodents—3-*N*-(1′,8′-Naphthalimido)propionic acid was suspended in 1% aqueous carboxymethylcellulose, homogenized, and administered to male CF_1 mice (~25 g) intraperitoneally for 16 d or male Holtzman rats (~350 g) orally by an intubation needle for 14 d. Serum cholesterol (4) and triglycerides[1] were determined as described previously (2).

Testing in Atherogenic Mice—Male CF_1 mice (~25 g) were placed on a commercial diet[2] to produce a hyperlipidemic state. After the cholesterol and triglyceride levels were elevated, the mice were administered the test drug at 20 mg/kg/d ip for an additional 14-d period, and serum cholesterol (4) and triglyceride[1] levels were determined (2).

Toxicity Studies—Periodic animal weights were obtained during the experiments and expressed as a percentage of the weight of the animal on day 0 (2). The acute toxicity (LD_{50} value) (5) was determined in male CF_1 mice (~25 g) by administering the test drug intraperitoneally from 100 mg to 2 g/kg as a single dose. The number of deaths were recorded over a 7-d period for each group.

Enzymatic Studies—*In vitro* enzymatic studies were determined using 10% homogenates of male CF_1 mouse liver with 2.5–10 μmol of test drug. *In vivo* enzymatic studies were determined with 10% homogenates of liver from male CF_1 mice obtained after administering the agent for 16 d at a dose ranging from 10 to 60 mg/kg/d ip. The enzyme activities were determined by previously described techniques: acetyl CoA synthetase (6); ATP-dependent citrate lyase (7); mitochondrial citrate exchange (8, 9); cholesterol side-chain oxidation (10); 3-hydroxy-3-methylglutaryl CoA reductase (HMG CoA reductase) (11, 12); acetyl CoA carboxylase activity (13); fatty acid synthetase (14); *sn*-glycerol-3-phosphate acyl transferase (15); and phosphatidate phosphohydrolase (16). Details of the enzymatic assays have been published previously (2).

Liver, Small Intestine, and Fecal Lipid Extraction—In male CF_1 mice that had been administered test drugs for 15 d, the liver, small intestine, and fecal materials (24-h collection) were removed, extracted (17, 18) and analyzed for cholesterol (4), triglycerides[1], neutral lipids (19), and phospholipids (20).

[^{3}H]Cholesterol Distribution in Rats—Male Holtzman rats (~350 g) were administered test agents for 14 d orally. On day 13, 10 μCi of [^{3}H]cholesterol was administerted intraperitoneally in mice and orally in rats by a previously described procedure (2).

Cholesterol Absorption Study—Male Holtzman rats (~400 g) were treated with test drugs at 20 mg/kg/d orally for 14 d. [1,2-^{3}H]Cholesterol (40.7 mCi/mmol) (10 μCi) was administered orally 18 h before surgery. After the rats were anesthetized and the bile ducts were canulated by a method described previously (2), the bile was collected over the next 6 h and analyzed for tritium content (21).

Plasma Lipoprotein Fractions—Male Holtzman rats (~400 g) were administered test drugs at 20 mg/kg/d for 14 d. Blood was collected from the abdominal aorta, and lipoprotein fractions were obtained by the methods of Havel and co-workers (22, 23). Each fraction was analyzed for cholesterol (4), triglycerides[1], neutral lipids (19), phospholipids (20), and protein levels (24).

[1] Hycel Triglyceride Test Kit; Fisher Scientific Co.

[2] Basal Atherogenic Test Diet; U.S. Biochemical Corp.

Table III—*In Vitro* Effects of 3-*N*-(1′,8′-Naphthalimido)propionic Acid on Male CF_1 Mouse Liver Enzyme Activities [a]

Drug, μmol	Mitochondrial Citrate Exchange	Acetyl CoA Synthetase	Citrate Lyase	HMG CoA Reductase	Cholesterol Side-Chain Oxidation
Control (1% carboxymethylcellulose)	100 ± 10[b]	100 ± 7[c]	100 ± 9[d]	100 ± 7[c]	100 ± 8[f]
Treated					
2.5	21 ± 9[j]	82 ± 6	73 ± 3[j]	93 ± 4	68 ± 9[j]
5.0	—	83 ± 7	69 ± 7[j]	83 ± 6[k]	—
10.0	—	86 ± 6	77 ± 4[j]	79 ± 6[j]	—

Drug, μmol	Acyl CoA-Cholesterol Acyl Transferase	Acetyl CoA Carboxylase	Fatty Acid Synthetase	*sn*-Glycerol-3-phosphate Acyl Transferase	Phosphatidate Phosphohydrolase
Control (1% carboxymethylcellulose)	100 ± 9[g]	100 ± 6[h]	100 ± 7[i]	100 ± 8[l]	100 ± 7[m]
Treated					
2.5	98 ± 6	58 ± 6[j]	100 ± 5	41 ± 7[j]	55 ± 3[j]
5.0	135 ± 9[j]	51 ± 3[j]	100 ± 6	50 ± 6[j]	45 ± 6[j]
10.0	134 ± 12[j]	46 ± 4[j]	98 ± 7	24 ± 4[j]	42 ± 7[j]

	Mitochondrial Oxidative Phosphorylation			
	α-Ketoglutarate		Succinate	
	State 4	State 3	State 4	State 3
Control (1% carboxymethylcellulose)	100 ± 6[n]	100 ± 7[o]	100 ± 5[p]	100 ± 6[q]
Treated	67 ± 5[j]	50 ± 3[j]	81 ± 6[j]	65 ● 4[j]

[a] Expressed as percent control (mean ± *SD*); $n = 6$. [b] 30.8% exchange of mitochondrial citrate. [c] 28.5 mg of acetyl CoA formed/g of wet tissue/20 min. [d] 30.5 mg citrate hydrolyzed/g of wet tissue/20 min. [e] 384,900 dpm of cholesterol formed/g of wet tissue/60 min. [f] 6080 dpm of CO_2 formed/g of wet tissue/18 h. [g] 4804 dpm/mg of microsomal protein/20 min. [h] 32,010 dpm/g of wet tissue/30 min. [i] 37,656 dpm/g of wet tissue/20 min. [j] $p \leq 0.001$. [k] $p \leq 0.010$. [l] 537,800 dpm/g of wet tissue/20 min. [m] 16.7 μg inorganic phosphate/g of wet tissue/15 min. [n] 3.51 μL of oxygen consumed/h/mg of tissue. [o] 5.21 μL of oxygen consumed/h/mg of tissue. [p] 5.92 μL of oxygen consumed/h/mg of tissue. [q] 11.31 μL of oxygen consumed/h/mg of tissue.

RESULTS

3-*N*-(1′,8′-Naphthalimido)propionic acid clearly demonstrated potent hypolipidemic activity in rodents. In rats, at 20 mg/kg/d orally, serum cholesterol levels were reduced 43% and serum triglyceride levels were reduced 34% (Table I). In mice, at 20 mg/kg/d ip, serum cholesterol levels were reduced maximally at 40 mg/kg/d by 36%, whereas serum triglyceride levels were reduced 42% at 20 mg/kg/d. In hyperlipidemic mice, serum cholesterol levels were elevated 183% (354 mg%) above normal control values (125 mg%). After treatment with 3-*N*-(1′,8′-naphthalimido)propionic acid for 14 d, serum cholesterol was elevated 23% above the control values, which were reduced 160% of the diet-induced cholesterol levels. The serum triglyceride levels in the hyperlipidemic mice were elevated 168% (367 mg/dL) above the control value (137 mg/dL). Drug treatment resulted in values 13% lower than the control. The drug had no effects on the consumption of food on a daily basis in the rats. Furthermore, the weight increase of the whole animal as well as the individual weights of the major organs were not affected by drug treatment for 2 weeks (Table II).

Examination of the *in vitro* effects of 3-*N*-(1′,8′-naphthalimido)propionic acid on the activities of enzymes required for *de novo* synthesis of cholesterol and triglycerides of mice showed that mitochondrial citrate exchange was markedly inhibited by drug treatment by 79% at 2.5 μmol (Table III). Citrate lyase, acetyl CoA synthetase, and HMG CoA reductase activities were inhibited marginally (20–30%).

Enzymes involved in the clearance of free cholesterol from the liver varied in the response to drug treatment. Cholesterol side-chain oxidation was inhibited at 2.5 μmol, and acyl CoA cholesterol acyl transferase activity was enhanced at 5 and 10 μmol (~35%). Activities of regulatory enzymes involved in fatty acid and triglyceride syntheses were significantly inhibited. Acetyl CoA carboxylase, *sn*-glycerol-3-phosphate acyl transferase, and phosphatidate phosphohydrolase activities were suppressed significantly, with the highest concentration (10 μmol) affording the maximum inhibition. Fatty acid synthetase activity was not affected by drug treatment.

Evaluation of some of the same enzymes after *in vivo* administration of 3-*N*-(1′,8′-naphthalimido)propionic acid demonstrated that citrate lyase was inhibited maximally at 20 mg/kg/d by 22% (Table IV). Acetyl CoA synthetase activity was suppressed 14% at 20 and 40 mg/kg/d. HMG CoA reductase activity was not altered by *in vivo* drug administration. 7-α-Hydroxylase, the regulatory enzyme for the conversion of cholesterol to bile acids, was significantly inhibited by drug administration, with 60 mg/kg producing the maximum effect (52% reduction). Acetyl CoA carboxylase activity was suppressed maximally 45% at 20 mg/kg/d. *sn*-Glycerol-3-phosphate acyl transferase activity was likewise inhibited maximally at 20 mg/kg/d by 51%. Phosphatidate phosphohydrolase activity was inhibited 31% at 20 mg/kg/d and 48% at 40 mg/kg/d. Fatty acid synthetase activity was marginally inhibited 19–20% at 20 and 40 mg/kg/d.

Lipid analysis of the liver of mice treated for 16 d with 3-*N*-(1′,8′-naphthalimido)propionic acid intraperitoneally showed that the number of milligrams per milliliter of total lipid was reduced 47 and 52% at 20 and 40 mg/kg/d, respectively (Table V). Cholesterol levels were reduced 20–30%. Triglyceride levels were markedly reduced (92–97%), whereas neutral lipid levels were reduced 72 and 84% at the same doses. At the dosage of 60 mg/kg/d, total lipid, cholesterol, triglycerides, and neutral lipids returned to more normal levels. The phospholipid levels were reduced at 10 mg/kg/d by 34%, but were significantly elevated at 20 and 40 mg/kg/d. Fecal samples obtained on day 16 demonstrated a marked increase in total lipid excreted as well as increases in cholesterol and neutral lipid levels; however, triglyceride and phospholipid levels were not elevated in the fecal samples.

Distribution studies in rats with [^{3}H]cholesterol showed that cholesterol absorption from the GI tract over a 24-h period was reduced 7%; however, excretion in the bile was accelerated 26% (Table VI). The milligram percent of cholesterol was elevated 38%, and the bile flow rate over 6 h was accelerated

Table IV—*In Vivo* Effects of 3-*N*-(1′,8′-Naphthalimido)propionic Acid on Male CF_1 Mouse Liver Enzyme Activities [a]

Enzyme	Control (1% Carboxymethylcellulose)	Enzyme Activity, % of Control			
		10 mg/kg/d	20 mg/kg/d	40 mg/kg/d	60 mg/kg/d
Citrate lyase	100 ± 8	79 ± 7[b]	78 ± 3[b]	83 ± 8	104 ± 9
Acetyl CoA synthetase	100 ± 7	91 ± 6	86 ± 7	86 ± 6[b]	92 ± 8
HMG CoA reductase	100 ± 6	95 ± 5	110 ± 7	112 ± 8	110 ± 6
7-α-Hydroxylase	100 ± 2	65 ± 14[b]	55 ± 13[b]	49 ± 4[b]	48 ± 5[b]
Acetyl CoA carboxylase	100 ± 5	85 ± 5[b]	55 ± 4[b]	73 ± 6[b]	110 ± 5
Fatty acid synthetase	100 ± 6	83 ± 6[c]	81 ± 5[b]	80 ± 7[b]	95 ± 5
sn-Glycerol-3-phosphate acyl transferase	100 ± 8	54 ± 5[b]	49 ± 4[b]	57 ± 4[b]	67 ± 7[b]
Phosphatidate phosphohydrolase	100 ± 5	87 ± 8	69 ± 7[b]	52 ± 6[b]	70 ± 8[b]

[a] After dosing for 16 d; expressed as percent of control (mean ± *SD*), $n = 6$. [b] $p \leq 0.0001$. [c] $p \leq 0.005$.

Table V—Effects of 3-*N*-(1′,8′-Naphthalimido)propionic Acid on Lipid Distribution of Mouse Liver and Feces [a]

	Lipid Extracted, mg	Cholesterol	Neutral Lipids	Triglycerides	Phospholipids	Protein
			Liver			
Control	100 ± 9	100 ± 7[b]	100 ± 4[c]	100 ± 5[d]	100 ± 8[e]	100 ± 4[f]
Treated						
10 mg/kg	97 ± 8	96 ± 8	33 ± 3[g]	23 ± 4[g]	66 ± 7[g]	101 ± 4
20 mg/kg	53 ± 7[g]	70 ± 6[g]	28 ± 2[g]	8 ± 2[g]	131 ± 7[g]	98 ± 2
40 mg/kg	48 ± 4[g]	71 ± 6[g]	16 ± 3[g]	3 ± 2[g]	127 ± 8[g]	95 ± 2
60 mg/kg	67 ± 6[g]	84 ± 7	23 ± 3[g]	10 ± 2[g]	108 ± 8	107 ± 3
			Feces			
Control	100 ± 6	100 ± 7[h]	100 ± 8[i]	100 ± 6[j]	100 ± 6[k]	
Treated						
10 mg/kg	145 ± 7[g]	145 ± 9[g]	197 ± 9[g]	100 ± 5	95 ± 7	
20 mg/kg	138 ± 6[g]	132 ± 3[g]	133 ± 7[g]	91 ± 6	95 ± 6	
40 mg/kg	150 ± 8[g]	163 ± 7[g]	153 ± 7[g]	91 ± 6	84 ± 5[g]	
60 mg/kg	145 ± 7[g]	139 ± 5[g]	161 ± 6[g]	102 ± 6	97 ± 7[g]	

[a] At 20 mg/kg/d; expressed as percent of control (mean ± *SD*). [b] 12.24 mg of cholesterol/g of tissue. [c] 28.35 mg of neutral lipid/g of tissue. [d] 4.77 mg of triglycerides/g of tissue. [e] 4.39 mg of phospholipid/g of tissue. [f] 4.5 mg of protein/g of tissue. [g] $p \leq 0.001$. [h] 7.81 mg of cholesterol/g of tissue. [i] 7.18 mg of neutral lipid/g of tissue. [j] 1.06 mg of triglycerides/g of tissue. [k] 2.02 mg of phospholipid/g of tissue.

Table VI—Effects of 3-*N*-(1′,8′-Naphthalimido)propionic Acid on Lipid Distribution in Rat Liver, Small Intestine, Bile, and Feces [a]

	Lipid, mg	Cholesterol	Neutral Lipid	Triglycerides	Phospholipids	Protein
Liver						
Control	100 ± 6	100 ± 7	100 ± 7	100 ± 8	100 ± 5	100 ± 6
Treated	78 ± 5[b]	83 ± 6[c]	74 ± 5[b]	28 ± 3[b]	107 ± 7	99 ± 6
Small Intestine						
Control	100 ± 5	100 ± 6	100 ± 8	100 ± 9	100 ± 10	—
Treated	45 ± 5[b]	82 ± 5[b]	97 ± 9	106 ± 8	120 ± 8[c]	—
Bile						
Control	—	100 ± 10	100 ± 9	100 ± 10	100 ± 8	—
Treated	—	138 ± 12[b]	135 ± 9[b]	72 ± 5[b]	127 ± 7[b]	—
Feces						
Control	100 ± 9	100 ± 10	100 ± 9	100 ± 8	100 ± 9	—
Treated	95 ± 7	128 ± 8[b]	127 ± 8[b]	90 ± 4	123 ± 5[b]	—

[a] After 14-d administration at 20 mg/kg/d; expressed as percent of control (mean ± *SD*), $n = 6$. [b] $p \leq 0.001$. [c] $p \leq 0.010$.

18% (Table VII). Phospholipid content of the bile was also elevated 27% compared with the control. The [^{3}H]cholesterol content in the small intestine, large intestine, chyme, and feces was elevated three, two, five, and two times, respectively, after drug treatment. The urine excretion of [^{3}H]cholesterol was lowered in treated animals, and higher levels of [^{3}H]cholesterol were found in major organs, *e.g.*, brain, heart, lung, liver, and stomach (Table VIII). Adrenal weights for the treated mice were identical to the control weights, indicating that the drug did not cause hypertrophy of the adrenal cortex as a compensatory mechanism of reduced serum cholesterol levels (Table VIII).

Rat lipoprotein fractions (Table IX) were analyzed, and drug treatment reduced cholesterol and neutral lipid in the chylomicron, low-, and high-density lipoprotein fractions of rats. Neutral lipids and triglyceride levels were reduced in the very low-density fractions. Phospholipid levels were reduced in the low-density lipoproteins, but elevated significantly in the high-density lipoprotein fraction. Protein content was essentially unchanged in all of the lipoprotein fractions of the rats. The calculation of the percentage of each lipid or protein concentration of the total lipoprotein particle (Table X) demonstrated slightly different distributions of the lipids. The cholesterol content was reduced in the chylomicron, the low-, and high-density lipoprotein fractions, but not in the very low-density fraction. The cholesterol reduction by drug was most dramatic in the low-density fraction. The cholesterol ratio of high-density/low-density lipoprotein fractions for the control was 0.394 and for the treated group was 0.449. Thus, drug treatment raises the value of this ratio. Triglyceride content was lowered significantly in the chylomicrons and very low-density lipoprotein fractions. Alterations of the low- and high-density lipoprotein fractions probably are not important, since triglyceride content is low in these fractions. Neutral lipids were lowered by drug administration in all four lipoprotein fractions; however, the magnitude of reduction was not as great as for cholesterol and triglycerides. Phospholipids were lowered in the low-density fraction but raised in the other three fractions. Protein content was elevated in all four fractions, probably due to the lipid lost from the lipoprotein fraction after drug treatment.

Data in Tables I–IX are expressed as percentage of control ± *SD*. The probable significant level (p) between each test group and the control group was determined by the Student's t test.

Table VII—Effects of 3-*N*-(1′,8′-Naphthalimido)propionic Acid on Rat Bile [a]

Drug	Bile Excretion Rate, mL/6 h	Total [^{3}H]Cholesterol, dpm/h	Cholesterol Conc., mg%	Phospholipid Conc., mg/mL
Control	100 ± 8[b]	100 ± 8[c]	100 ± 7[d]	100 ± 3
Treated	118 ± 8[e]	126 ± 9[f]	138 ± 12[f]	127 ± 7[f]

[a] After dosing at 20 mg/kg/d for 14 d; expressed as percent of control (mean ± *SD*), $n = 6$. [b] 93 μL of bile eluted/min. [c] 6279 dpm/mL. [d] 111 mg%. [e] $p \leq 0.010$. [f] $p \leq 0.001$.

DISCUSSION

3-*N*-(1′,8′-Naphthalimido)propionic acid was shown to be a potent hypolipidemic agent both by oral and intraperitoneal administration in normal and hyperlipidemic rodents. The suppression of serum lipids appeared to be related to the *in vitro* and *in vivo* inhibition of *de novo* cholesterol and triglyceride

Table VIII—Effects of 3-*N*-(1′,8′-Naphthalimido)propionic Acid after 14 d of Dosing on [^{14}C]Cholesterol Distribution in Holtzman Rats 24 h after Administration

	Control		3-*N*-(1′,8′-Naphthalimido)-propionic Acid	
Organ[a]	Total Organ, dpm	Recovery, %	Total Organ, dpm	Recovery, %
Brain	8,382	0.13	11,609	0.197
Heart	7,344	0.114	10,717	0.182
Lung	22,052	0.342	47,488	0.804
Liver	46,578	0.723	70,986	1.202
Spleen	13,770	0.214	37,900	0.642
Kidney	18,920	0.294	23,114	0.392
Stomach	9,270	0.144	21,552	0.365
Small intestine	174,705	2.711	539,857	9.145
Large intestine	33,413	0.518	56,376	0.955
Chyme	33,605	0.521	163,836	2.775
Feces	193,230	2.998	340,704	5.771

[a] $n = 6$.

Table IX—Effects of 3-*N*-(1′,8′-Naphthalimido)propionic Acid on Rat Serum Lipoprotein Fractions[a]

	Chylomicrons					Very Low-Density Lipoprotein				
	Cholesterol	Neutral Lipid	Triglyceride	Phospholipid	Protein	Cholesterol	Neutral Lipid	Triglyceride	Phospholipid	Protein
Control	100 ± 9[b]	100 ± 8[c]	100 ± 6[d]	100 ± 10[e]	100 ± 7[f]	100 ± 8[g]	100 ± 9[h]	100 ± 7[i]	100 ± 7[j]	100 ± 8[k]
Treated	58 ± 5[l]	66 ± 6[l]	47 ± 5[l]	90 ± 9	90 ± 8	92 ± 8	58 ± 6[l]	46 ± 3[l]	101 ± 9	96 ± 7
	Low-Density Lipoprotein					High-Density Lipoprotein				
	Cholesterol	Neutral Lipid	Triglyceride	Phospholipid	Protein	Cholesterol	Neutral Lipid	Triglyceride	Phospholipid	Protein
Control	100 ± 9[m]	100 ± 7[n]	100 ± 8[o]	100 ± 7[p]	100 ± 8[q]	100 ± 8[r]	100 ± 9[s]	100 ± 4[t]	100 ± 6[u]	100 ± 8[v]
Treated	45 ± 5[l]	49 ± 4[l]	36 ± 4[l]	54 ± 6[l]	91 ± 7	66 ± 8[l]	76 ± 7[l]	48 ± 4[l]	185 ± 10[l]	93 ± 7

[a] After administration of 20 mg/kg/d ip for 14 d; expressed as percent of control (mean ± *SD*), $n = 6$. [b] 337 μg/mL. [c] 67 μg/mL. [d] 420 μg/mL. [e] 149 μg/mL. [f] 184 μg/mL. [g] 190 μg/mL. [h] 98 μg/mL [i] 221 μg/mL. [j] 26 μg/mL. [k] 50 μg/mL. [l] $p \leq 0.001$. [m] 210 μg/mL. [n] 10 μg/mL. [o] 45 μg/mL. [p] 41 μg/mL. [q] 122 μg/mL. [r] 544 μg/mL. [s] 620 μg/mL. [t] 27 μg/mL. [u] 153 μg/mL. [v] 657 μg/mL.

Table X—Effects of 3-*N*-(1′,8′-Naphthalimido)propionic Acid on the Percent Composition of Each Lipid and Protein Fraction of the Total Lipoprotein Particle

	Cholesterol	Triglycerides	Phospholipids	Proteins	Total, μg/mL
			Chylomicrons		
Control	30.92	38.53	13.67	16.88	1090
Treated	28.22	28.51	19.39	23.88	691
			Very Low-Density Lipoproteins		
Control	39.01	45.38	5.34	10.27	487
Treated	49.86	29.06	7.41	13.68	351
			Low-Density Lipoproteins		
Control	50.24	10.77	9.81	29.19	418
Treated	38.73	6.64	9.07	45.49	244
			High-Density Lipoproteins		
Control	39.39	1.96	11.08	47.57	1581
Treated	28.35	1.03	22.35	48.26	1266

syntheses in the liver. 3-*N*-(1′,8′-Naphthalimido)propionic acid lowered the cytoplasmic level of acetyl CoA *via* mitochondrial citrate exchange, citrate lyase, and acetyl CoA synthetase inhibition. Acetyl CoA is a key intermediate precursor required for both cholesterol and triglyceride syntheses. Reduction of its level should lower serum levels of both lipids. The regulatory enzyme of cholesterol synthesis, HMG CoA reductase activity, was not inhibited by the drug; although clofibrate inhibits this enzyme (25), phthalimide (2) and saccharin (3) have been shown to have no effect on its activity. 3-*N*-(1′,8′-Naphthalimido)propionic acid did suppress the activity of the regulatory enzymes for fatty acid synthesis (acetyl CoA synthetase) and the regulatory enzymes for triglyceride synthesis (*sn*-glycerol-3-phosphate acyl transferase and phosphatidate phosphohydrolase). These enzyme activities were also reduced by saccharin and phthalimide derivatives (2, 3). Furthermore, the magnitude of the reduction is of a level to account for the reduction of serum triglyceride levels.

The interference with liver cholesterol synthesis by 3-*N*-(1′,8′-naphthalimido)propionic acid was not significant enough to explain the lowering of serum cholesterol levels. Thus, the distribution and excretion of cholesterol after drug treatment was evaluated. First, the drug did not accelerate cholesterol conversion to bile acid; in fact, the regulatory enzyme for this conversion, 7-α-hydroxylase, was inhibited by drug treatment *in vivo*. Side-chain degradation of cholesterol also was not increased, as measured *in vitro*. Second, [^{3}H]cholesterol excretion was accelerated in the bile and feces, indicating that the drug was accelerating clearance of cholesterol from the blood *via* biliary excretion. High contents of labeled material could be observed in the small intestine and large intestine, as well as the chyme and fecal material, suggesting that the extrahepatic circulation of cholesterol was reduced by the drug. Cholesterol absorption from the intestine after oral administration was reduced 7%. Higher levels of [^{3}H]cholesterol were observed in some of the major organs; however, when the lipid content of the liver and small intestine was examined, there was a decrease in cholesterol, triglycerides, and neutral lipids. Phospholipid content was elevated in both the liver and bile. Clofibrate treatment results in an increase in phospholipid content in the liver and serum (26). Fecal lipid levels reflect the increase in bile cholesterol and neutral lipid levels, which were elevated compared with the control values.

The lipid content of the blood lipoprotein fractions was lowered. Chylomicrons, which normally contain large concentrations of triglycerides, showed lower levels of cholesterol, neutral lipid, and triglyceride. Low-density lipoproteins, which normally contain a high concentration of cholesterol had markedly reduced cholesterol, neutral lipids, triglycerides, and phospholipid levels after drug treatment. Low-density lipoproteins are responsible for lipid deposition in atherosclerotic plaque in blood vessels. Reducing lipid levels in this lipoprotein fraction should also show reduction in the quantity of lipids being deposited in these plaques. Although it is difficult to extrapolate data from lipoprotein studies in rats to lipoprotein levels in humans, since the lipoprotein fractions are different, these studies demonstrate that 3-*N*-(1′,8′-naphthalimido)propionic acid modulated the lipid levels in the lipoprotein fraction and that 3-*N*-(1′,8′-naphthalimido)propionic acid shows promise as a potential hypolipidemic agent.

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High-Performance Liquid Chromatographic Determination of Metoprolol and α-Hydroxymetoprolol Concentrations in Human Serum, Urine, and Cerebrospinal Fluid

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Abstract □ A sensitive and simplified high-performance liquid chromatographic procedure was developed for the simultaneous quantification of metoprolol and α-hydroxymetoprolol in human serum, as well as cerebrospinal fluid and urine. Following protein precipitation with trichloroacetic acid, the sample was alkalinized with 1 M NaOH and extracted with dichloromethane. The mobile phase consisted of acetonitrile–water (50:50) containing 0.005 M 1-heptanesulfonic acid in 0.001% acetic acid. Using pronetalol as an internal standard, compounds were quantitated using fluorescence detection at 230 nm with a 300-nm emission filter and 0.02 AUFS. Extraction recovery is ~80% for both compounds. The lower limits of detection are 5 ng/mL and 4 ng/mL for metoprolol and α-hydroxymetoprolol, respectively.

Keyphrases □ Metoprolol—HPLC, α-hydroxymetoprolol, human serum, urine, and cerebrospinal fluid □ α-Hydroxymetoprolol—HPLC, metoprolol, human serum, urine, and cerebrospinal fluid

Metoprolol, a selective β-1-receptor blocker with an active hydroxy metabolite, is widely used in the treatment of several cardiovascular and neurological disorders (1–3). Many patients treated with standard oral doses of β-blockers experience either adverse toxic effects or receive no therapeutic benefit (4). Evaluation of the serum concentration profile of metoprolol may enable optimization of therapy for some patients. Several recent reports suggest that many of the pharmacological actions of metoprolol are paralleled by serum concentrations of metoprolol and/or α-hydroxymetoprolol (5, 6). Although α-hydroxymetoprolol may have relatively weak β-adrenergic blocking potency, its contribution to the other pharmacological actions of metoprolol remain to be determined (1, 2, 7). It is known that α-hydroxymetoprolol accumulates in the serum of patients with certain disease states, such as renal failure (7).

The current assay methods for the analysis of metoprolol include GC–MS (8), GC–EC detection (9), as well as high-performance liquid chromatography (HPLC) (10, 11). Most were designed primarily for quantification of only the parent drug. The assay procedure reported here is sensitive and specific for both metoprolol and the active hydroxy metabolite. Additionally, this procedure is useful for quantification of both compounds from serum and cerebrospinal fluid (CSF).

EXPERIMENTAL SECTION

Reagents—All chemicals and reagents were analytical grade unless otherwise indicated. Metoprolol tartrate[1], α-hydroxymetoprolol[2], and pronetalol[3] were all received as powders; stock solutions were prepared in methanol. Dichloromethane, acetonitrile[4], 30% trichloroacetic acid solution, sodium hydroxide[5], and sodium-1-heptanesulfonate[6] were used as received.

The HPLC system consisted of a delivery system[7], universal injector data module[7], and a variable-wavelength fluorescence detector[8]. Separation was conducted on a reverse-phase C_{18} column, 3.9 mm × 30 cm[9]. The mobile phase consisted of 25 mL of 0.005 M sodium-1-heptanesulfonate in 1.0% acetic acid diluted to 1 L with distilled deionized water (solvent A). The same amount of heptanesulfonic acid solution (25 mL) was diluted to 1 L with acetonitrile (solvent B). The mobile phase was a mixture of solvents A and B (50:50). A flow rate of 2 mL/min was maintained. The effluent was monitored at an excitation wavelength of 230 nm with a 300-nm UV interference filter, to set the emission wavelength, with an attenuation of 0.02.

Extraction Procedure—To 1 mL of serum (in a glass tube) was added 10 μL of a 3.0-μg/mL solution of the internal standard (pronetalol) and 200 μl of 30% trichloroacetic acid solution. This mixture was vortexed and then centrifuged[10] at 5000 rpm for 10 min. The clear supernatant (750 μL) was transferred and 375 μL of 1 M NaOH and 4.0 mL of dichloromethane were added. The mixture was vortexed for 30 s, then centrifuged for 10 min at 5000 rpm. The upper aqueous phase was aspirated and discarded; the lower organic phase was transferred, evaporated to dryness under a stream of nitrogen at 30°C, and reconstituted in 100 μL of the mobile phase. The extraction procedure for urine and (CSF) is exactly the same as described for serum.

Recovery Studies—Human serum and methanol samples were each spiked with two different concentrations of metoprolol and α-hydroxymetoprolol. The methanol samples were evaporated under a stream of nitrogen at 30°C, with internal standard added just prior to injection. The serum samples were extracted as described, with the exception that internal standard was again added prior to injection. Percent recovery was determined by comparison of peak height ratios between extracted samples and methanol samples. Five replicate determinations were made at each concentration. Similar studies were conducted using blank urine and CSF. To determine the within-day

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[2] Hassle Subsidiary, Molndal, Sweden.
[3] Imperial Chemical Industries, Hurdsfield Indust. Estate, Macclesfield, Cheshire, U.K.
[4] Burdick and Jackson Laboratories, Muskegon, Mich.
[5] Fisher Scientific Co., Fair Lawn, N.J.
[6] Eastman Kodak Co., Rochester, N.Y.
[7] Waters Associates, Milford, Mass.
[8] Model FS970; Schoeffel Instruments, Westwood, N.J.
[9] μBondapak C_{18}; Waters Associates, Milford, Mass.
[10] GLC-1 Centrifuge; Sorvall, Newton, Conn.

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