Effect of Diphenhydramine on Methaqualone Metabolism: An *In Vitro* Study

K. W. HINDMARSH ˣ, N. W. HAMON, D. F. LeGATT, and S. M. WALLACE

Abstract □ A GLC assay to quantitate the methaqualone metabolite 2-methyl-3-(2-hydroxymethylphenyl)-4(3H)-quinazolinone was developed. Standard curves were linear, and recovery of the metabolite from tissue homogenates averaged 89%. *In vitro* metabolism of methaqualone by the 10,000×g supernatant fraction of rat liver homogenate was measured by monitoring metabolite formation with the GLC assay. Diphenhydramine inhibited the *in vitro* metabolism of methaqualone. The percentage inhibition increased with increasing diphenhydramine concentration. The significance of this inhibition in relation to use and abuse of methaqualone–diphenhydramine combinations is discussed.

Keyphrases □ Diphenhydramine—effect on methaqualone metabolism in rat liver homogenate □ Methaqualone—metabolism, effect of diphenhydramine in rat liver homogenate □ Metabolism—methaqualone, effect of diphenhydramine in rat liver homogenate □ GLC—analysis, methaqualone metabolite in rat liver homogenate □ Antihistaminics—diphenhydramine, effect on methaqualone metabolism in rat liver homogenate □ Hypnotic–sedatives—methaqualone, metabolism, effect of diphenhydramine in rat liver homogenate

The abuse of methaqualone [2-methyl-3-O-tolyl-4(3H)-quinazolinone], a nonbarbiturate hypnotic, has been recognized as a serious problem among drug addicts (1). Methaqualone also was reported to be a street drug (2–6). The abuse of methaqualone and diphenhydramine in combination (7–9) and poisoning cases arising from such abuse have been reported (10–13). A combination pharmaceutical product containing both drugs emerged as an abuse drug among soldiers stationed in West Germany (14, 15). There has been no clearcut rationale for the combination of diphenhydramine and methaqualone in commercial drug preparations or for supplementation of methaqualone with diphenhydramine by abusers.

In vitro, the 10,000×g supernatant fraction of rat liver homogenate metabolizes methaqualone to 2-methyl-3-(2-hydroxymethylphenyl)-4(3H)-quinazolinone (16). This metabolite is also one major metabolite identified in human and rat urine (17). The monohydroxy metabolites do not show any marked biological activity (18, 19). Inhibition of metabolism may result in increased pharmacological activity of methaqualone.

The objective of this present study was to determine the effect of diphenhydramine on the metabolism of methaqualone *in vitro* by the rat hepatic 10,000×g supernatant fraction. Metabolism of methaqualone to 2-methyl-3-(2-hydroxymethylphenyl)-4(3H)-quinazolinone was monitored by GLC.

EXPERIMENTAL

Materials—Methaqualone hydrochloride[1], diphenhydramine hydrochloride[2], 1-chlorobutane[3], glucose 6-phosphate monosodium salt[4],

nicotinamide adenine dinucleotide phosphate[4], sucrose[5], semicarbazide hydrochloride[5], concentrated hydrochloric acid[5] (reagent grade), and magnesium chloride[6] were obtained .commercially, and appropriate aqueous solutions were prepared. Tris(hydroxymethyl)aminomethane hydrochloride (pH 7.4) buffer containing sucrose was prepared using 25 ml of 3.4 mM tris(hydroxymethyl)aminomethane, 20.7 ml of 3.4 mM HCl, and 5.58 g of sucrose. This mixture was diluted to 100 ml with distilled water.

Tris(hydroxymethyl)aminomethane hydrochloride (pH 5.5) buffer containing semicarbazide hydrochloride was prepared from 25 ml of 0.5 M tris(hydroxymethyl)aminomethane, 15 ml of 0.5 M HCl, and 0.17 g of semicarbazide hydrochloride. This solution was diluted to 100 ml with distilled water and heated for 1 hr at 40° prior to use. 2-Methyl-3-(2-hydroxymethylphenyl)-4(3H)-quinazolinone was synthesized according to previously described methods (16, 20).

Preparation of 10,000×g Supernatant Fraction—Adult male albino rats of the Wistar strain, 250 g, were fasted for 18 hr and then sacrificed by decapitation. The livers were removed and immediately placed in 15 ml of an ice-cold solution of tris(hydroxymethyl)aminomethane hydrochloride buffer and sucrose. The tissue was minced, weighed, and homogenized[7] in a volume of the same buffer equal to two times the liver weight. The homogenate was centrifuged[8] at 600×g for 10 min, and the resulting supernate was centrifuged twice at 10,000×g for 20 min.

The protein concentration of the 10,000×g supernate was determined using the colorimetric method described by Lowry *et al.* (21) and modified by Miller (22). Protein standards were prepared using bovine serum albumin.

In Vitro **Metabolism of Methaqualone**—*Control Studies*—The following reagents were added in the order given to a series of 25-ml erlenmeyer flasks: 1 ml of tris(hydroxymethyl)aminomethane hydrochloride buffer containing semicarbazide hydrochloride, 2 ml of 0.07 M magnesium chloride, 1 ml of 21.3 mM glucose 6-phosphate, 1 ml of 4.4
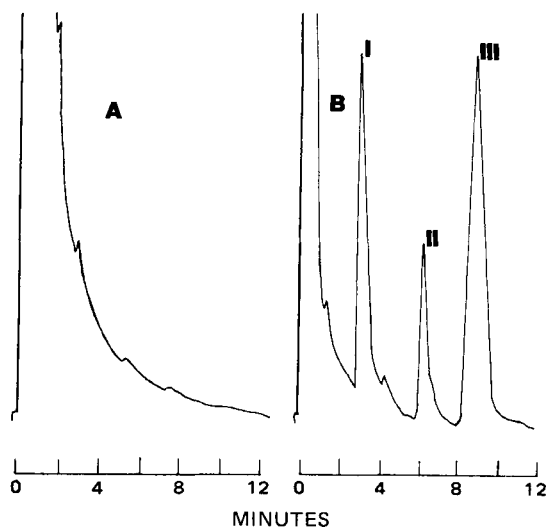

Figure 1—*Gas chromatograms of: A, control homogenate; and B, methaqualone (peak I), trimethylsilyl derivative of 2-methyl-3-(2-hydroxymethylphenyl)-4-(3H)-quinazolinone (peak II), and trimethylsilyl derivative of codeine (peak III).*

[1] William H. Rorer (Canada) Ltd., Bramalea, Ontario, Canada.
[2] Parke-Davis and Co., Brockville, Ontario, Canada.
[3] J. T. Baker Chemical Co., Phillipsburg, N.J.
[4] Sigma Chemical Co., St. Louis, Mo.
[5] Fisher Scientific Co., Fair Lawn, N.J.
[6] British Drug Houses (Canada) Ltd., Toronto, Ontario, Canada.
[7] Virtis "45," Virtis Co., Gardiner, N.Y.
[8] Model B-20, International Equipment Co., Needham Heights, Mass.

Table I—GLC Estimation of 2-Methyl-3-(2-hydroxymethylphenyl)-4(3H)-quinazolinone ($n = 3$)

Concentration of Metabolite, μg/μl	Peak Height Ratio	SD
5	0.89	0.008
10	2.22	0.006
15	2.69	0.02
20	3.97	0.02
30	6.39	0.006
40	8.37	0.02

Table II—Recovery of 2-Methyl-3-(2-hydroxymethylphenyl)-4(3H)-quinazolinone from Incubation Media Determined by GLC Assay

Concentration Added, μg/μl	Concentration Recovered, μg/μl	Percentage Recovery, mean
5	4.2, 4.4, 4.6	88.0
10	8.6, 8.6, 8.6	86.0
15	13.6, 13.6, 14.0	91.5
20	17.8, 18.0, 18.2	90.0
30	25.0, 25.0, 25.0	83.3
40	37.0, 37.5, 40.0	95.4
Mean ± SD		89.0 ± 4.5

mM nicotinamide adenine dinucleotide phosphate, and the 10,000×g supernatant fraction. Varying amounts of methaqualone hydrochloride (2.6–26.4 μmoles) were added to each flask, and the flasks were then incubated at 37° for 20 min in a metabolic shaker bath[9]. Three separate flasks were prepared for incubation at each methaqualone concentration.

Inhibition Studies—A 1-ml aliquot of 1, 3, or 5 mM diphenhydramine hydrochloride was added to an incubation mixture similar to that already described. To maintain the same total volume of incubation mixture as in the control studies, the volume of the magnesium chloride solution added was decreased to 1 ml.

Extraction Procedure—At the completion of the 20-min incubation period, 1 N NaOH (1 ml) was added to each flask. The flask contents were extracted separately with 4 × 6-ml portions of 1-chlorobutane using a shaker[10]. The shaker was maintained at a constant speed (15 oscillations/5 sec) for 10 min. The organic extracts from each flask were pooled and centrifuged[11] at 2500 rpm for 10 min. The upper organic layer was evaporated to dryness at room temperature under a gentle flow of air.

The residues were transferred quantitatively to 1-ml vials[12] using a small quantity of 1-chlorobutane. A 55-μl aliquot of aqueous codeine stock solution (1 μg/ml) was added to each vial, and the solvent was removed using a gentle flow of air. Silyl ethers were prepared by adding 15 μl of pyridine[12] (silylation grade), 30 μl of bis(trimethylsilyl)acetamide[12], and 10 μl of trimethylchlorosilane[12] to the resulting residue in each vial. Then the vials were stoppered and incubated at 100° for 1 hr. Aliquots (2–3 μl) of the silylated solution were injected into the gas chromatograph.

GLC Analysis—A gas chromatograph[13] equipped with a flame-ionization detector was employed. The chromatographic column was 1.2-m × 3.1-mm o.d., coiled, stainless steel tubing packed with 3% OV-101 on 80–100-mesh, high performance Chromosorb W[14]. The column was conditioned by maintaining the oven at 290° for 18 hr with low nitrogen flow. Operating temperatures were: injection port, 255°; column, 180°; and detector, 255°. The nitrogen flow rate was 80 ml/min. Hydrogen and compressed air flow rates were adjusted to give maximum response.

The metabolite concentration in the incubation medium was calculated
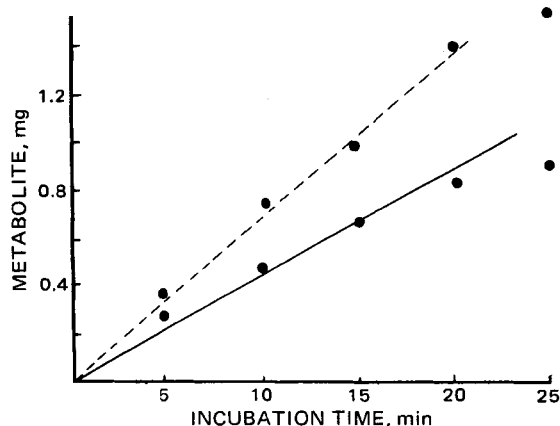

Figure 2—*2-Methyl-3-(2-hydroxymethylphenyl)-4(3H)-quinazolinone formation as a function of incubation time at methaqualone concentrations of 2.18 (●- -●) and 0.43 (●—●) mM.*

[9] Dubnoff incubator, GCA Precision Scientific, Chicago, Ill.
[10] Eberbach, Ann Arbor, Mich.
[11] International clinical centrifuge, International Equipment Co., Needham Heights, Mass.
[12] Reacti vials, Pierce Chemical Co., Rockford, Ill.
[13] Model 5750B, Hewlett-Packard, Avondale, Pa.
[14] Chromatographic Specialties, Brockville, Ontario, Canada.

from a standard curve plotted as the peak height ratio of the metabolite to the internal standard against known metabolite concentrations. Standard curves consisted of five concentrations of the metabolite ranging from 5 to 40 μg/μl.

RESULTS AND DISCUSSION

A method suitable for quantitation of the major *in vitro* metabolite was developed. Under the GLC conditions described, the retention times were 3.2 min for methaqualone (peak I), 6.5 min for the silyl derivative of 2-methyl-3-(2-hydroxymethylphenyl)-4(3H)-quinazolinone (peak II), and 9.2 min for silylated codeine (peak III) (Fig. 1B). No interfering peaks were observed on chromatographic analysis of an extract of control liver homogenate (Fig. 1A). The standard curves for the methaqualone metabolite were linear (r^2, coefficient of determination, 0.994) over the range of 5–40 μg/μl of the standard (Table I). Recovery of the metabolite from the homogenate was 89.0 ± 4.5% when compared to samples chromatographed directly without extraction (Table II).

A preliminary study indicated that diphenhydramine inhibited methaqualone metabolism when added to the incubation mixture. Three different diphenhydramine concentrations (1, 3, and 5 mM) were added to incubation mixtures containing the same amount of methaqualone (2.6 μmoles), and the peak heights for metabolite formation were compared to control samples to which no diphenhydramine was added. The percentage inhibition increased (45.9, 75.4, and 96.1%, respectively) as the diphenhydramine concentration increased. Neither diphenhydramine nor methaqualone affected the generation of reduced cofactor from the nicotinamide adenine dinucleotide phosphate added to the incubation mixture (as monitored spectrophotometrically at 340 nm).

Further experiments to investigate the kinetics of the enzymatic degradation of methaqualone and the effect of diphenhydramine on the reaction were initiated. A standard incubation time of 20 min was chosen after verifying that the conversion rate of the substrate, methaqualone, to the metabolite was constant for that time period under the study conditions. At methaqualone concentrations of 0.43 and 2.18 mM, the conversion of substrate to metabolite was linear for the 20 min (Fig. 2).

The velocity of the metabolite formation increased with increasing substrate concentration over a range of 0.43–4.4 μmoles of methaqualone/ml of incubation mixture. At substrate concentrations greater than 2.15 μmoles/ml, velocity increased only minimally (Fig. 3), suggesting
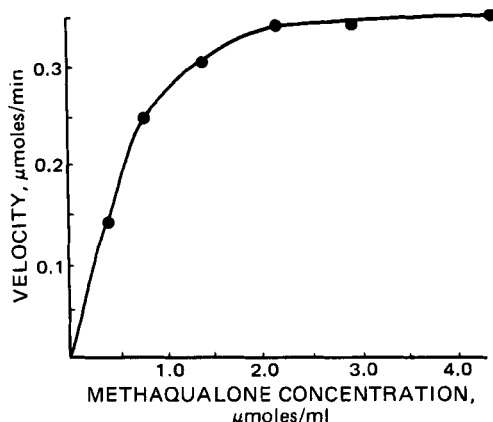

Figure 3—*Effect of increasing concentrations of methaqualone on the velocity of metabolite formation at a 20-min incubation time.*

Table III—Effect of Diphenhydramine on the Conversion of Methaqualone to 2-Methyl-3-(2-hydroxymethylphenyl)-4-(3H)-quinazolinone by Rat Liver Homogenate

Methaqualone Concentration, μmoles/ml	Inhibition of Metabolism, %	
	0.5 mM Diphenhydramine	0.83 mM Diphenhydramine
0.43	40.63	59.38
0.72	32.35	47.06
1.01	35.90	46.15
1.43	26.20	38.10
2.15	26.00	40.00

that the enzyme is saturated at concentrations greater than 2.15 μmoles/ml.

In the presence of diphenhydramine at concentrations of 0.5 and 0.83 mM, the velocity of metabolite production decreased (Fig. 4). Inhibition of conversion of methaqualone to 2-methyl-3-(2-hydroxymethylphenyl)-4(3H)-quinazolinone increased with increasing diphenhydramine concentration (Table III).

To evaluate maximal velocity, V_m, the Michaelis constant, K_m, and the inhibitory constant, standard methods for linearizing enzyme kinetic data were applied. Data for the velocity of metabolite formation at various substrate concentrations were plotted as $1/V$ against $1/S$ (Fig. 5) and as V against V/S. Although the correlation coefficients, r, for all data sets were significant ($p < 0.05$), systematic deviations from linearity were apparent. Curvature was more obvious without inhibitor than with 0.83 mM diphenhydramine and in plots of V against V/S than in plots of $1/V$ against $1/S$.

While recognizing the difficulties in interpreting nonlinear plots, K_m and V_m were estimated from the slope and intercept of the double-reciprocal plots (as calculated by least-squares linear regression) for five substrate and two inhibitor concentrations. The maximal velocity altered only slightly from 0.26 mmoles/min without diphenhydramine to 0.23 and 0.22 mmole/min with 0.5 and 0.83 mM diphenhydramine, respectively. The K_m as estimated from data obtained without inhibitor added was 0.30 mM. The apparent K_m with diphenhydramine concentrations of 0.5 and 0.83 mM increased to 0.62 and 1.06, respectively. The similarity in V_m values and the significant difference in apparent K_m values determined in the presence and absence of inhibitor suggest that diphenhydramine inhibition of methaqualone metabolism is competitive rather than noncompetitive.

One may speculate that such nonlinearity is due to independent binding sites with different binding affinities for the substrate (e.g., isoenzymes) or to cooperative binding of more than one substrate molecule. Isoenzymes have been demonstrated for a number of enzyme systems (23). Methaqualone metabolism is catalyzed by liver microsomal oxidoreductases (24). Functionally independent isoenzymes of micro-
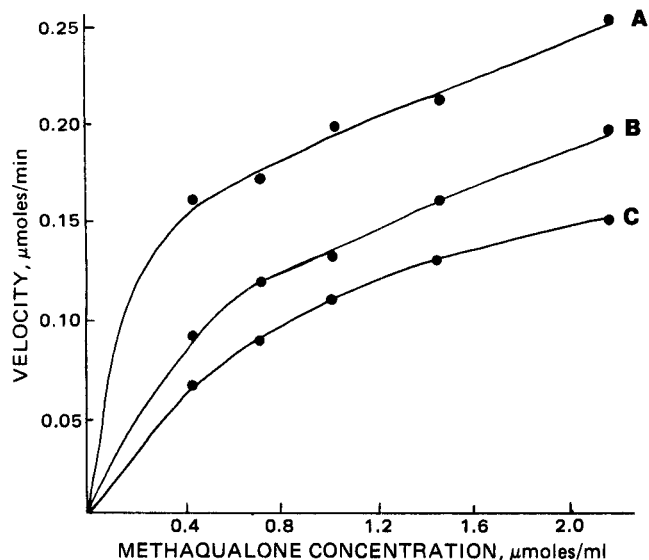

Figure 5—*Double-reciprocal plot of the effect of methaqualone (substrate) concentration on the initial velocity of metabolite formation in the presence of diphenhydramine at concentrations of 0 (●), 0.5 (■), and 0.83 (○) mM.*

somal oxidoreductases with different binding affinities for the substrate may exist. Naturally occurring enzymes appear to consist of a small number of similar subunits (22). Binding of a single substrate molecule may enhance or inhibit binding of subsequent substrate molecules. Therefore, affinity of binding appears to change as the substrate concentration is increased.

In conclusion, it has been demonstrated *in vitro* that diphenhydramine inhibits the metabolism of methaqualone by rat liver homogenate. The inhibition of the conversion of the pharmacologically active methaqualone to an inactive metabolite (18, 19) may explain the enhanced effect claimed by drug abusers for a methaqualone–diphenhydramine combination.

The significance of inhibition of methaqualone metabolism by diphenhydramine detected *in vitro* is presently being investigated *in vivo*.

Figure 4—*Effect of diphenhydramine on the velocity of metabolite formation at varying methaqualone (substrate) concentrations. Key: A, 0.00 mM diphenhydramine; B, 0.50 mM diphenhydramine; and C, 0.83 mM diphenhydramine.*
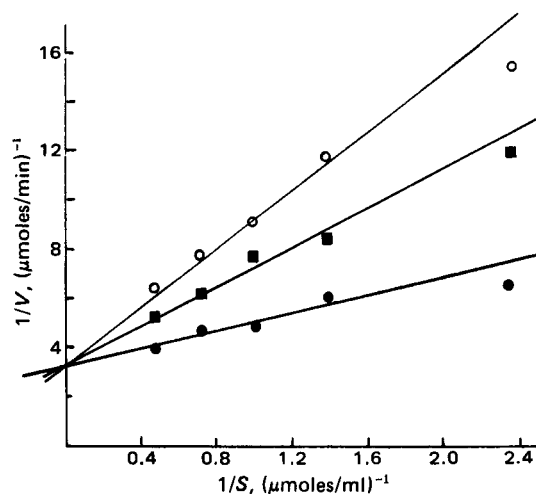
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Potential Antitumor Agents *via* Inhibitors of L-Asparagine Synthetase: Substituted Sulfonamides and Sulfonyl Hydrazides Related to Glutamine

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Abstract □ A series of 4-(substituted aminosulfonyl)- and 4-(substituted hydrazinosulfonyl)-2-aminobutanoic acids, compounds structurally related to glutamine, was synthesized as potential inhibitors of L-asparagine synthetase and subjected to screening as antitumor agents. Target amino acids were obtained by condensation of a blocked reactive sulfonyl chloride with the appropriate amine or hydrazide, followed by deblocking with hydrogen–palladium or liquid hydrogen fluoride–anisole. Neither the target compounds nor their protected precursors inhibited the enzyme from L5178Y/AR or prolonged the life of mice with P-388 lymphocytic leukemia. However, DL-4,4′-dithiobis[2-(benzyloxycarbonylamino)butanoic acid], an intermediate in the synthesis of the target amino acids, exhibited 90% inhibition of L-asparagine synthetase at 10 mM.

Keyphrases □ Sulfonamides, various—synthesized, evaluated for antineoplastic activity in mice and effect on L-asparagine synthetase activity *in vitro* □ Sulfonyl hydrazides, various—synthesized, evaluated for antineoplastic activity in mice and effect on L-asparagine synthetase activity *in vitro* □ Antineoplastic activity—various sulfonamides and sulfonyl hydrazides evaluated in mice □ L-Asparagine synthetase activity—effect of various sulfonamides and sulfonyl hydrazides evaluated *in vitro* □ Enzyme activity—effect of various sulfonamides and sulfonyl hydrazides on L-asparagine synthetase evaluated *in vitro* □ Structure-activity relationships—various sulfonamides and sulfonyl hydrazides evaluated for antineoplastic activity in mice and effect on L-asparagine synthetase activity *in vitro*

Asparaginase, the enzyme responsible for hydrolyzing L-asparagine to L-aspartic acid, is therapeutically useful in the treatment of certain tumors (1). However, its effectiveness is diminished by several factors, including an induced resistance by formerly susceptible tumor cells. Various reports (2–4) indicated that such resistance is attributable to the appearance of high levels of the biosynthetic enzyme L-asparagine synthetase (I), which catalyzes the transfer of the amide nitrogen of L-glutamine to L-aspartic acid to form L-asparagine (5). The relation between resistance to asparaginase and high levels of I and sensitivity to asparaginase and absence of I provides a distinct biochemical difference between certain tumors (high levels of I) and normal tissue (low levels of I), which should be capable of being exploited chemotherapeutically.

In studies aimed at developing inhibitors of I and potential antitumor agents, Brynes *et al.* (6) recently de-

scribed the synthesis and biological activity of asparagine analogs characterized by replacement of the amide carbonyl with a sulfonyl group. The compounds generally produced moderate (30–40% at 2 mM) inhibition of I isolated from Novikoff hepatoma, although 3-(hydroxysulfamoyl)-L-alanine displayed quantitative inhibition at 2 mM (6). Noting the importance of glutamine as a substrate for I (as well as for other significant biochemical events such as the biosynthesis of purines), this report describes the preparation and biochemical testing of several similar sulfonamides and sulfonyl hydrazides (VIa–VIg, Scheme I).

EXPERIMENTAL[1]

Chemistry—The route employed in obtaining target compounds VIa–VIg was analogous to that established previously (6) for a series of L-asparagine analogs. Generally, homocystine was fully protected by treatment first with benzyloxycarbonyl chloride and then with benzyl bromide in the presence of dicyclohexylamine. Subsequent cleavage of the blocked homocystine (III) with chlorine in aqueous acetic acid provided the reactive sulfonyl chloride IV, which was, in turn, allowed to react with the appropriate amine or hydrazide, yielding the blocked intermediates Va–Vg. Hydrogenolysis or treatment with hydrogen fluoride gave the desired free amino acids (Scheme I). All compounds were characterized by their melting points, elemental analyses, IR spectra, and homogeneity on TLC (visualization with ninhydrin).

DL-4,4′-*Dithiobis[2-(benzyloxycarbonylamino)butanoic Acid]* (*II*)
—This protected amino acid was prepared from DL-homocystine and benzyloxycarbonyl chloride in exactly the same manner as described for N,N′-di(benzyloxycarbonyl)-L-cystine (7). The use of 5.3 g (0.02 mole) of homocystine afforded 9.2 g (86%) of crystalline product from chloroform, mp 119–127°. Recrystallization from ethanol–water yielded an analytical sample, mp 138–139.5°.

Anal.—Calc. for $C_{24}H_{28}N_2O_8S_2$: C, 53.72; H, 5.26; N, 5.22. Found: C, 53.90; H, 5.44; N, 5.36.

Dibenzyl DL-4,4′-*Dithiobis[2-(benzyloxycarbonylamino)butanoate]* (*III*)—Diacid II (26.8 g, 0.05 mole), benzyl bromide (13.6 ml, 0.10 mole), and dicyclohexylamine (21.6 ml, 0.11 mole) were combined in 500 ml of dimethylformamide and allowed to stir overnight at room temperature. Following removal of the solvent *in vacuo*, the white solid was thoroughly

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