Studies on the Antioxidant Action of Thyroxine and Related Compounds[1]

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Thyroxine affords a high degree of protection against lipid peroxidation induced by Fe(II) or by a mixture of Fe(II) and ascorbate in isolated rat liver mitochondria, in rat liver homogenates, and in methyl arachidonate suspensions. The hormone also prevents spontaneous and CCl_4-accelerated peroxidation in 9000*g* supernatant fractions of rat liver. 3,5,3′-Triiodothyronine, 3,5,3′,5′-tetraiodothyropropionic acid, 3,5,3′-triiodothyroformic acid, and 2,6-diiodohydroquinone, a compound bearing a close structural resemblance to the prime ring of thyroxine, protect against peroxidation to about the same degree as the hormone. 3,5-Diiodothyronine is moderately efficient as an antioxidant, whereas thyronine and 3,5-diiodotyrosine are much less efficient. These findings suggest that the antioxidant action of thyroxine resides in its hydroquinone moiety, that the hormone acts as a typical phenolic antioxidant, and that the phenoxy free radical of thyroxine is involved in the molecular mechanisms underlying the antioxidant action.

The antioxidant action of thyroxine (T_4) has been recognized since De Caro[3] observed over three decades ago that the hormone and related compounds decrease oxygen uptake by solutions of unsaturated fatty acids. Subsequent investigators have confirmed these observations[4] and have demonstrated that the hormone also prevents lipid peroxidation in isolated erythrocytes,[5] in liver homogenates,[5b,6] and in isolated mitochondria.[7] In some of the studies with tissue preparations, prior injection of T_4 into animals from which the tissues were obtained protected against peroxidation.[5b,6b]

We recently observed[8] that T_4 protects isolated rat liver mitochondria against Fe(II)-induced swelling and lipid peroxidation. Some of our results indicated that the hormone exerts its protective effect by chelating Fe(II), a suggestion made by De Caro[3c,d] and strengthened by later reports on T_4 chelation of iron.[9] Other observations could not be explained in terms of a chelating mechanism. However, all of our findings were consistent with the suggestion of Bunyan and coworkers[5b] that the quinol monoether structure of T_4 enables it to act like the well-known phenolic antioxidants. The latter are believed to break peroxidation chain reactions by donating a hydrogen atom to chain-propagating peroxy free radicals to form peroxide molecules and relatively stable phenoxy free radicals.[10]

Wynn and co-workers[11] have pointed out that certain structural features of the T_4 molecule should enable it to form a stable phenoxy free radical. Borg[12] has demonstrated by epr spectroscopy that the hormone produces a stable free radical when treated with 1 equiv of oxidant. Furthermore, several investigators have postulated that a T_4 free radical plays a role in molecular mechanisms underlying certain *in vitro* effects of thyroid hormones.[13] This mounting interest in a T_4 free radical and its probable involvement in preventing lipid peroxidation prompted us to extend our studies on the antioxidant action of the hormone. We have, therefore, investigated the protective action of T_4 and a variety of related compounds against peroxidation in isolated rat liver mitochondria, in rat liver homogenates, and in methyl arachidonate suspensions. We have also tested T_4 for its ability to prevent spontaneous and CCl_4-accelerated peroxidation in the 9000*g* supernatant fraction of rat liver. The results of these studies are reported here.

Experimental Section

Materials.—L-Thyroxine, L-3,5,3′-triiodothyronine, L-3,5-diiodothyronine, 3,5,3′,5′-tetraiodothyropropionic acid, and L-3,5-diiodotyrosine were purchased as the free acids from Sigma Chemical Co. 3,5,3′-Triiodothyroformic acid was obtained from Cyclo Chemical Corp. 2,6-Diiodohydroquinone (2,6-DIHQ) was purchased from K and K Laboratories, Inc., and was purified by a procedure described in a recent paper from this laboratory.[14] Hydroquinone and 4-methoxyphenol were secured from Eastman Chemical Products, Inc.; *t*-butylhydroquinone from Aldrich Chemical Co., Inc.; butylated hydroxyanisole (a mixture of 2- and 3-*t*-butyl-4-methoxyphenol) from Sigma Chemical Co.; and 4,4′-dihydroxydiphenyl ether from K and K Laboratories, Inc. Reagent grade sucrose from Fisher Scientific Co., Baker and Adamson biological grade KCl from Allied Chemical Corp., and A grade Tris with a heavy metal content of less than 1 ppm from CalBiochem were used to prepare sucrose and KCl–Tris solutions. Methyl arachidonate was purchased from Applied Science Laboratories, Inc. Sodium ascorbate was obtained from

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TABLE I

PROTECTIVE EFFECT OF THYROXINE AND RELATED COMPOUNDS AGAINST LIPID PEROXIDATION AND ATTENDANT SWELLING IN EXPERIMENTS WITH ISOLATED RAT LIVER MITOCHONDRIA[a]

Compd	% inhib of peroxidation[b] at 5 μM	% inhib of peroxidation[b] at 1 μM	% inhib of swelling[c] at 5 μM
Thyroxine	100	88	100
3,5,3'-Triiodothyronine	94	47	100
3,5-Diiodothyronine	35	24	17
Thyronine	0	0	0
3,5,3',5'-Tetraiodothyropropionic acid	100	50	100
3,5,3'-Triiodothyroformic acid	100	45	94
2,6-Diiodohydroquinone	100	90	100
Hydroquinone	12	6	9
4-Methoxyphenol	19	6	4
3,5-Diiodotyrosine	0	0	0

[a] Peroxidation and swelling were initiated by 100 μM ascorbate plus 0.2 μM Fe(II). The degree of peroxidation was determined at the end of a 40-min incubation period by the TBA color reaction. Mitochondrial swelling was assessed by determining the absorbance change of the incubation mixture during the 40-min period. For other experimental conditions, see the text. [b] The TBA color (absorbance at 532 mμ) was 0.17 when ascorbate and Fe(II) were present and 0.02 when absent. The latter value was also obtained when mitochondria were incubated in the absence of ascorbate and Fe(II) but in the presence of the compound being tested for inhibitory activity. [c] The change in absorbance was 0.53 when ascorbate and Fe(II) were present. When these substances were absent, there was no change in absorbance. Some of the compounds being tested for inhibitory activity caused swelling themselves. At a concentration of 5 μM, swelling was produced consistently by 3,5,3',5'-tetraiodothyropropionic acid, 3,5,3'-triiodothyroformic acid, 2,6-diiodohydroquinone, and occasionally by T_4. Before per cent inhibition of ascorbate-Fe(II) swelling was calculated for these compounds, the change in absorbance caused by the compound alone was subtracted from that caused by the compound plus ascorbate and Fe(II). The swelling action of 2,6-DIHQ was blocked by ascorbate and Fe(II).

Sigma Chemical Co. Ferrous ammonium sulfate hexahydrate was used as the source of Fe(II). This salt and all other chemicals were reagent grade commercial preparations.

All solutions were prepared with deionized water (Continental Water Conditioning Corp.) with a specific resistance of 2,000,000 ohms cm or greater as it flowed from the resin bed.

T_4 and all the other compounds containing the diphenyl ether structure, except 4,4'-dihydroxydiphenyl ether, were dissolved initially in 0.1 *N* NaOH (0.052 ml/μmole), and the resulting solutions were diluted with water. The amounts of dilute NaOH added to the buffers along with the compounds being tested did not significantly change the pH of the buffers. All other substances tested for antioxidant activity were dissolved in water, some with the aid of heat.

Methods.—Previously described procedures[8,15] were followed in preparing rat liver mitochondria and in the peroxidation and swelling studies. Experiments were performed at 20–21° in 0.125 *M* KCl–0.02 *M* Tris, pH 7.4. The degree of peroxidation was assessed by the 2-thiobarbituric acid (TBA) color reaction.

Liver homogenates were prepared by the following procedure. A male Sherman rat (200–300 g) that had been allowed free access to food and water was decapitated. The liver was removed immediately and washed briefly with cold 0.125 *M* KCl–0.02 *M* Tris, pH 7.4. It was then placed in 40 ml of cold buffer and chopped into small pieces with stainless steel scissors. The mixture was transferred to a precooled Waring blendor jar, sufficient cold buffer was added to make a 5% homogenate, and the mixture was homogenized for 1 min at 19,000 rpm. The homogenate was transferred to a flask, placed in an ice bath, and used within 1 hr.

Peroxidation studies were performed with liver homogenates by procedures described previously for experiments with mitochondria suspensions.[8] The substances being tested were added to 5.50 ml of 0.125 *M* KCl–0.02 *M* Tris, pH 7.4. Instead of mitochondria stock solution, 0.50 ml of liver homogenate was added. After incubation for 30 min at 20–21°, a 2-ml portion of the mixture was removed for TBA color development by the procedure described for 2-ml volumes of mitochondria incubation mixture.

Peroxidation studies with methyl arachidonate suspensions were performed in essentially the same manner. Instead of mitochondria stock solution, 0.05 ml of 0.012 *M* methyl arachidonate in ethanol was added to 6 ml of 0.125 *M* KCl–0.02 *M* Tris, pH 7.4. After incubation for 20 min at 20–21°, a 2-ml portion of the mixture was removed for TBA color development.

The procedures employed by Ghoshal and Recknagel[16] were followed closely in preparing and studying the 9000*g* supernatant fraction of rat liver. A male Sherman rat (400–450 g) that had been allowed free access to food and water was decapitated. The liver was removed immediately and washed briefly with cold 0.112 *M* NaCl–0.05 *M* sodium phosphate buffer, pH 5.6. It was then placed in a fresh portion of cold buffer (2 ml/g of liver), chopped into small pieces with stainless steel scissors, and homogenized in a cooled Teflon-pestle homogenizer (clearance, 1524–2286 cm^{-1}). The homogenate was centrifuged at 9000*g* for 12 min at 0°. The supernatant was removed, placed in a flask surrounded by ice, and used within 1 hr.

Peroxidation was measured by the TBA color reaction after incubation of portions of the supernatant fraction in 20-ml beakers in a Dubnoff metabolic shaker. Duplicate 0.5-ml portions of supernatant were added to 5.5-ml vol. of 0.112 *M* NaCl–0.05 *M* sodium phosphate buffer, pH 5.6, containing the substances being tested. The suspensions were shaken gently in air at 37° for the desired period of time. Then a 2-ml portion was removed from each beaker, and the TBA color was determined exactly as described previously for 2-ml volumes of mitochondria incubation mixture.[8] Separate duplicate incubations were performed for each time interval studied.

Each experiment reported in this paper was performed in duplicate at least four times, and results were essentially the same.

Results

T_4 protected isolated rat liver mitochondria against lipid peroxidation induced by a mixture of ascorbate and Fe(II) (Table I). The hormone retarded Fe(II)-induced peroxidation in whole rat liver homogenates (Table II) and in methyl arachidonate suspensions (Table III). It also inhibited spontaneous and CCl_4-accelerated peroxidation in 9000*g* supernatant fractions of rat liver (Table IV). In liver homogenate experiments, T_4 exerted about the same degree of antioxidant activity on a molar basis as butylated hydroxyanisole and *t*-butylhydroquinone (Table II), both of which are highly efficient lipid antioxidants.[17] In liver homogenate experiments, T_4 was 50–100 times more efficient than the antioxidants hydroquinone and 4-methoxyphenol (Table II). The hormone was also much more effective than the latter compounds in preventing peroxidation in isolated mitochondria (Table I).

All of the T_4 analogs tested whose prime ring contained either one or two iodine substituents *ortho* to the phenolic hydroxyl group prevented peroxidation to about the same degree as T_4 (Tables I–III). These compounds included 3,5,3'-triiodothyronine, 3,5,3',5'-tetraiodothyropropionic acid, and 3,5,3'-triiodothyroformic acid. 3,5-Diiodothyronine and thyronine were considerably less active antioxidants, the latter being weaker than the former (Tables I–III). 3,5-Di-

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TABLE II

PROTECTIVE EFFECT OF THYROXINE AND RELATED COMPOUNDS AGAINST LIPID PEROXIDATION IN WHOLE RAT LIVER HOMOGENATE[a]

Compd	% inhib of peroxidation[b] at							
	1 μM	5 μM	10 μM	25 μM	50 μM	100 μM	250 μM	500 μM
Thyroxine	38	78	96					
3,5,3′-Triiodothyronine		66	90					
3,5,3′-Triiodothyroformic acid		76	100					
3,5-Diiodothyronine		38	52	64	76			
Thyronine		6	12	15	26	29		
2,6-Diiodohydroquinone	42	100	100					
4,4′-Dihydroxydiphenyl ether			60	80	98			
Butylated hydroxyanisole	50	100	100					
t-Butylhydroquinone	20	100	100					
Hydroquinone					18	36	82	100
4-Methoxyphenol					42	55	73	97

[a] Peroxidation was initiated by 5 μM Fe(II). The degree of peroxidation was determined at the end of a 30-min incubation period by the TBA color reaction. For other experimental conditions, see the text. [b] The TBA color (absorbance at 532 mμ) was 0.56 when Fe(II) was present and 0.01 when absent. The latter value was also obtained after incubation in the absence of Fe(II) but in the presence of the compound being tested for inhibitory activity.

TABLE III

PROTECTIVE EFFECT OF THYROXINE AND RELATED COMPOUNDS AGAINST PEROXIDATION IN METHYL ARACHIDONATE SUSPENSIONS[a]

Substance added[b]	Intensity of TBA color[c]
None	+5
Thyroxine, 1 μM	+1
3,5,3′-Triiodothyronine, 1 μM	+1
3,5-Diiodothyronine, 1 μM	+3
3,5-Diiodothyronine, 10 μM	+1
Thyronine, 1 μM	+5
Thyronine, 10 μM	+5
3,5,3′-Triiodothyroformic acid, 1 μM	+1
2,6-Diiodohydroquinone, 1 μM	+1

[a] Peroxidation was initiated by 2 μM Fe(II). The degree of peroxidation was determined at the end of a 20-min incubation period by the TBA color reaction. Since cloudiness in the final colored solutions prevented accurate determination of absorbance, the pink TBA color was estimated visually and, when present, assigned an intensity of +1 to +5. For other experimental details, see the text. [b] In addition to Fe(II). [c] In experiments in which Fe(II) was omitted, there was no detectable pink color, either in the absence or presence of the substances being tested for inhibitory activity.

iodotyrosine was ineffective in preventing peroxidation in isolated mitochondria (Table I).

2,6-Diiodohydroquinone (2,6-DIHQ) can be regarded as a T_4 analog in which the diiodophenyl ring bearing the alanine side chain is replaced by a hydrogen atom. This compound exhibited about the same degree of antioxidant action on a molar basis as T_4 (Tables I–III).

4-Phenoxyphenol and 4,4′-dihydroxydiphenyl ether can be regarded as thyronine analogs, the alanine side chain being replaced by a hydrogen atom in the former and by a hydroxyl group in the latter. Bunyan and co-workers[5b] observed that 4-phenoxyphenol was one-sixth as active as T_4 in protecting erythrocytes from vitamin E deficient rats against dialuric acid induced hemolysis. This hemolysis is accompanied by peroxidation and is inhibited by antioxidants.[5] Under the same experimental conditions, thyronine did not prevent hemolysis.[5b] In the present experiments with liver homogenates, 4,4′-dihydroxydiphenyl ether surpassed both thyronine and 3,5-diiodothyronine in antioxidant efficiency, the compound being 0.2–0.6 times as active as T_4 (Table II).

Peroxidation of mitochondrial lipids induced by such agents as Fe(II) and ascorbate is attended by mitochondrial swelling.[18] When peroxidation is blocked, mitochondrial swelling is also prevented.[18] In the present experiments with isolated mitochondria, prevention of swelling paralleled closely prevention of peroxidation (Table I).

Discussion

The results of this study confirm earlier observations that T_4 acts as a highly efficient antioxidant under a variety of experimental conditions. Bunyan, *et al.*,[5b] and Green, *et al.*,[6a] noted that on a molar basis T_4 affords about the same degree of protection as α-tocopherol against ascorbate-induced lipid peroxidation in liver homogenates from vitamin E deficient rats. T_4 and α-tocopherol are about equally effective in protecting isolated rat liver mitochondria against Fe(II)-induced peroxidation and swelling.[8,18a] The present findings indicate that T_4 prevents peroxidation in rat liver homogenates to about the same extent as the highly efficient antioxidants butylated hydroxyanisole and *t*-butylhydroquinone.

The antioxidant action of T_4 has not been shown to have physiological significance. However, Bunyan and co-workers[5b] found that administration of T_4 to weanling rats on a vitamin E deficient diet reduces the susceptibility of their erythrocytes to dialuric acid induced hemolysis. Lejsek and Šimek[6b] observed that homogenates of livers from T_4-pretreated undernourished rats undergo less spontaneous peroxidation than those from undernourished rats not pretreated with T_4. According to the report of Aragona and Barone[19] rats are rendered more susceptible to CCl_4-induced liver lesions by treatment with methylthiouracil and more resistant by T_4 treatment. These observations may reflect the antioxidant action of T_4 *in vivo*, since CCl_4 liver injury is currently believed to result from lipid peroxidation initiated during metabolism of CCl_4 by liver cells.[16,20] As observed in the present study with 9000*g* supernatant fractions

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TABLE IV

PROTECTIVE EFFECT OF THYROXINE AGAINST LIPID PEROXIDATION IN THE 9000*g* SUPERNATANT FRACTION OF RAT LIVER[a]

Additions	TBA value[b] after an incubation period of 15 min	20 min	25 min	30 min	35 min
None (control)	0.278	0.88	~1.2	~1.6	
CCl_4, 10 μl	0.59	~1.2			
Thyroxine, 2.5 μ*M*		0.052	0.220	0.51	
Thyroxine, 5 μ*M*		0.035	0.042	0.057	0.066
CCl_4, 10 μl, plus thyroxine, 2.5 μ*M*		0.084	0.280	0.70	
CCl_4, 10 μl, plus thyroxine, 5 μ*M*		0.137	0.042	0.053	0.083

[a] The degree of peroxidation was determined at the end of the desired incubation period by the TBA color reaction. For other experimental conditions, see the text. [b] Absorbance at 532 mμ.

of rat liver, CCl_4-accelerated lipid peroxidation is blocked by T_4.

T_4 appears to exert its antioxidant effect by intervening directly in peroxidation reactions. This is indicated by the observation that T_4 prevents peroxidation of methyl arachidonate suspensions and by similar observations with solutions of other unsaturated lipids.[3,4] Thus, in contrast to most other known thyroid hormone effects, it is not necessary to postulate preliminary interaction of the hormone with membranes, receptor sites, enzymes, or the like.

The results of the analog study demonstrate clearly that the antioxidant action of T_4 is not dependent upon the alanine side chain or upon the presence of both prime ring iodine substituents. For example, 3,5,3′-triiodothyroformic acid, an analog that contains hydrogen instead of iodine in the 5′ position and a carboxyl group in place of the alanine side chain, is almost as efficient an antioxidant as T_4. However, if both the 3′- and 5′-iodine substituents are absent, as in 3,5-diiodothyronine, the antioxidant efficiency is considerably lower. The finding that 2,6-DIHQ is fully as effective as T_4 in protecting against peroxidation suggests that the antioxidant property of the hormone resides in the hydroquinone moiety. We have not tested derivatives of 2,6-DIHQ in which simple ether substituents instead of the hydroxyl group are present in the 4 position. However, such compounds would be analogous to 4-methoxyphenol, butylated hydroxyanisole, and 4,4′-dihydroxydiphenyl ether, all of which in the present study equal or surpass the corresponding hydroquinones in antioxidant efficiency. Bunyan and co-workers[5b] noted that the monophenyl ether of hydroquinone is much more effective than hydroquinone itself in preventing hemolysis of erythrocytes from vitamin E deficient rats.

The efficiency of phenolic antioxidants, such as quinols and quinol monoethers, depends upon their tendency to form relatively stable phenoxy free radicals by donating a hydrogen atom to peroxy free radicals.[10] The observation that T_4 and 2,6-DIHQ are as effective as the highly efficient phenolic antioxidants butylated hydroxyanisole and *t*-butylhydroquinone suggests that T_4 and 2,6-DIHQ form free radicals with relative ease. This suggestion is in agreement with predictions based upon structural features[11] and with actual demonstration of a T_4 free radical[12] and a 2,6-DIHQ free radical[21] by electron spin resonance spectroscopy.

Possible involvement of the phenoxy free radical, or semiquinone, of T_4 in thyroid hormone action was suggested as early as 1941 by Niemann and Redemann.[22] The free radical has since been implicated in several *in vitro* actions of the hormone. These include stimulation of ascorbic acid oxidase,[13a] inhibition of Cu(II)-catalyzed oxidation of ascorbic acid,[13a] reduction of Cu(II),[13b] uncoupling of oxidative phosphorylation in isolated mitochondria,[13c,d] and stimulation of peroxidase-catalyzed reactions.[13f,g] Lein[13e] has suggested that photochemical activation of T_4 is attributable to formation of the phenoxy free radical and that the latter may be the active form of the hormone. Wynn and Gibbs[11a] postulated that the phenoxy free radical is the form in which T_4 binds to liver microsomes in initial degradation reactions. Barker and Shimada[23] have also postulated that a protein-bound quinoid free radical is an intermediate in the degradation of the hormone. Björkstén[24] has recently suggested that thionamide antithyroid compounds act by scavenging T_4 free radicals. This series of observations is extended by the present results, which indicate that the T_4 free radical is involved in the antioxidant action of the hormone.

The chemical structural requirements for the manifestation of T_4-like activity in intact animals have recently been summarized and reassessed by Jorgensen.[25] On the basis of structure–activity relationships established to date, he has advanced the hypothesis that the biological activity of T_4 depends upon certain stereochemical features of the molecule that enable the prime ring to function as a hydroquinone, with the ether oxygen assisting the release of electrons through the 4′-hydroxyl group. With respect to the antioxidant action of T_4, this hypothesis is supported by the present results, particularly by the observation that 2,6-DIHQ exerts the same degree of protection as T_4.

2,6-DIHQ has been included in very few analog studies on T_4 effects. Wynn and Fore[11b] recently demonstrated that this compound and its monomethyl ether, 2,6-diiodo-4-methoxyphenol, cause T_4-like uncoupling of oxidative phosphorylation in isolated mitochondria. Wolff and Wolff[26] noted that the hydroquinone exhibits about one-fourth as much inhibitory activity toward glutamic dehydrogenase as does T_4. We have observed that 2,6-DIHQ is somewhat more potent than T_4 as a mitochondrial swelling agent.[14] The hydroquinone also inhibits malic dehy-

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drogenase but somewhat less strongly than does T_4.[27] These observations suggest that 2,6-DIHQ merits further attention as a tool for elucidating molecular mechanisms underlying thyroid hormone action. Furthermore, the possibility should not be overlooked that 2,6-DIHQ may be a physiologically important thyroactive compound. It has been isolated from the thyroid gland[28] and has been produced from iodinated thyronines[29] and from 3,5-diiodotyrosine[28b,30] under conditions that could prevail *in vivo*.

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5,6-Dihydro-4H-1,3,4-thiadiazines. III. Chemistry and Pharmacology of a Series of Basic Derivatives[1]

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A series of basically substituted 5,6-dihydro-4H-1,3,4-thiadiazines was synthesized and tested for central nervous system depressant activity in mice. None of the compounds showed significant activity in the maximal electroshock, hydrochloric acid writhing, strychnine lethality, or the metrazol seizure threshold tests. All the compounds in the series showed some activity in the hexobarbital sleep time test.

Prior to the start of this work a series of substituted 5,6-dihydro-4H-1,3,4-thiadiazines had been synthesized and tested for pharmacologic activities.[3,4] These compounds were, in general, devoid of pharmacological activity in the mouse.[5] Certain members of this previous series of compounds inhibited monoamine oxidase activity of the brain tissue of the rat and possessed antimicrobial activity against certain types of bacteria and fungi.[4] Because of the novelty of the 5,6-dihydro-4H-1,3,4-thiadiazine heterocycle and because previously we had observed that certain pyridyl-substitued 5,6-dihydro-4H-1,3,4-oxadiazines exhibited central nervous system depressant activity in mice,[6] we decided to synthesize and test pharmacologically a series of basically substituted 5,6-dihydro-4H-1,3,4-thiadiazines. This paper reports the results of this study.

Chemistry.—The two synthetic methods used to prepare the members of the previously reported[3,4] series of thiadiazines are the treatment of a 2-(β-hydroxyalkyl)carboxylic acid hydrazide with P_2S_5 and the concentrated H_2SO_4 cyclodehydration of a 2-(β-hydroxyalkyl)thiocarboxylic acid hydrazide. Because the treatment of 2-methyl-2-(β-hydroxypropyl)isonicotinic acid hydrazide with P_2S_5 gave 5,6-dihydro-4,6-dimethyl-2-(4-pyridyl)-4H-1,3,4-thiadiazine (isolated as the dihydrochloride) in only 6% yield, and because previously[6] we had encountered difficulty in preparing the variously substituted 2-(β-hydroxyalkyl)nicotinic, -isonicotinic, and -picolinic acid hydrazides, we used four different synthetic methods to prepare the basically substituted thiadiazines listed in this paper. These methods are condensation of a β-hydrazinoalkylthiol with either (A) a nitrile, (B, C) an imino ester, (D) an aldehyde, or (E) cyanogen bromide (see Chart I).

Method A is straightforward and of wide scope as evidenced by the fact that it yielded a thiadiazine from aromatic nitrile,[1] aliphatic nitrile,[1] tertiary aminoalkylnitrile, and pyridylnitrile. This method, which involves heating at the reflux temperature for 18 hr a mixture of nitrile, β-hydrazinoalkylthiol, and ethanol and then distilling the mixture *in vacuo*, yielded an oil composed of the desired basically substituted thiadiazine plus impurities that exhibit infrared absorption at 2.9, 3.0, 3.1 (m, broad), and 6.16 μ (m). The oil was easily purified by treating it with alumina (Merck No. 71695) in benzene. Methods B and C demonstrate the ability of the imino ester derivative of a cyanopyridine to condense with a β-hydrazinoalkylthiol. The condensation of 1-(1-methylhydrazino)-2-propanethiol with 3-pyridinecarboxaldehyde (method D) in refluxing ethanol in the presence of pyridine gave tetrahydro-4,6-dimethyl-2-(3-pyridyl)-2H-1,3,4-thiadiazine (III). In contrast, we previously[1] had observed that condensation of 1-(1-methylhydrazino)-2-propanethiol with benzaldehyde in refluxing ethanol in the presence of pyridine gave the (2-mercaptopropyl)methylhydrazone of benzaldehyde. Method E illustrates condensation of a cyanogen halide with a β-hydrazinoalkylthiol to give an aminothiadiazine directly.

The structures assigned to the compounds in Table I were substantiated by elemental, infrared, and nmr analyses.

Pharmacology.—These thiadiazines were evaluated for central nervous system depressant activity using a battery of screening methods; the data are summarized in Table I. None showed significant activity in the maximal electroshock, strychnine lethality, hydrochloric acid writhing, or the metrazol seizure threshold tests.

(1) Paper II: *J. Heterocyclic Chem.*, **4**, 254 (1967).

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(3) D. L. Trepanier, W. Reifschneider, W. Shumaker, and D. S. Tharpe, *J. Org. Chem.*, **30**, 2228 (1965).

(4) D. L. Trepanier, U. S. Patent 3,290,303 (1966).

(5) In mice, these compounds did not show significant activity in the following standard screening tests: hexobarbital sleep time, HCl writhing, tremorine antagonism, maximal electroshock, strychnine protection, *d*-amphetamine aggregation, and monoamine oxidase inhibiting.

(6) D. L. Trepanier, P. E. Krieger, and J. N. Eble, *J. Med. Chem.*, **8**, 802 (1965).