TABLE I

Compound	Average M concentration required to depolarize to 45 mV	Equipotent M ratio
Acetylcholine	5×10^{-6}	1
Acetylthiolcholine	5×10^{-5}	17
Acetylthionocholine	5×10^{-6}	1.7

enzyme formed during the hydrolysis of either AcCh or AcSCh.

Electric eel acetylcholinesterase was used for these studies. The K_m of the thiono ester was found to be 6×10^{-4} M, compared with a K_m of 1×10^{-4} for AcCh and 0.6×10^{-4} for AcSCh. The V_{max} of the hydrolysis of acetylthionocholine was significantly lower than that of AcCh or AcSCh.

Experimental Section

Acetylthionocholine Bromide.—Na (200 mg) was dissolved in 5.0 g of 2-dimethylaminoethanol, followed by the addition of a solution of 8.0 g of ethyl thionoacetate[18] in 100 ml of toluene. A slow stream of N_2 was passed into the mixture which was heated at 97–98° for 2 hr. The reaction mixture was concentrated *in vacuo* in a 50° bath. The residue was acidified with 5 ml of concentrated HCl in 70 ml of ice-cold H_2O and the mixture was filtered. The filtrate was extracted with 200 ml of Et_2O to remove unreacted ethyl thionoacetate. The aqueous layer was shaken with 35 ml of ice-cold saturated Na_2CO_3, followed by extraction with 40-ml portions of Et_2O. The organic extracts were washed with H_2O and dried ($MgSO_4$). Addition of 2.0 ml of MeBr to the Et_2O led, after refrigeration, to the formation of 2.4 g of light yellow crystals. The product was recrystallized from 2:1 Me_2CO–EtOH; mp 151–152°;[19] uv λ_{max} (EtOH) 237 mμ (ϵ_m x 8440).

Anal. ($C_7H_{16}BrNOS$) C, 34.71; H, 6.65; S, 13.23. Found: C, 34.81; H, 6.68; S, 12.82.

Acetylthionothiolcholine Bromide.—This compound was obtained in 20% yield by the reaction of 6.0 g of 2-dimethylaminoethanethiol and 8.0 g of ethyl thionoacetate in toluene followed by quaternization with MeBr. After three recrystallizations from EtOH the product melted at 169°; uv (EtOH) λ_{max} 298 mμ (ϵ_{max} 7830).

Anal. ($C_7H_{16}BrNS_2$) C, H, S.

Depolarizing Activity.—The depolarizing activity of acetylthionocholine was measured in the isolated single cell electroplax preparation, using cells from the electric organ of *E. electricus*.[20,21] Eserine was added to prevent hydrolysis by acetylcholinesterase present in the tissue.

Hydrolysis by Acetylcholinesterase.—Highly purified electric eel acetylcholinesterase was used for these studies. Enzyme assays were carried out titrimetrically, using a Radiometer autotitrator. A constant pH of 7.5 ± 0.02 was maintained during enzymatic hydrolysis by the automatic addition of 0.01 N NaOH to neutralize the acetic acid produced by substrate hydrolysis. Initial rates were used, the rate being constant for at least 2 min.

Acknowledgments.—We are indebted to Mrs. Eva Bartels of Columbia University for the electroplax assays and to Professor David Nachmansohn of Columbia University for a gift of the acetylcholinesterase used in these studies. This work was supported by grants from the National Science Foundation (GB-6835) and the National Institute for Neurological Diseases (NB-07835).

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(21) H. B. Higman and E. Bartels, *ibid.*, **54**, 543 (1962).

In Vitro Inhibition of Cholesterolgenesis by Various Thyroid Hormone Analogs

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Nineteen thyroid hormone analogs were tested in an *in vitro* cholesterolgenic liver homogenate system obtained from rats. ^{14}C-Acetate was used as substrate, DMSO was used as a solvent for adding the thyroid hormone analogs to the system. Of those compounds tested, L-triiodothyronine, D-thyroxine, DL-triiodothyronine, and DL-3,5-diiodo-3′-phenylthyronine at 1.0×10^{-4} M inhibited cholesterolgenesis from ^{14}C-acetate substrate. No effect was elicited by T_3 or T_4 when mevalonate was substrate. These studies indicated that the 3′-I or a bulky 3′-Ph associated with either D- or L-thyronine is necessary for inhibition of *in vitro* cholesterolgenesis. The D isomer is more active than the L isomer in this system. The need for higher than physiological levels of the thyroid hormones (triiodothyronine or thyroxine) in this system is in part a result of the nonspecific binding of the hormones to inert proteins in the homogenate system. When enough hormone is present to saturate the binding sites on the inert proteins the remaining hormone binds to active proteins contained in the microsomes resulting in an inhibition of cholesterolgenesis.

The alteration of blood cholesterol concentrations associated with thyroid function is well known. The hypocholesterolemia associated with thyroxine administration is attributed in part to an increased conversion of cholesterol into bile acids which overrides the increased cholesterolgenesis caused by thyroxine.[1] In thyroidectomized animals a decrease in the level of β-hydroxy-β-methylglutaryl-coenzyme A reductase (HMG-reductase) occurs whereas the administration of thyroxine increases the levels of HMG-reductase resulting in increased cholesterolgenesis.[2,3] In addition to the studies above, a new parameter was recently reported in which the addition of L-triiodothyronine (T_3) and L-thyroxine (T_4) to an *in vitro* cholesterolgenic rat liver homogenate from euthyroid rats resulted in decreased cholesterolgenesis.[4] The studies presented here are an extension of those studies and indicate that a structural specificity similar to that of T_3 and T_4 is

(1) D. Kritchevsky, *Metabolism*, **9**, 984 (1964).

(2) W. Gruder, I. Nolte, and O. Wieland, *Eur. J. Biochem.*, **4**, 273 (1968).

(3) F. A. Gries, F. Matschinsky, and O. Wieland, *Biochim. Biophys. Acta*, **56**, 615 (1962).

(4) C. D. Eskelson, *Life Sci.*, 467 (1968).

generally necessary for the decreased cholesterolgenic response. Evidence is presented indicating that the hormone response resides primarily in the microsomes and that the response occurs only after all the T_3 or T_4 binding sites on the soluble proteins and most of the binding sites on microsomal proteins are satisfied.

Methods

The methods have been described earlier[4] and consist of a broken cell preparation of liver containing only microsomes and the soluble cellular fraction prepared with modification according to the procedure of Bucher and McGarrahan.[5] Either ^{14}C-acetate or ^{14}C-mevalonate was employed as substrate. Cofactors and pH 7.0 phosphate buffer described by Knauss, *et al.*,[6] were also added to the enzymic system. All of the thyroid analogs were added to the system in 0.2 ml of DMSO. The final total volume of the system was adjusted to 5.0 ml and incubated for 1 hr in a water bath maintained at 37°. The *de novo* synthesized cholesterol was isolated from the system by extraction with petroleum ether and precipitation with tomatine. The radiometric determination was made according to the procedure of Kabara, *et al.*[7]

The disintegration rate/min (dpm), mean, standard deviation (std dev), and student "t" tests between means were computed by an IBM 1620 computer. The tabular and graphical representations are expressed as the average of four determinations ± the standard deviation. Only P values equal to or greater than 98% level were accepted as significant.

Many of the enzyme preparations used in these studies were stored up to 7 weeks at Dry Ice temperatures which does not alter enzymic activity in this system.[8]

Experimental Section

T_3 and T_4 Binding Studies.—The binding of T_3 and T_4 to the proteins and microsomes in the enzymic system was determined in the regular incubation mixture containing nonradioactive acetate substrate.

The thyroid hormones were added to the liver homogenate system at 1.0×10^{-3}, 1.0×10^{-4}, and 1.0×10^{-5} M, with a final T_4 concentration of 4.4×10^{-8} M and a final T_3 concentration of 4×10^{-9} M. There were 5.86 μCi of radioactive T_4 and 5.68 μCi of radioactive T_3 in the respective flasks.

The studies were done in duplicate by incubating the enzymic system containing the various concentrations of hormones for 1 hr. Each flask's contents was cooled to ice temperatures and then transferred to individual centrifuge tubes by rinsing with 6 ml of 3:1 H_2O solution-pH 7.0 buffer. The solution was centrifuged at 0°, 104,000g for 30 min and the supernatant was poured off and diluted to 15 ml with H_2O. The supernatant was allowed to drain for 10 min from the microsome button in the bottom of the centrifuge tube. The microsomes were then dissolved in 2 ml of 5 N NaOH. This solution (1 ml) was counted in a well-type scintillation counter as was 1 ml of the diluted supernatant. To 1 ml of the diluted supernatant was added 2 ml of 0.6 N $HClO_4$ and the protein button obtained by centrifugation. The excess supernatant fluid was allowed to drain from the protein button for 3 min. The radioactivity in the protein button was determined by means of a well-type scintillation counter. The values in Figures 3 and 4 are expressed as the per cent of the total radioactive T_3 or T_4 added to the enzymic system.

Determination of Thyroid Hormone Site of Action.—To determine the site of action of the thyroid hormones in this system ^{14}C-acetate was used as substrate. The experiment was conducted using nine groups. The enzyme pool (liver homogenate) was divided into three equal volumes. To the control enzyme pool was added 0.1 ml of DMSO/ml of enzyme solution. To the second enzyme pool was added 0.1 ml of DMSO/ml of enzyme solution and containing enough L-T_3 so that the final concentration in the enzyme mixture is 2.5×10^{-4} M. To the third enzyme pool was added 0.1 ml of DMSO/ml of enzyme solution and containing enough L-T_4 so that the final concentration in the enzyme mixture is 2.5×10^{-4} M. These enzymic pools were then incubated for 15 min at 37° and then rapidly cooled to 5°. The microsomes and soluble proteins were next separated from each pool by centrifugation at 0° and at 104,000g. The microsomes from 60 ml each of the pretreated homogenate were resuspended in 20 ml of a 50:50 dilution (pH 7.0) phosphate incubation buffer with H_2O. Crossover studies using microsomes (M) which were pretreated with DMSO, those pretreated with L-T_4 (M_{T_4}), and those pretreated with L-T_3 (M_{T_3}) were done by adding the various soluble enzymes which were obtained from the pretreated enzymic pool, *i.e.*, those soluble enzymes (E) that were pretreated with DMSO, with L-T_4 (E_{T_4}), and with L-T_3 (E_{T_3}). The first group which served as the control group contained ten samples. The remaining groups each consisted of four samples. To the samples of the first, second, and third groups was added 1 ml of the microsomal suspension from the enzymic preparation pretreated with DMSO.

To the samples of the first, second, and third groups was added 2 ml of the enzyme solutions which were pretreated with DMSO, L-T_3, and L-T_4, respectively. To all of the samples of groups 4, 5, and 6 was added 1 ml of microsomal suspension obtained from the enzymic pool pretreated with L-T_3. To the samples of the fourth, fifth, and sixth groups was added 2 ml of enzyme solutions which were pretreated with L-T_3, L-T_4, and DMSO, respectively. To all of the samples of groups 7, 8, and 9 was added 1 ml of microsomal solution obtained from the enzymic pool pretreated with L-T_4. To the samples of the seventh, eighth, and ninth groups was added 2 ml of enzyme solutions which were pretreated with L-T_3, L-T_4, and DMSO, respectively. The results are expressed as described earlier (see Table IV).

Results

The decreased rate of cholesterolgenesis by T_3 is illustrated in Figure 1. The complete system using acetate as substrate with DMSO added as a control proceeds with a linear rate, after a 15-min lag. It is believed that the lag is due to the equilibration of the system to substrates, temperature, etc. T_3 added at 1.0×10^{-4} M depresses the rate of cholesterol synthesis to about 50% of its normal rate. No inhibition is observed in the cholesterol biosynthesis system with T_3 when mevalonate is used as substrate (Table I).

The inhibition of cholesterolgenesis from acetate by T_3 is proportional to the added T_3 concentration. Figure 2 depicts the sensitivity of T_3's inhibition as beginning at the level of 2.5×10^{-5} M.

The results of the binding experiments are shown in Figures 3 and 4. It was previously reported[9,10] that T_3, T_4, and other thyroid analogs bind to subcellular components: microsomes, mitochondria, soluble proteins, etc. T_3 is bound to both microsomes (M) and soluble cellular proteins (E) of this system. The relative binding of the two fractions for T_3 varies with the T_3 concentration; for the microsomes, the per cent binding of the total ^{131}I-T_3 added to the enzymic system increases from 32% at 4×10^{-9} M to 61% at 1×10^{-3} M,

(5) N. L. R. Bucher and K. McGarrahan, *J. Biol. Chem.*, **222**, 1 (1956).

(6) J. B. Knauss, J. W. Porter, and G. Wasson, *ibid.*, **234**, 2835 (1959).

(7) J. J. Kabara, J. T. McLaughlin, and C. A. Riegel, *Anal. Chem.*, **33**, 305 (1961).

(8) C. D. Eskelson, Ph.D. Thesis, University of Nebraska, Lincoln, Neb., Jan 1967.

(9) J. R. Tata, L. Ernster and E. M. Suranyi, *Biochim. Biophys. Acta*, **60**, 480 (1962).

(10) J. R. Tata, L. Ernster, and E. M. Suranyi, *ibid.*, **60**, 461 (1962).

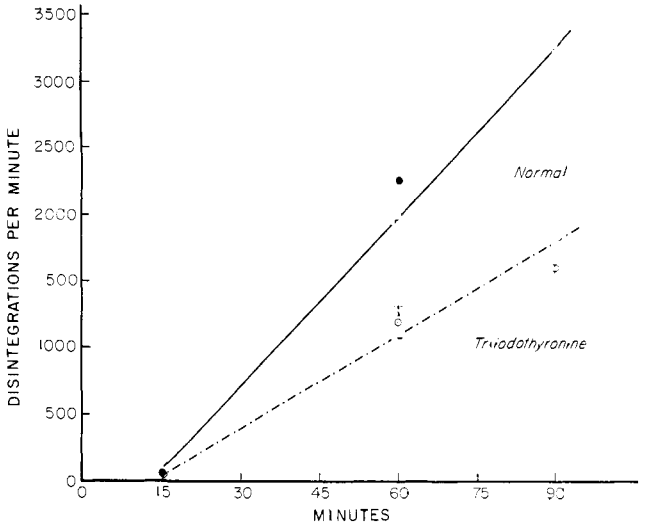

Figure 1.—The time course of ^{14}C-acetate incorporation into cholesterol and the effect of L-triiodothyronine on cholesterol synthesis. The ordinate represents corrected DPM observed in the isolated cholesterol tomatinide. Each point of the curves represents the average of four replicates ± the standard deviation: control line (●); T_3 line (ϕ).

whereas the percentage binding to the protein fraction decreases from 44% to 8% at the respective T_3 concentrations.

In this system most of the T_4 binding sites on the soluble proteins are saturated at 1×10^{-3} *M* T_4, since the total T_4 bound to the proteins drops off significantly at this hormone concentration. The data for T_4 binding in Figure 4 are generally similar to those for the T_3 binding curves. T_4 binds to both microsomes and the soluble proteins, the degree of binding varying with the T_4 concentration. However some differences are noted. At 1.0×10^{-5} *M* T_4 and T_3, 30% of the T_4 and 15% of the T_3 are bound to soluble protein, whereas 52% T_4 and 60% T_3 are bound to the microsomes, respectively. The binding of sodium radioiodide to the soluble protein and microsomes was also studied in this system. At a NaI concentration of 1.0×10^{-3} *M*, 1.8% of radioiodide was bound to microsomes; 5.1% was bound to the soluble proteins; and 93.0% was found in the supernatant fluid. The high levels of binding of T_3 and T_4 to the microsomes plus the very low level of binding of radioiodide to the microsomes lends credence to the metabolic stability of the T_3 and T_4 in this system. Tata, *et al.*,[9] in their studies have shown by chromatography that 76–83% of the radioactivity present in the various subcellular components is undegraded T_3 and T_4.

The 19 thyroid hormone analogs were tested in the standard system using ^{14}C-acetate as the precursor. These results are expressed in Table I. Also tested was the effect of T_3, D-T_4, L-thyronine, and DL-T_3 on cholesterolgenesis using ^{14}C-mevalonate as the precursor. Because of the limitations of the number of samples to be assayed, the testing had to be conducted in a series of four experiments. In each case a group of samples to which T_3 was added was included to compare T_3's action for that preparation with the other analogs. All precursors were tested at 1.0×10^{-4} *M* concentration. Experiment 1 indicates that the solvent, DMSO, used for dissolving the analogs enhances cholesterol synthesis from acetate but not from mevalonate. This

TABLE I

THE EFFECT OF VARIOUS THYROID ANALOGS ON *in Vitro* CHOLESTEROLGENESIS[a]

Group No.	Additions to the enzymic system	From ^{14}C-acetate substrate, dpm ± std dev		From ^{14}C-mevalonate substrate, dpm ± std dev	
		Expt 1			
1	Control	2388[b]	185		
2	0.2 ml of DMSO	4006[c]	180	18,977	895
3	L-T_3	2117[d]	186	18,872	507
4	D-T_4	2638[d]	915	18,717	739
5	L-Thyronine	5228	1318	19,428	937
6	DL-T_3	1813[d]	125	19,740	794
		Expt 2			
1	Control	4121	400		
2	L-T_3	3021[c]	261		
3	3,5-Diiodo-3′-L-ethylthyronine	3786	793		
4	3,5-Diiodo-3′-L-isopropylthyronine	4105	593		
5	3,5-Diiodo-3′-L-phenylthyronine	1930[c]	199		
6	3,5-Diiodo-3′-L-isobutylthyronine	4442	882		
7	3,5-Diiodo-3′-L-*t*-butylthyronine	3966	495		
		Expt 3			
1	Control	3465	441		
2	L-T_3	1633[c]	71		
3	3,5-Diiodothyronine	2861	374		
4	3-Iodothyronine	3760	396		
5	3,3′,5′-Trichlorothyronine	3421	20		
6	3,3′-Dichlorothyronine	3152	344		
7	3-Chlorothyronine	3641	62		
8	Thyroxamine	3585	221		
9	Each of 3-iodothyronine and L-T_3	2661	184		
		Expt 4			
1	Control	20,864	1549		
2	T_3	18,181[c]	1002		
3	3,5,3′,5′-Tetraiodothyropropionic acid	23,507	1525		
4	3,5,3′,5′-Tetraiodoformic acid	23,013	854		
5	3,5,3′-Triiodothyropropionic acid	22,125	788		
6	3,5,3′-Triiodothyroacetic acid	23,221	1153		

[a] Nineteen analogs were tested in a series of four experiments. All analogs were added in 0.2 ml of DMSO solvent so that the final concentration was 1.0×10^{-4}*M*. The first four analogs listed in expt 1 were also tested in this system using ^{14}C-mevalonate as the precursor for cholesterol synthesis. [b] The results are expressed as the average of four replicates of the corrected dpm ± the standard deviation of the isolated cholesterol tomatinide. [c] Significant from group 1. [d] Significant from group 2.

has been previously reported and observed faithfully for the system[8] (see Table I). As a consequence, all testing included a control group to which DMSO was added. All analogs added were dissolved in the same volume of DMSO (0.2 ml) as was added to the control. We have also observed the inhibition of T_3 on cholesterol synthesis using acetate as precursor when aqueous solvents were used in the control and as the solvent vehicle for T_3.[4] The basal rate at which cholesterol synthesis proceeds in the standard system using ^{14}C-acetate as precursors varies with each preparation. The variability is known to be related in part to the nutritional status of the animals prior to sacrifice as well as to other factors of preparation of the enzyme extracts.[5,6,8,11]

Of the analogs tested, none affected synthesis of cholesterol from mevalonate. Whether this is true for

(11) T. A. Miettinen, *J. Lab. Clin. Med.*, **71**, 537 (1968).

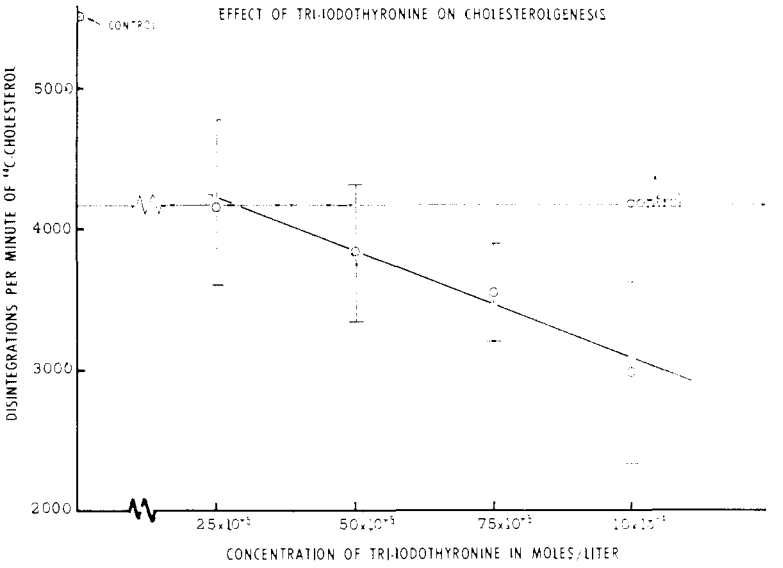

Figure 2.—Effect of increasing concentration of T_3 on cholesterolgenesis from acetate. Control line is the negative of the standard deviation from the control value (see arrow on ordinate). The control value and each point on the curve with added T_3 (⊕) represents the average of four replicates ± standard deviation.

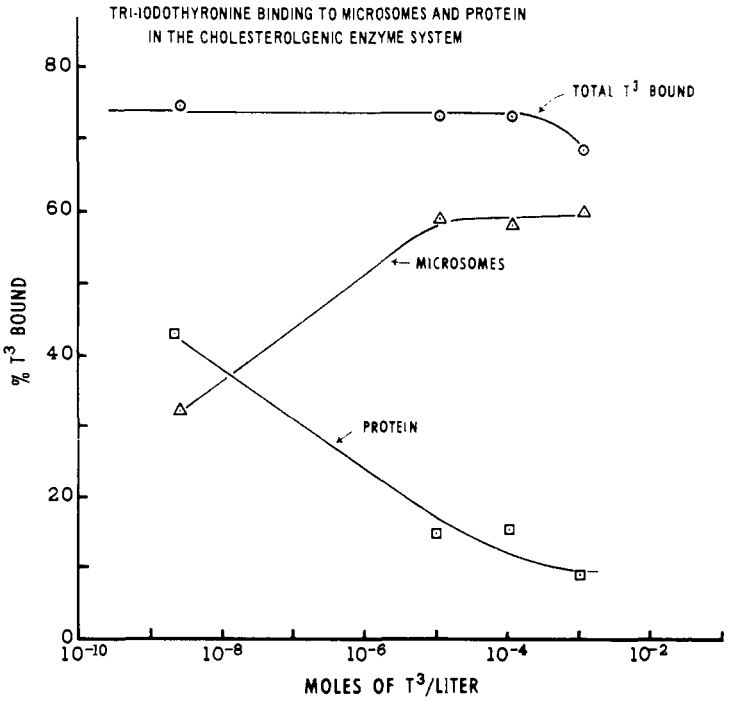

Figure 3.—Per cent of total [^{131}I]-labeled T_3 added to the enzymic system which is bound to microsomes (△) and soluble protein (⊡) fractions. Each point represents the average of duplicate determinations. The total T_3 bound fraction is equal to the sum of the T_3 bound to protein and microsomal fractions (⊕).

the other analogs, which were not tested, is not known.

Of the analogs tested, T_3, DL-T_3, D-T_4, and DL-3,5-diiodo-3′-phenylthyronine were effectively inhibiting cholesterol synthesis from acetate precursor.

Table II indicates the results of a study on the effect of adding both T_3 and T_4 at 0.5×10^{-4} M each as well as 2.5×10^{-6} M each. At the former concentration a significant inhibition of cholesterol synthesis is observed, indicating a synergism between the two compounds. It should be noted that T_3 added at 0.5×10^{-4} M lowers cholesterolgenesis, but by our statistical analyses fails to record significance (P's are for the 98% level).

The data in Table III indicate that D-T_4 inhibits cholesterolgenesis significantly, whereas at the same concentration L-T_4 does not. The data suggest that D-T_4 is more effective than L-T_3, but the differences between these groups (3 and 4) are not significant. Once again a nonsignificant depression of synthesis is observed when L-T_3 is used at 1.0×10^{-4} M. At higher concentrations L-T_3 inhibits this system.[4]

It is conceivable that many of the thyroid analogs tested here could have an effect on the cholesterolgenic

Table II

Synergism of 3,5,3′-Triiodothyronine and Thyroxine on Cholesterolgenesis from ^{14}C-Acetate

Group no.	Additions to the enzymic system	Dpm ± std dev	
1	Control	1884[a]	323
2	0.2 ml of DMSO	3404[b]	1790
3	0.2 ml of DMSO T_3 and T_4 each at $0.5 \times 10^{-4} M$	1812[c]	1337
4	0.2 ml of DMSO T_3 and T_4 each at $2.5 \times 10^{-6} M$	4042	239

[a] The results are expressed as the average of four replicates of the corrected dpm ± the standard deviation of isolated cholesterol tomatinide. [b] Significant from group 1. [c] Significant from group 2.

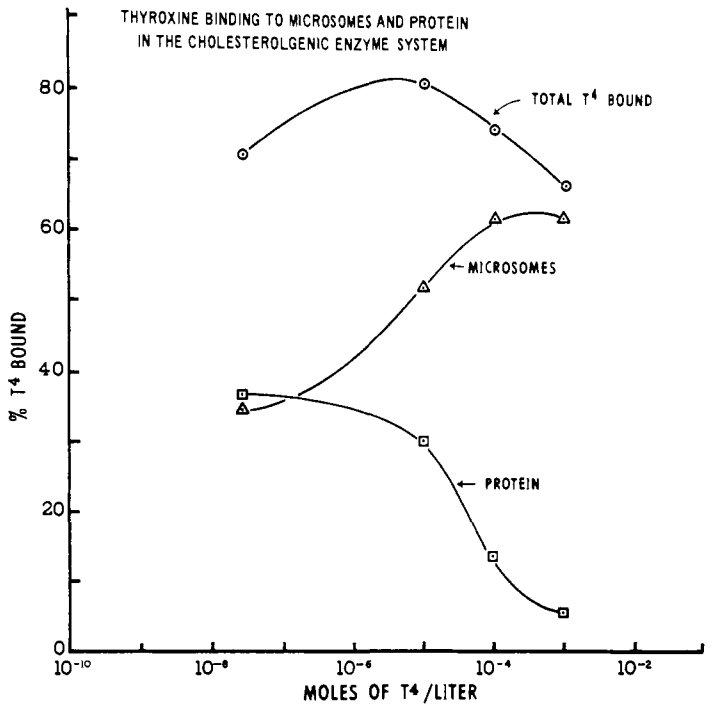

Figure 4.—Binding of ^{131}I-labeled T4 to the microsomes (△) and soluble protein fractions (□). Conditions were the same as for Figure 3.

TABLE III

COMPARISON OF THE EFFECTS OF L-T_3, L-T_4, AND D-T_4 ON CHOLESTEROLGENESIS FROM ^{14}C-ACETATE

Group no.	Additions to the enzymic system	Expt 1 dpm ± std dev		Expt 2 dpm ± std dev	
1	0.2 ml of DMSO Control	2249[a]	325	5526	1317
2	0.2 ml of DMSO $1.0 \times 10^{-4}M$ L-T_4	1824	178	4290	526
3	0.2 ml of DMSO $1.0 \times 10^{-4}M$ D-T_4	846[b]	307	2348[b]	553
4	0.2 ml of DMSO $1.0 \times 10^{-4}M$ L-T_3	1185[b]	126	2976[b]	643

[a] The results are expressed as the average of four replicates of the corrected dpm ± the standard deviation of isolated cholesterol tomatinide. [b] Significant from groups 1 and 2.

TABLE IV

THE EFFECT ON CHOLESTEROL BIOSYNTHESIS OF PRETREATING MICROSOMES OR SOLUBLE PROTEINS WITH T_3 OR T_4

Group no.	Pretreatment of microsomes and/or soluble enzymes	dpm ± std dev	
1	M[a] + E[b]	262[c]	40
2	M + E_{T_3}[d]	176[f]	28
3	M + E_{T_4}[e]	172[f]	50
4	M_{T_3}[g] + E_{T_3}	106[f,h]	17
5	M_{T_3} + E_{T_4}	100[h,i]	9
6	M_{T_3} + E	122[f]	32
7	M_{T_4}[j] + E_{T_3}	109[f]	23
8	M_{T_4} + E_{T_4}	105[f]	34
9	M_{T_4} + E	100[f]	27

[a] M (Microsomes). [b] E (soluble cellular enzymes). [c] Each value represents the mean of four replicate determinations, except for group 1, of the corrected dpm ± the standard deviation of the isolated cholesterol tomatinide. Group 1 was replicated ten times. [d] E_{T_3} (soluble cellular enzymes pretreated with T_3). [e] E_{T_4} (soluble cellular enzymes pretreated with T_4). [f] Significant from 1. [g] M_{T_3} (microsomes pretreated with T_3). [h] Significant from 2. [i] Significant from 3. [j] M_{T_4} (microsomes pretreated with T_4).

system. For example, the possibility exists that if they were more avidly bound to inert proteins than T_3 or T_4 their influences would not be elicited at the hormone concentrations studied here.

The data for the pretreatment of the enzymic system with T_3 and T_4 followed by separation and recombination are shown in Table IV. The enzymic activity of the reconstituted system is severely depressed. This may be due to the various manipulative stages involved. The isolated microsomes are sticky and difficult to resuspend, and were subjected to considerable trauma. It is known that simply overhomogenizing the initial preparation leads to loss of activity[6] suggesting that the integrity of the microsomal structure is an important consideration for an active preparation.

In all cases pretreatment of either or both fractions with T_3 or T_4 leads to significant lowering of cholesterol formation. It was not possible to discern whether pretreating the microsomes alone with either T_3 or T_4 was more effective in lowering cholesterol formation than with pretreatment of the E fraction. The data suggest that this is true, but significance at 98% level was not achieved. Pretreatment and combination of both fractions appear to inhibit maximally, especially when the microsomes had been pretreated with T_3 (groups 4 and 5). Undoubtedly the reconstituted systems undergo a reequilibration of the T_3 and T_4 for the various binding sites on both the microsomes and protein fraction. The data suggest that the inhibition observed with pretreated E fractions (groups 2 and 3) may be due to reassociation with the untreated microsomes by the T_3 or T_4 bound to the E fraction. The reverse may also be true (groups 6 and 9) but the indication is that the microsomes bind T_3 and T_4 more firmly than does the E fraction (Figure 4).

Discussion

The rate-determining step of cholesterolgenesis occurs in the microsomes and is concerned with the reduction

of HMG-coenzyme A to form mevalonic acid.[12] When mevalonic acid is used as substrate, the rate-determining cholesterolgenic reaction is bypassed. The results in Table I, expt 1, indicate that the thyroid hormones are influencing cholesterolgenesis from ^{14}C-acetate and not from ^{14}C-mevalonate. This suggests that the rate-determining reaction for cholesterolgenesis is under thyroid hormone influence. This concept is further supported by the data recorded in Table IV in which microsomes exposed to T_3 or T_4 have the lowest ability for cholesterolgenesis. The studies of Fletcher and Myant[13] using liver homogenates from rats pretreated with high levels of T_4 also support these concepts. The decreased cholesterolgenesis observed (see Table IV) when microsomes are incubated with soluble enzymes which were exposed to the thyroid hormones is probably a reflection of the transference of T_3 or T_4 due to binding competition (see Figures 3 and 4) between the cell sap proteins and microsomal proteins.

The rate of cholesterolgenesis is decreased by T_3 and is proportional to the amount of T_3 present in the *in vitro* system (see Figures 1 and 2). The high concentrations of the thyroid hormones necessary to elicit the response in this system certainly suggest pharmacological levels are necessary, and may be necessary because of a nonspecific binding of T_3 and T_4 to inert proteins, effectively removing them from the active sites on the cholesterolgenic enzymes (see Figures 3 and 4). Since "T_3 and T_4 activity" resides in the microsomes (see Table IV), and from the data in Figures 3 and 4 it may be concluded that either T_3 is twice as effective as T_4 in decreasing cholesterolgenesis or that twice as many T_3 binding sites exist on the microsomes.

Many of the binding sites for T_3 and T_4 are identical as illustrated in Table III in which a synergistic action between T_3 and T_4 is demonstrated. Since T_4 seems more avidly bound by the system it may be suggested that T_4 is occupying some of the binding sites of T_3, thus freeing T_3 to react with enzymes being influenced by the thyroid hormone. 3-Iodothyronine does not seem to bind the proteins of the system as does T_4 (see Table I, expt 3, group 9), since no synergism is elicited between 3-iodothyronine and T_3.

Specific structural requirements seem necessary for the action elicited by the thyroid hormones. The carboxyl group is essential since thyroxamine does not elicit a response. The carboxyl group must be α to an amino group since 3,5,3′-triiodothyropropionic acid does not elicit a response (see Table I, expt 3 and 4). The D isomer appears more active than the L isomer and may be explained on the basis that it does not bind to proteins or subcellular particles[10] as much as the L-isomer (see Tables I and III, expt 1). Evidence suggests that the 3,5-diiodo groups are not necessary; however, the 3′-iodo group or a similar bulky constituent whose molecular weight is greater than 77, *i.e.*, Ph, is essential for inhibitory cholesterolgenic activity (see Table I). The PhO grouping is at least spatially essential since diiodotyrosine is not active in the system.[4] From the data presented here it may be postulated that the most active anticholesterolgenic compound would be a D-3′-iodo- (or bulky constituent) thyronine. It is suggested that the enzyme which is affected by T_3 or T_4 binds the hormone at three points, *i.e.* carboxyl group, amino group, and 3′-iodo group.

Attempts to demonstrate altered microsomal structure by thyroid hormones, using the methods of Tedeschi, *et al.*,[14] have failed to indicate this process occurs as suggested earlier.[4]

It is probable that the HMG-CoA reductase is being inhibited by these hormones. This possibility is interesting since HMG-CoA reductase synthesis seems to be controlled by the thyroid hormones.[2,3] These facts are significant in that the influenced enzyme is rate determining[12] and catalyzes an irreversible reaction. It is conceivable therefore, if the HMG-CoA reductase activity was not controlled and the enzyme concentration increased, that many metabolic processes would decrease due to the shunting of acetyl-CoA from the general body pool. An important principle may be implicated here, since an enzyme whose synthesis requires a small amount of the hormone's presence may also have its activity controlled by larger amounts of the same hormone. In the case of the thyroid hormones indications are that various protein binding sites must be filled, and thus greater concentrations of hormone are needed, before the hormone elicits an inhibition on the active enzyme.

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