Site of the *in Vitro* Inhibition of Cholesterol Biosynthesis by Tolbutamide and Phenethylbiguanide*

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ABSTRACT: The site of inhibition of cholesterol biosynthesis from [2-^{14}C]mevalonate by tolbutamide and phenethylbiguanide has been investigated *in vitro* using a rat liver enzyme system. Tolbutamide does not inhibit the formation of either the isoprenols or squalene but it does inhibit CO_2 evolution from [2-^{14}C]mevalonate and lanosterol formation when arsenite is present in the incubation mixture. The data, therefore, show that tolbutamide stops the biosynthetic pathway at the cyclization of squalene. Phenethylbiguanide, on the other hand, inhibits the formation of both cholesterol and nonsaponifiable lipids, resulting in the accumulation of isoprenol compounds. Reverse-phase chromatography showed that all the radioactivity in the isoprenol fraction was with farnesol and its acid hydrolysis product, nerolidol. Phenethylbiguanide, therefore, inhibits cholesterol biosynthesis between farnesyl pyrophosphate and squalene.

The authors have previously reported that the hypoglycemic compounds phenethylbiguanide (*N'*-β-phenethylformamidinyliminourea hydrochloride), tolbutamide (*N*-butyl-*N'*-*p*-toluenesulfonylurea), metahexamide (*N*-cyclohexyl-*N'*-(3-amino-4-toluenesulfonyl)urea), and chlorpropamide (*N*-propyl-*N'*-*p*-chlorobenzenesulfonylurea) inhibit cholesterol biosynthesis from [1-^{14}C]acetate (Dalidowicz and McDonald, 1962a,b; McDonald and Dalidowicz, 1962) in liver homogenates prepared by the method of Bucher (see Frantz and Bucher, 1954). The maximum inhibition with any particular hypoglycemic compound was obtained when the concentration of the inhibitor was 4×10^{-3} M.

When [2-^{14}C]mevalonate was used as the substrate, the hypoglycemic compounds were again found to inhibit cholesterol biosynthesis in a manner similar to that observed when [1-^{14}C]acetate was used as the substrate. Further experiments have shown that the inhibition of cholesterol biosynthesis by phenethylbiguanide took place between isopentenyl pyrophosphate and the formation of squalene. The inhibition of cholesterol biosynthesis by the arylsulfonylurea compounds, on the other hand, was found to occur after the formation of squalene (McDonald and Dalidowicz, 1962). The present paper presents evidence regarding the site of inhibition of cholesterol biosynthesis by tolbutamide and phenethylbiguanide.

Experimental Procedure

Animals. The animals selected for use in the experiments were Sprague-Dawley male rats obtained from Abrams Small Stock Breeders, Chicago, Ill. The rats, weighing approximately 200 g, were kept on a regular diet. Sacrificing was done by decapitation.

Chemicals. Tolbutamide, as the sodium salt, was supplied by the Upjohn Co., Kalamazoo, Mich. Phenethylbiguanide hydrochloride was supplied by U.S. Vitamin Corp., New York.

Nicotinamide–adenine dinucleotide, reduced, disodium salt (NADH),[1] nicotinamide–adenine dinucleotide phosphate, reduced, sodium salt (NADPH), and adenine triphosphate, disodium salt (ATP), were obtained from Pabst Laboratories, Milwaukee, Wis. Glutathione, reduced (GSH), was obtained from California Corp. for Biochemical Research, Los Angeles.

DL-[2-^{14}C]Mevalonic acid as the *N,N*-dibenzylethylenediammonium bis(3,5-dihydroxy-3-methylpentanoate) salt (DBED salt) was obtained from New England Nuclear Corp., Boston. The mevalonic acid (1.19 mc/mmole) was diluted to a particular level (shown in the tables of results under Conditions) with unlabeled DBED salt of mevalonic acid obtained from Mann Research Laboratories, New York.

Preparation of the Enzyme System. Liver homogenates were prepared according to Frantz and Bucher (1954). The homogenates thus obtained were used for the preparation of the enzymes necessary for the biosynthesis of cholesterol from mevalonic acid by the combined procedure of Popjak *et al.* (1958) and Popjak (1959a). This

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[1] Abbreviations used in this work: NADH and NADPH reduced form of nicotinamide–adenine dinucleotide and nicotinamide–adenine dinucleotide phosphate, respectively; ATP, adenosine triphosphate; GSH, reduced glutathione; DBED, *N,N*-dibenzylethylenediammonium bis(3,5-dihydroxy-3-methylpentanoate).

procedure has been described previously (McDonald and Dalidowicz, 1962).

The Incubation Mixture. Each incubation vessel contained 1.0 ml of dialyzed soluble enzyme (17–21 mg of protein), 0.2 ml of microsomes, 1.0 ml of substrate solution, and the hypoglycemic compound in the specified amount. The volume was made up to 4 ml with potassium phosphate buffer. The substrate solution contained 2 μmoles of NADH, 1 μmole of NADPH, 30 μmoles of ATP, 30 μmoles of GSH, 4 μmoles of Mn^{2+}, and 1.0 μmole of DL-[2-^{14}C]- or [1-^{14}C]mevalonate (0.37–0.74 μc of ^{14}C) in 1 ml of phosphate buffer (0.1 M, *p*H 7.4) containing 16 μmoles of Mg^{2+} and 0.03 M nicotinamide. The protein contained in the dialyzed soluble fraction was measured by the biuret reaction (Gornall *et al.*, 1949).

Isolation of Nonsaponifiable Lipids. The nonsaponifiable lipid and cholesterol were isolated as described previously (McDonald and Dalidowicz, 1962). Cholesterol was determined as the digitonide after alumina chromatography.

Isolation and Identification of the Isoprenol Fraction. The isolation of the isoprenols, presqualene branched-chain alcohol intermediates of cholesterol, was done by the method of Popjak (1959b) and determined in a scintillation counter. Identification of the [^{14}C]isoprenol substances was done by reverse-phase paper chromatography of the 3,5-dinitrobenzoyl derivatives of the alcohols. The derivatives were made by the method of Cheronis and Entrikin (1957).

The petroleum ether fractions obtained were evaporated to a volume of 1 ml. To this 1 mg of authentic samples of geraniol, farnesol, and nerolidol, respectively, 40 mg of 3,5-dinitrobenzoyl chloride (recrystallized from CCl_4), 5 ml of isopropyl ether (free from alcohol), and 1 drop of pyridine were added and the mixture was refluxed for 2 hours in a water bath at 60°. The tubes were then cooled and the contents were washed once with 4 ml of dilute sulfuric acid (2%) to remove the pyridine, once with 10% NaOH, and once with water to remove the 3,5-dinitrobenzoic acid. The ether layer thus obtained was evaporated to dryness. The residue was dissolved in 0.2 ml of chloroform and 100 μl was then streaked on Whatman No. 1 filter paper impregnated with 10% mineral oil. The paper strips were placed in a chromatography jar and equilibration was allowed to take place overnight. The strips were then developed for 12–16 hours with 75% acetic acid. Strips of authentic samples of geraniol, farnesol, and nerolidol as the 3,5-dinitrobenzoates (prepared as before) were developed by the same procedure whenever the compounds were identified.

Following the chromatography, the strips were dried. The nonradioactive derivatives were stained with 5% naphtholamine. The movement of the derivatives was revealed as dark-orange spots. The radioactive strips were scanned for radioactivity and the compounds were identified by comparison with the movement of the authentic samples of the alcohols. Geranyl-3,5-dinitrobenzoate gave an R_F value of 0.64, while farnesyl and nerolidyl-3,5-dinitrobenzoates had identical R_F values (0.32) in every case. Both isopentenyl and dimethylallyl-3,5-dinitrobenzoates gave R_F values of 0.81–0.83. These figures agree closely with those reported by Holmes and Bentz (1960).

Isolation of Squalene. This was done according to the method of Langdon and Bloch (1953) by chromatography of the nonsaponifiable lipid fraction on alumina columns. Squalene was eluted with 100 ml of petroleum ether after which cholesterol was eluted with 50 ml of diethyl ether. The squalene fraction was then evaporated to 10 ml, 1 ml of this solution was put directly into 10 ml of scintillation solution, and radioactivity was determined in a scintillation counter. In some of the experiments squalene was further characterized through the thiourea adduct. To the chromatographed specimen, after evaporation of the solvent, a few milliliters of a saturated methanolic solution of thiourea, a few grains of finely powdered thiourea, and 10 mg of carrier squalene were added. After thorough shaking, the mixture was left at room temperature for a few days. The crystals of the clathrate were then filtered and decomposed with water, squalene was extracted with petroleum ether, and radioactivity was determined in a scintillation counter. The cholesterol fraction was treated in a similar way. Part of the fraction was further characterized by precipitation with digitonin and determination of the cholesterol-digitonide in a gas-flow counter.

Measurement of Radioactivity. The radioactivity in the nonsaponifiable lipid, isoprenols, squalene, and in some cases cholesterol was determined with a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc., La Grange, Ill.) with the photomultiplier tube voltage set at 1020 v and a discriminator setting of 10–100 v. The composition of the liquid scintillator that was used has been described by Bray (1960).

Radioactivities of solid samples ($BaCO_3$ and digitonides) were determined with a Tracerlab gas-flow assembly. Self-absorption corrections were made on all solid samples. In both scintillation and gas-flow counting, a sufficient number of counts was taken to reduce the statistical error of counting to less than 5%. The scanning of radiochromatograms was done by a Tracerlab SC-55 chromatogram scanner.

Results

Site of the in Vitro Inhibition of Cholesterol Biosynthesis by Tolbutamide. When [2-^{14}C]mevalonate is incorporated into cholesterol by liver homogenates or liver enzyme systems, one of the labels from [2-^{14}C]-mevalonate is found to reside in one of the three methyl groups of lanosterol not present in the cholesterol molecule. When lanosterol is demethylated by stepwise oxidation of the methyl groups and subsequent elimination as CO_2 (Olson *et al.*, 1957), one molecule of $^{14}CO_2$ is obtained for every molecule of lanosterol demethylated.

Since tolbutamide inhibits cholesterol biosynthesis after the formation of squalene, the $^{14}CO_2$ obtained from incubations with [2-^{14}C]mevalonate was collected and determined as $Ba^{14}CO_3$. Table I shows that tolbuta-

TABLE I: Effect of Tolbutamide on the Formation of CO_2 from [2-^{14}C]Mevalonate.[a]

Measurements of ^{14}C	Control	Tolbutamide	Per Cent Inhibition
Nonsaponifiable lipid (cpm)	106,750	93,170	12.7
Cholesterol (cpm/mg)	2,580	365	85.8
CO_2 as $BaCO_3$ (cpm)	609	128	79.1

[a] Each flask contained 1.0 ml of dialyzed soluble enzymes (17.5 mg of protein), 0.2 ml of microsomal preparation, NADH (2 μmoles), NADPH (1 μmole), ATP (30 μmoles), reduced glutathione (30 μmoles), $MgCl_2$ (16 μmoles), $MnSO_4$ (4 μmoles), and mevalonic acid (0.5 μmole, 0.76 μc of ^{14}C). Aerobic incubations were at 37° for 2 hours. The concentration of tolbutamide was 4×10^{-3} M. The data presented in this and subsequent tables is that of a typical experiment. In all cases it is representative of four or five experiments done with different enzyme preparations, in triplicate.

TABLE II: Effect of Tolbutamide on the Formation of Lanosterol from [2-^{14}C]Mevalonate.[a]

Measurements of ^{14}C	Control	Tolbutamide	Per Cent Inhibition
Nonsaponifiable lipid (cpm)	46,800	43,770	6.5
Lanosterol (cpm/mg)	1,820	460	74.1
CO_2 as $BaCO_3$ (cpm)	169	18	89.3

[a] The conditions were the same as in Table I except that each flask was preincubated with 0.001 M arsenite and the enzyme system before other additions were made. The enzyme system contained 23.4 mg of protein. The concentration of tolbutamide was 4×10^{-3} M. Aerobic incubations at 37° for 2 hours.

mide inhibits the evolution of $^{14}CO_2$ approximately to the same degree as it inhibits the formation of cholesterol. To ascertain whether the inhibition occurred before or after the formation of lanosterol, the effect of tolbutamide on the formation of lanosterol from [2-^{14}C]-mevalonate was studied by using arsenite to stop the biosynthetic sequence at lanosterol as reported by Moller and Tchen (1961).

The liver enzyme system, capable of synthesizing cholesterol, was first preincubated with 10^{-3} M arsenite for 1 hour at 0°. After the preincubation period was terminated, [2-^{14}C]mevalonate, NADH, NADPH, ATP, GSH, Mg^{2+}, Mn^{2+}, and tolbutamide (4×10^{-3} M) were added and the mixture was incubated at 37° for 2 hours. The reactions were then stopped and the nonsaponifiable lipid was extracted. The 3-β-hydroxy steroids were then precipitated by digitonin after addition of trace amounts of unlabeled lanosterol. The results in Table II show that lanosterol formation from [2-^{14}C]mevalonate is inhibited by tolbutamide to the same extent as cholesterol was in the previous experiments.

Tolbutamide, therefore, also inhibits the formation of lanosterol from [2-^{14}C]. Previously it was shown that squalene synthesis was not inhibited by tolbutamide in anaerobic incubations (McDonald and Dalidowicz, 1962). The biosynthetic pathway, therefore, must be interrupted by tolbutamide at the cyclization of squalene. This would indicate that if the incorporation of [2-^{14}C]-mevalonate were allowed to proceed to cholesterol (aerobic incubation with the addition of glutathione), an increase in the accumulation of squalene should be observed in the presence of tolbutamide. The results in Table III show that if the metabolic sequence is not terminated until cholesterol is formed, there is a concomitant increase in the accumulation of squalene with the drop in cholesterol synthesized in incubations containing tolbutamide.

Site of the in Vitro Inhibition of Cholesterol Biosynthesis by Phenethylbiguanide. Data previously reported showed that phenethylbiguanide decreased the formation of squalene resulting in the accumulation of the isoprenols (McDonald and Dalidowicz, 1962). This suggested that the inhibition was at one of the isoprenol pyrophosphates in the biosynthesis of cholesterol. To obtain further support for this general site of inhibition, incubations were started without the addition of microsomes to the system. This omission stops the biosynthetic pathway at the C_{15}-isoprenol pyrophosphate, farnesol pyrophosphate (Goodman and Popjak, 1960). At the end of the first hour of incubation some reactions were stopped by KOH and the isoprenol fraction was extracted and analyzed. To some of the other incubations 0.2 ml of the microsomal preparation was added, while others were left without microsomes. The incubations were then allowed to proceed for another hour. At the end of the second hour the reactions were stopped and the isoprenol fraction was analyzed.

The data in Table IV show that when the microsomes were omitted from the incubation mixtures the formation of the isoprenols from [2-^{14}C]mevalonate in both controls and incubations containing phenethylbiguanide was the same. This was true whether the incubation period was 1 or 2 hours. The incorporation of [2-^{14}C]mevalonate into the isoprenol fraction after 2 hours was much higher than after 1 hour but nevertheless the same for both controls and phenethylbiguanide containing incubations.

If microsomes were added at the end of the first hour, the isoprenol pyrophosphates in the controls were

TABLE III: Effect of Tolbutamide on the Formation of Squalene from [2-^{14}C]Mevalonate.[a]

Type of Incubation	Measurements of ^{14}C	Mevalonate Incorporated (μmoles) Control	Tolbutamide	Change
Anaerobic[b]	Nonsaponifiable lipid	0.042	0.042	
	Squalene[c]	0.040 (0.039)	0.041 (0.041)	
	Cholesterol	0	0	
Aerobic	Nonsaponifiable lipid	0.224	0.212	
	Squalene[c]	0.134 (0.133)	0.161 (0.159)	+0.027 ± 0.003
	Cholesterol	0.082	0.046	−0.036 ± 0.004

[a] The conditions were the same as described in Table I with the noted exceptions. The concentration of tolbutamide was 2.5×10^{-3} M. All incubations were carried out at 37° for 2 hours. [b] Glutathione was not present in the incubation mixture. [c] The figures in the parentheses were taken after the squalene was passed through the thiourea adduct.

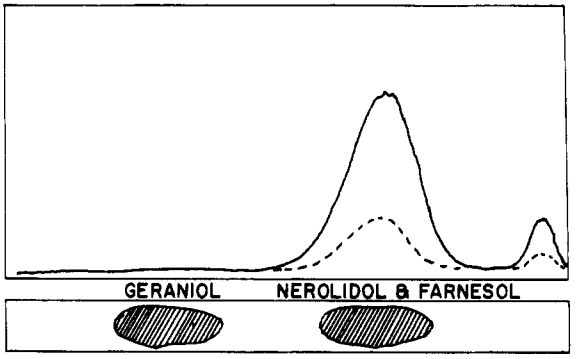

FIGURE 1: Radiochromatograms of the isoprenol-3,5-dinitrobenzoates. Reverse-phase chromatography on Whatman No. 1 paper impregnated with 10% medium mineral oil. Developing solvent, 75% acetic acid. Top graph: reproduction of a radiochromatogram of isoprenol-3,5-dinitrobenzoates obtained from incubation mixtures; broken line, control; solid line, incubations containing phenethylbiguanide. Full-scale deflection = 100 cpm. Bottom graph: reproduction of the separation obtained from 3,5-dinitrobenzoyl derivatives of authentic samples of the isoprenols. The origin is at the right, the solvent front at the left.

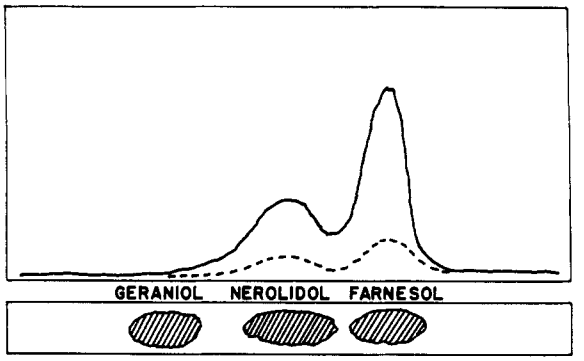

FIGURE 2: The separation of farnesyl- and nerolidyl-3,5-dinitrobenzoates. Reverse-phase chromatography on Whatman No. 1 paper impregnated with 10% medium mineral oil. Developing solvent, 75% acetic acid. Top graph: reproduction of a radiochromatogram of isoprenol-3,5-dinitrobenzoates obtained from the incubation mixtures; broken line, control; solid line, incubations containing phenethylbiguanide. Full-scale deflection = 250 cpm. Bottom graph: reproduction of the separation obtained from 3,5-dinitrobenzoyl derivatives of authentic samples of the isoprenols. The origin is at the right; the solvent front was run off the paper in order to separate the farnesyl and nerolidyl derivatives.

further metabolized to cholesterol as evidenced by the decrease in radioactivity of the isoprenol fraction after 2 hours (Table V) and the appearance of synthesized cholesterol. Incubations containing phenethylbiguanide, however, had the same amount of radioactivity associated with the isoprenols whether the microsomes were present or not. The cholesterol formed in the phenethylbiguanide-containing incubations when the microsomes were added was much less than when phenethylbiguanide was absent from the incubation medium. The enzymes contained in the microsomes that transform the isoprenols into squalene and subsequently into cholesterol, therefore, were unable to metabolize further the isoprenols when phenethylbiguanide was present. In order to determine which compound or compounds contained this high radioactivity when phenethyl-

TABLE IV: Effect of Phenethylbiguanide on the Formation of Isoprenol Pyrophosphates from [2-^{14}C]Mevalonate.[a]

Conditions	Isoprenol Pyrophosphates		Cholesterol	
	Control	Phenethyl-biguanide	Control	Phenethyl-biguanide
Complete system 1 hr. incubation	20,800	20,200	0	0
Complete system 2 hr. incubation	42,600	43,050	0	0
Complete system	17,500	45,600	10,020	810
Microsomes added after 1 hr. of incubation. Incubation continued for an additional hour				

[a] Each flask contained 1.0 ml of dialyzed soluble enzymes (18.0 mg of protein), NADH (2 μmoles), NADPH (1 μmole), ATP (30 μmoles), reduced glutathione (30 μmoles), $MgCl_2$ (16 μmoles), $MnSO_4$ (4 μmoles), and mevalonic acid (0.5 μmole, 0.25 μ of ^{14}C). Aerobic incubations were at 37°.

biguanide is present, the isoprenols were separated and identified.

Figure 1 shows the radiochromatograms obtained from the reverse-phase chromatography of the isoprenol-3,5-dinitrobenzoates. There was a large accumulation of a compound having an R_F value of 0.32 in incubations containing phenethylbiguanide. This corresponds to farnesyl- and nerolidyl-3,5-dinitrobenzoates as shown by the movement of authentic samples of the derivatives. Furthermore, the addition of authentic samples of geraniol, farnesol, and nerolidol to the radioactive isoprenol fraction and subsequent reaction with 3,5-dinitrobenzoyl chloride and chromatography produced dark-orange spots with 5% naphtholamine corresponding to the R_F values of geraniol and farnesol + nerolidol-3,5-dinitrobenzoates. Scanning for radioactivity, it was found that only the dark-orange spots contained the radioactivity and that this corresponded to the R_F values of farnesol- and nerolidol-3,5-dinitrobenzoates. A small radioactive peak was also obtained at the origin, which again was larger in incubations that contained phenethylbiguanide as compared with the ones that did not. This is thought to be free nerolidol, since authentic samples of free nerolidol stayed at the origin in the system that was used. Also, if the refluxing during the making of the derivatives was increased from 1 to 2 hours, the radioactive peak at the origin disappeared and a concomitant rise in radioactivity with an R_F value of 0.32 was observed.

In order to separate the farnesol- and nerolidol-3,5-dinitrobenzoates, the solvent front was allowed to run off the paper. By this method, the two C_{15}-isoprenol-3,5-dinitrobenzoates were reasonably separated. The farnesyl-3,5-dinitrobenzoate came first and resolved into a sharp peak, followed by a large drawn-out band of radioactivity corresponding to nerolidyl-3,5-dinitrobenzoate. The results in Figure 2 show that the radiochromatograms of the isoprenol derivatives from incubations containing phenethylbiguanide had an accumulation of farnesol and nerolidol as compared to incubations in which phenethylbiguanide was not present.

Discussion

The action of the hypoglycemic compounds, tolbutamide, chlorpropamide, metahexamide, and phenethylbiguanide, on the biosynthesis of cholesterol has been investigated *in vitro* with [2-^{14}C]mevalonate using a rat liver enzyme system. The results show that the incorporation of [2-^{14}C]mevalonate into cholesterol is inhibited by all compounds studied. The maximal inhibition occurs when the concentration of hypoglycemic compound is 4×10^{-3} M (McDonald and Dalidowicz, 1962).

The manner in which these compounds inhibit cholesterol biosynthesis, however, is quite different. Phenethylbiguanide decreases the formation of the nonsaponifiable lipids and cholesterol by approximately the same amount from [2-^{14}C]mevalonate. This implies that phenethylbiguanide inhibits the biosynthesis of cholesterol before the formation of the cholesterol intermediate found in the nonsaponifiable lipid fraction. This was substantiated by the inhibition of squalene formation from [2-^{14}C]mevalonate (McDonald and Dalidowicz, 1962). Furthermore, there is an accumulation of the isoprenols, presqualene cholesterol intermediates. Reverse-phase chromatography of the 3,5-dinitrobenzoate derivatives of the alcohols showed that the accumulation of radioactivity was in the farnesyl and nerolidyl derivatives.

Allyl pyrophosphates, in common with other substances in which a potentially anionic group is in the α position to a double bond, are electrophilic compounds and can be expected to lose the pyrophosphate ion rather readily. The electron-deficient species resulting

from the elimination of the pyrophosphate ion may be partially stabilized by resonance between two equivalent forms. In the presence of water the two forms may yield, by the addition of a hydroxyl ion, either a primary or a tertiary alcohol. This is the mechanism of allylic rearrangement, and was found by Goodman and Popjak (1960) and Lynen *et al.* (1958) to be responsible for the conversion of a large part of farnesol pyrophosphate to nerolidol during acid hydrolysis of the isoprenol pyrophosphates.

The nerolidol obtained in Figures 1 and 2, therefore, actually comes from the rearrangement of farnesyl pyrophosphate during the isolation procedure. The measurement of the radioactivity of both farnesyl- and nerolidyl-3.5-dinitrobenzoates is actually an indication of the radioactivity originally present in farnesyl pyrophosphate. Phenethylbiguanide, then, increases the formation of farnesyl pyrophosphate. From data previously reported, it was seen that phenethylbiguanide also decreases the incorporation of [2-^{14}C]-mevalonate into squalene (McDonald and Dalidowicz, 1962). The site of inhibition of cholesterol biosynthesis by phenethylbiguanide is therefore between farnesyl pyrophosphate and squalene, stopping the biosynthetic pathway of cholesterol from mevalonate at the C_{15} alcohol pyrophosphate.

The arylsulfonylureas, tolbutamide, chlorpropamide, and metahexamide, however, are different as regards their effect on the biosynthesis of cholesterol. They do not inhibit the incorporation of [2-^{14}C]mevalonate into the nonsaponifiable lipid and cholesterol by the same magnitude. Starting with [2-^{14}C]mevalonate, there is almost no difference between the nonsaponfiable lipid formed when the arylsulfonylurea compounds are present or absent from the incubation mixture, and yet cholesterol biosynthesis is inhibited. When the isoprenols and squalene are isolated, again there is no difference (McDonald and Dalidowicz, 1962). When lanosterol was isolated, however, there was a decrease in its formation corresponding to the decrease obtained in cholesterol synthesized from [2-^{14}C]mevalonate. And finally, when squalene was isolated after aerobic incubations, there was an increase in its formation when tolbutamide was present in the incubation mixtures. The increase in squalene formation from [2-^{14}C]-mevalonate could be explained by the decrease in the biosynthesis of cholesterol in the same incubation. It follows, therefore, that tolbutamide inhibits cholesterol biosynthesis from [2-^{14}C]mevalonate between squalene and lanosterol, or more precisely, at the cyclization of squalene.

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