

In vitro* Inhibition of Cholesterol Biosynthesis from Acetate-1-C¹⁴ and Mevalonate-2-C¹⁴ by Hypoglycemic Compounds

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The action of the hypoglycemic compounds, tolbutamide, chlorpropamide, metahexamide, and phenethylbiguanide, on the biosynthesis of cholesterol has been investigated *in vitro* with both acetate-1-C¹⁴ and mevalonate-2-C¹⁴. The results show that the incorporation of acetate-1-C¹⁴ and mevalonate-2-C¹⁴ into cholesterol is inhibited by all compounds studied. The maximal inhibition occurs when the concentration of hypoglycemic compound is 4×10^{-3} M. It has been found that the inhibition of cholesterol biosynthesis by phenethylbiguanide takes place between isopentenyl pyrophosphate and the formation of squalene. The inhibition of cholesterol biosynthesis by the arylsulfonylurea compounds, on the other hand, takes place after the formation of squalene.

Although the arylsulfonylureas exert their hypoglycemic activity through the mediation of insulin, many metabolic effects which accompany arylsulfonylurea-induced hypoglycemia are different from those which accompany insulin-induced hypoglycemia.

Tolbutamide and chlorpropamide, added *in vitro* to rat liver slices, have been found to decrease epinephrine-induced glycogenolysis (Vaughan, 1957) and ketogenesis (Boshell *et al.*, 1960). When rat liver homogenates were used, tolbutamide was found to alter a number of enzymatic activities, including at least one transaminase (Bornstein, 1957), and the phosphor-ylase-reactivating system (Berthet *et al.*, 1956).

The mode of action of the hypoglycemic compound phenethylbiguanide is different from that of the arylsulfonylureas, as the compound produces hypoglycemia in pancreatectomized animals (Nielsen *et al.*, 1958) and in animals with severe alloxan diabetes (Ungar *et al.*, 1957). The difference is also seen in its effect on other metabolic pathways. Phenethylbiguanide inhibits the oxidation of glucose, acetate, and succinate and considerably reduces fat synthesis (Wick *et al.*, 1958) and increases blood pyruvate and lactate levels in man (Fajans *et al.*, 1958).

Since the hypoglycemic compounds affect several metabolic pathways involving acetate, they might be expected to affect the biosynthesis of cholesterol. In this paper the authors report the effect of tolbutamide, chlorpropamide, metahexamide, and phenethylbiguanide on the *in vitro* biosynthesis of cholesterol from acetate-1-C¹⁴ and mevalonate-2-C¹⁴.

EXPERIMENTAL

Homogenates.—Sprague-Dawley female rats, weighing approximately 200 g and kept on a stock diet, were killed by decapitation. The

livers were removed rapidly, blotted, weighed, and ground gently at 700 rpm for two minutes in a Potter-Elvehjem homogenizer with 1.25 volumes of 0.08 M phosphate buffer, pH 7.4, containing 0.03 M nicotinamide and 0.0048 M MgCl₂. Unbroken cells and tissue debris were eliminated by centrifugation at $700 \times g$ for 15 minutes. The supernatant homogenate obtained was used as the source of enzymes in the biosynthesis of cholesterol from acetate-1-C¹⁴ (Frantz and Bucher, 1954).

In experiments in which mevalonate-2-C¹⁴ was used, the combined procedures of Popjak *et al.* (1958) and Popjak (1959a) were used for the separation of enzymes necessary for the biosynthesis of cholesterol from mevalonic acid. Homogenates prepared as above, with the exception that 2.5 ml of 0.1 M phosphate buffer, pH 7.4, per gram of liver was used, were centrifuged at $10,000 \times g$ for 25 minutes. After the cell debris and the mitochondria were discarded, the supernatant was recentrifuged at $104,000 \times g$ in a Spinco Model L preparative high-speed centrifuge for 60 minutes. The S₁₀₄ supernatant was pipetted off, care being taken not to disturb the sedimented particles.

The sediment, which contains microsomes, was resuspended in a volume of buffer equal to about one-quarter of the original volume of the S₁₀ and sedimented again at $104,000 \times g$ for 60 minutes. This time the supernatant was discarded and the washed microsomes were diluted to 0.1 volume of the S₁₀ supernatant from which they were separated. The microsomes thus prepared were kept at -15° until used in the experiments.

The supernatant from S₁₀₄ was saturated with solid (NH₄)₂SO₄ (6.3 g/10 ml of supernatant). The precipitate was filtered off, dissolved in 0.02 M KHCO₃ (12 ml/3 g of precipitate), and dialyzed for 3 hours against 0.02 M KHCO₃. The dialyzed solution was then fractionated with (NH₄)₂SO₄. The precipitate obtained from 0–30% saturation

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was discarded and the solution was saturated to 60% $(\text{NH}_4)_2\text{SO}_4$. The precipitate obtained after the second precipitation was stored at -15° until the day of the experiment, when the precipitate was again dissolved in 0.02 M KHCO_3 and dialyzed for 3 hours against 0.02 M KHCO_3 . The dialyzed material was then treated with protamine sulfate (2 mg/ml) and the precipitate obtained was discarded. The supernatant was used in the experiments as a source of enzymes and will be referred to as dialyzed soluble enzymes.

Chemicals.—Tolbutamide and metahexamide were supplied by The Upjohn Company, Kalamazoo, Mich.; phenethylbiguanide, by U. S. Vitamin Corporation, New York; chlorpropamide, by Charles Pfizer and Company, Inc., New York; and insulin (Iletin), by Eli Lilly and Company, Indianapolis, Ind. DPN, TPN, and ATP were purchased from Sigma Chemical Company, St. Louis, Mo., and DPNH and TPNH from Pabst Laboratories, Milwaukee, Wis. The sodium salt of acetate-1- C^{14} was purchased from Volk Radio-Chemical Company, Skokie, Ill. The original specific activity of the radioacetate, 7.0 mC/mm, was diluted to 1 $\mu\text{C}/20$ μmoles by unlabeled sodium acetate. DL-Mevalonic acid lactone-2- C^{14} was obtained from Nuclear Chicago Corporation, Chicago. The original specific activity of the mevalonate, 3.35 mC/mm, was diluted with unlabeled mevalano lactone to either 0.15 $\mu\text{C}/\mu\text{M}$ or 0.25 $\mu\text{C}/\mu\text{M}$.

Incubations.—In incubations where acetate-1- C^{14} was used, the standard incubation mixture was composed of 0.5 ml of rat liver homogenate (approximately 800 mg of liver), the particular hypoglycemic compound in the specified amount in 1 ml of standard buffer solution, and 1 ml of substrate solution. The final volume of the incubation mixture was 2.5 ml. The substrate solution contained 1.8 μmoles of DPN, 1.8 μmoles of TPN, and 3.3 μmoles of ATP in each milliliter of substrate solution. In several tables this will be referred to as the "acetate incubation mixture."

In incubations where mevalonate-2- C^{14} was used, the incubation mixture was composed of 1.0 ml of the dialyzed soluble enzymes, 0.2 ml washed microsomes, and the following co-factors: DPNH (2 μmoles), TPNH (1 μmole), ATP (30 μmoles), Mn^{++} (4 μmoles) with 0.1 M phosphate buffer (pH 7.4), 0.03 M nicotinamide, and 14 μmoles of MgCl_2 . In several tables this will be referred to as the "mevalonate incubation mixture" to which additions were made as indicated. The final volume of the incubation mixture was 4 ml.

Studies on the incorporation of acetate-1- C^{14} and mevalonate-2- C^{14} into the nonsaponifiable lipid were carried out aerobically in conical flasks at 37° in a metabolic shaker. The experiments on the effect of the hypoglycemic compounds on the biosynthesis of squalene were carried out anaerobically by continuously flushing the incubation flasks with nitrogen. After

the specified incubation period, the reactions were stopped by the addition of 3 ml of 15% KOH in 50% ethanol to each incubation vessel followed by heating for 10 minutes at 37° .

Isolation and Extraction of Nonsaponifiable Lipids.—The partially saponified reaction mixtures were transferred to 25-ml screw-capped culture tubes. The reaction vessel was rinsed with 3 ml of the alcoholic KOH solution and the rinse added to the contents of the culture tubes. Saponification was completed by heating the tightly capped tubes in a water bath at $75-80^\circ$ for one hour. The saponified reaction mixtures were extracted four times with 10-ml portions of light petroleum ether ($30-60^\circ$ boiling fraction). This solution contained the total nonsaponifiable lipid.

In experiments where only the cholesterol was determined, the petroleum ether was evaporated to dryness in a heavy-duty centrifuge tube. The residue was dissolved in 3 ml of acetone-ethanol (1:1 v/v), and 1.0 mg of carrier cholesterol in 0.5 ml of ethanol was added to this solution. The 3- β -hydroxy sterols were precipitated by the addition of 7 ml of 0.5% digitonin in 50% ethanol. After standing at room temperature for 16 hours, the digitonide precipitate was recovered by centrifugation, washed once with 90% ethanol, once with acetone, once with acetone-ether (1:2 v/v), and finally with petroleum ether.

In experiments where both the cholesterol and the total nonsaponifiable lipid were determined, only a suitable aliquot of the petroleum ether extract was used for cholesterol determination. Squalene was isolated by chromatography on alumina columns (Langdon and Bloch, 1953).

Measurements of Radioactivity.—The radioactive cholesterol digitonide was determined with a Tracerlab SC-16 Windowless Flow Assembly attached to a Tracerlab Autoscaler (Tracerlab Inc., Boston) and the results were corrected for self-absorption. The radioactivity in the nonsaponifiable lipid, isoprenols, and squalene was determined with a Tri-Carb Liquid Scintillation Spectrometer (Packard Instrument Co., Inc., Chicago), with the photomultiplier tube voltage set at 1020 v and a discriminator setting of 10–100 v.

The composition of the liquid scintillator which met the criteria of the highest counting efficiency was described by Bray (1960). A sufficient number of counts was taken to reduce the statistical error of counting to less than 5%.

RESULTS

Effect of Tolbutamide and Phenethylbiguanide on Cholesterol Biosynthesis from Acetate-1- C^{14} .—Early experiments showed that tolbutamide was able to inhibit the conversion of acetate-1- C^{14} to cholesterol in concentrations of less than 0.4×10^{-3} M (Dalidowicz and McDonald, 1962). Further experiments, shown in Figure 1, indicate

that the inhibition is proportional to the concentration of tolbutamide in the incubation mixture. A maximum inhibition was obtained when the concentration of tolbutamide was between 4 and 5×10^{-3} M. Beyond this range the incorporation of acetate-1- C^{14} into cholesterol was almost zero.

Although phenethylbiguanide is different from tolbutamide in its structure and hypoglycemic action, the compound inhibits the conversion of acetate-1- C^{14} into cholesterol to about the same extent as tolbutamide. Figure 2 shows that when the concentration of phenethylbiguanide was 5×10^{-3} M, the compound almost completely inhibited the biosynthesis of cholesterol. The effect of the concentration of phenethylbiguanide present in the incubation mixture was again proportional to the decrease in radioactivity in the cholesterol synthesized. The degree of inhibition of different concentrations of phenethylbiguanide on the biosynthesis of cholesterol from acetate-1- C^{14} compared favorably with that of tolbutamide.

Effect of Hypoglycemic Compounds on the Incorporation of Acetate-1- C^{14} into the Total Nonsaponifiable Lipids.—In order to obtain a clearer picture of the inhibition, a study was made of the distribution of radioactivity between the digitonide-precipitable sterols and the other components of the nonsaponifiable lipid fraction. Tolbutamide, phenethylbiguanide, chlorpropamide, metahexamide (4.0×10^{-3} M), and insulin (0.1, 2.0, and 10.0 USP units) were incubated with rat liver homogenates and acetate-1- C^{14} . After the incubation, the cholesterol and the nonsaponifiable lipid were isolated and the activity determined. Table I shows that both the digitonide-precipitable cholesterol and the total nonsaponifiable lipid were inhibited by all compounds, but not to the same extent. The arylsulfonyleurea compounds had a lesser effect than either phenethylbiguanide or insulin on the incorporation of acetate-

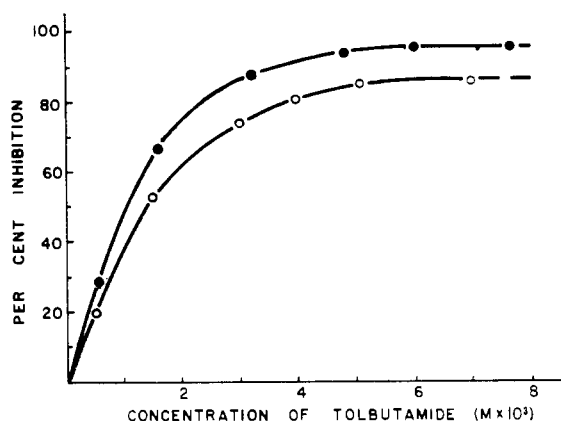


FIG. 1.—Effect of tolbutamide on the biosynthesis of cholesterol from (a) acetate-1- C^{14} (●) (each flask contained the acetate incubation mixture and 20 μ moles of acetate-1- C^{14} [1.0 μ C of C^{14}], and (b) mevalonate-2- C^{14} (○) (each flask contained the mevalonate incubation mixture, 1 μ mole of mevalonate-2- C^{14} [0.25 μ C of C^{14}], and 30 μ moles of glutathione). Aerobic incubations at 37° for 2 hours.

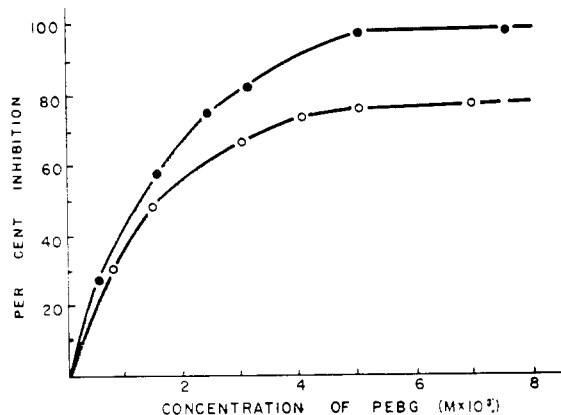


FIG. 2.—Effect of phenethylbiguanide on the biosynthesis of cholesterol from (a) acetate-1- C^{14} (●) and (b) mevalonate-2- C^{14} (○). Incubations carried out as noted in Figure 1.

TABLE I

DISTRIBUTION OF RADIOACTIVITY BETWEEN FRACTIONS

Each flask contained the acetate incubation mixture with 10 μ moles of acetate (0.5 μ C of C^{14}) and the indicated hypoglycemic compounds (4.0×10^{-3} M). The amounts of insulin used were as indicated. Aerobic incubations at 37° for 2 hours.

Hypoglycemic Compound	Specific Activity of Cholesterol (cpm/mg)	Non-saponifiable Lipid (total cpm)
None	1550	16,060
Tolbutamide	69	9,030
Chlorpropamide	204	9,150
Metahexamide	208	9,720
Phenethylbiguanide	36	4,610
Insulin (10 units)	24	4,920
Insulin (2 units)	35	6,850
Insulin (0.1 unit)	32	8,180

1- C^{14} into the total nonsaponifiable lipid. Furthermore, chlorpropamide and metahexamide had a lesser effect on the biosynthesis of cholesterol than the other compounds that were tested. Insulin, in the amount of 0.1 USP units, completely inhibited the biosynthesis of cholesterol. Moreover, insulin in the amount of 10 USP units had the same effect on the formation of the nonsaponifiable lipid as phenethylbiguanide. When the concentration of insulin was reduced to 2.0 USP and 0.1 USP units the formation of the nonsaponifiable lipid was increased, although it never was higher than the nonsaponifiable lipid obtained when the arylsulfonyleureas were used.

Attempts at Localization of the Site of Action.—In an attempt to localize the site of action of the hypoglycemic compounds in the inhibition of cholesterol biosynthesis, the total nonsaponifiable

lipid fraction was partitioned into petroleum ether from the alkaline incubation mixture after saponification with KOH. The aqueous residue was then acidified to pH 1.0 with H₂SO₄. After the solution had stood at room temperature for 1 hour, the pH was readjusted to 10.0 with KOH and the neutral C¹⁴-labeled substances were extracted with petroleum ether and the activity was determined in a scintillation counter. The data, shown in Table II, indicate that there is

TABLE II
EFFECT OF HYPOGLYCEMIC AGENTS
ON NEUTRAL-C¹⁴-COMPOUNDS

Each flask contained the acetate incubation mixture and 10 μ moles of acetate (0.5 μ C of C¹⁴). Aerobic incubations at 37° for 2 hours.

Hypoglycemic Agent	Conc. (M $\times 10^3$)	Specific Activity of Sterol (cpm/mg)	Neutral Compounds (total cpm)
None		1030	678
Chlorpropamide	4	136	9,100
Insulin (10 units)		16	9,900
Phenethylbiguanide	4	24	10,290
Metahexamide	4	139	11,640
Tolbutamide	4	46	12,110

a large accumulation of the neutral petroleum ether-soluble fraction in the inhibited mixtures. Since the nature of these neutral compounds could not be ascertained, mevalonic acid, a more direct precursor of cholesterol than is acetate (Tavormina *et al.*, 1956, and Popjak *et al.*, 1958) was used in further experiments.

Effect of Hypoglycemic Compounds on the Incorporation of Mevalonate-2-C¹⁴ into the Nonsaponifiable Lipids.—Figures 1 and 2 show that the hypoglycemic compounds tolbutamide and phenethylbiguanide also inhibited the incorporation of mevalonate-2-C¹⁴ into cholesterol. The maximal inhibition was obtained with the same concentrations of the hypoglycemic compounds as when acetate-1-C¹⁴ was used (4×10^{-3} M). The maximal inhibition, however, was not the same as

when the crude homogenate preparation and acetate-1-C¹⁴ were used. The difference is probably due to the better preparation of the enzyme system used in this series of experiments.

When the total nonsaponifiable lipid was determined, it was found that tolbutamide, chlorpropamide, and metahexamide had no effect on the incorporation of mevalonate-2-C¹⁴ into this fraction, but that the formation of cholesterol was still suppressed. Phenethylbiguanide, on the other hand, inhibited the incorporation of mevalonate-2-C¹⁴ into both cholesterol and the total nonsaponifiable lipids to approximately the same extent (Table III).

The Site of Action of the Hypoglycemic Compounds.—After incubation of mevalonate-2-C¹⁴ with the mevalonate incubation mixture under air, the isoprenols, presqualene branched chain alcohol intermediates of cholesterol, were isolated by the method of Popjak (1959b). The results in Table IV show that phenethylbiguanide increases the formation of these compounds, whereas the arylsulfonyleureas have no effect.

When the incubations were carried out anaerobically under nitrogen, the formation of the nonsaponifiable lipid from mevalonate-2-C¹⁴ was

TABLE IV
EFFECT OF HYPOGLYCEMIC COMPOUNDS ON THE FORMATION OF ISOPRENOL-PP FROM MEVALONATE-2-C¹⁴

Each incubation flask contained mevalonate incubation mixture, glutathione (30 μ moles), and mevalonate-2-C¹⁴ (1 μ mole, 0.25 μ C of C¹⁴). The concentration of each hypoglycemic compound was 2.5×10^{-3} M. Aerobic incubations at 37° for 2 hours.

Hypoglycemic Compound	Specific Activity of Cholesterol (cpm/mg)	Isoprenol-PP (cpm)
None	11,960	3,360
Phenethylbiguanide	6,320	10,040
Tolbutamide	3,980	3,370
Chlorpropamide	7,350	3,100
Metahexamide	6,020	3,870

TABLE III
EFFECT OF HYPOGLYCEMIC COMPOUNDS ON THE INCORPORATION OF MEVALONATE-2-C¹⁴ INTO THE NONSAPONIFIABLE LIPIDS

Each flask contained 1.0 ml of dialyzed soluble enzymes, suspension of washed microsomes 0.2 ml, 1.0 ml of mevalonate incubation mixture, mevalonate-2-C¹⁴ (1 μ mole, 0.25 μ C of C¹⁴), and glutathione (30 μ moles). The concentration of each hypoglycemic compound was 2.5×10^{-3} M. Aerobic incubations at 37° for 1 hour.

Hypoglycemic Compound	Nonsaponifiable Lipid (total cpm)	% Inhibition	Specific Activity of Cholesterol (cpm/mg)	% Inhibition
None	77,780		5260	
Phenethylbiguanide	47,990	38.3	2760	47.4
Tolbutamide	73,860	5.0	1970	62.5
Chlorpropamide	71,060	8.6	2730	48.0
Metahexamide	72,290	7.0	3440	34.6

again inhibited by phenethylbiguanide but not by the arylsulfonylureas (Table V). The same table also shows that phenethylbiguanide lowered the formation of squalene and increased isoprenol production from mevalonic acid, whereas the arylsulfonylureas had no effect.

TABLE V
EFFECT OF HYPOGLYCEMIC COMPOUNDS ON THE BIOSYNTHESIS OF SQUALENE FROM MEVALONATE-2-C¹⁴

Each incubation flask contained mevalonate incubation mixture with mevalonate-2-C¹⁴ (1 μ mole, 0.15 μ C of C¹⁴). The concentration of each hypoglycemic compound was 4×10^{-3} M. Anaerobic incubations under nitrogen for 1 hour.

Hypoglycemic Compound	Nonsaponifiable Lipid (total cpm)	Isoprenol-PP (cpm)	Squalene (cpm)
None	39,100	3350	23,750
Phenethylbiguanide	24,100	7270	14,310
Tolbutamide	37,700	2950	24,230
Chlorpropamide	39,300	3360	21,440

DISCUSSION

The hypoglycemic compounds, tolbutamide, chlorpropamide, metahexamide, and phenethylbiguanide, inhibit the incorporation of both acetate-1-C¹⁴ and mevalonate-2-C¹⁴ into cholesterol. 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 both acetate-1-C¹⁴ and mevalonate-2-C¹⁴. This would imply that phenethylbiguanide inhibits the biosynthesis of cholesterol before the formation of the cholesterol intermediate found in the nonsaponifiable lipid fraction. This is substantiated by the inhibition of squalene formation from mevalonate-2-C¹⁴. Furthermore, since there is an accumulation of the isoprenols, it follows that phenethylbiguanide interrupts the biosynthesis of cholesterol at the level of some isoprenol intermediate.

The arylsulfonylurea compounds, tolbutamide, chlorpropamide, and metahexamide, however, are different as regards their effect on the biosynthesis of cholesterol. They do not inhibit the incorporation of either acetate-1-C¹⁴ or mevalonate-2-C¹⁴ into the nonsaponifiable lipid and cholesterol by the same magnitude. Starting with mevalonate-2-C¹⁴, there is almost no difference between the nonsaponifiable 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. The arylsulfonylurea compounds, therefore, inhibit cholesterol biosynthesis after the formation of squalene.

The increase in the neutral-C¹⁴ compounds obtained with acetate-1-C¹⁴ as the precursor of cholesterol cannot be explained at the present time. The explanation will have to await the elucidation of the nature of this fraction, which does no doubt contain the isoprenols, but since acetate was used in the biosynthesis of cholesterol, the formation of other compounds is possible.

The suppression of cholesterol biosynthesis by the hypoglycemic compounds, as reported in this paper, might partially explain the lowering of blood cholesterol by tolbutamide (Munroe and Shipp, 1962; Owen, 1962) and chlorpropamide (Owen, 1962). The decrease of the level of circulating blood cholesterol by insulin as reported by Nitzescu *et al.* (1924) also could be explained partially by the inhibition of cholesterol biosynthesis.

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