*Atherosclerosis*
Elsevier Publishing Company, Amsterdam – Printed in The Netherlands



# SPECIFIC INHIBITION OF CHOLESTEROL ABSORPTION BY SULFAGUANIDINE

H. J. EYSSEN, J. F. VAN DEN BOSCH, G. A. JANSSEN AND H. VANDERHAEGHE
*University of Leuven, Rega Institute for Medical Research, B-3000 Louvain (Belgium)*
(Revised, received January 7th, 1971)

SUMMARY

(1) Feeding of 1% sulfaguanidine to mice on a cholesterol-supplemented diet lowered liver cholesterol concentrations about 50%. This phenomenon was obtained with commercial diet, and with formula diets containing different carbohydrates.

(2) Single oral doses of sulfaguanidine promoted the fecal excretion of simultaneously given [4-$^{14}$C]cholesterol.

(3) Fecal excretion of fatty acid was not affected by sulfaguanidine. Fecal excretion of bile salts was slightly depressed.

(4) The effect of sulfaguanidine was most pronounced when the drug and cholesterol were fed simultaneously. Prior feeding of sulfaguanidine for 1 week did not alter cholesterol absorption after withdrawal of sulfaguanidine.

(5) The inhibition of cholesterol absorption does not depend on the antibacterial action of sulfaguanidine, since it also reduced liver cholesterol in germfree mice.

(6) Compounds with chemical structures resembling that of sulfaguanidine were inactive.

Key words: *Cholesterol absorption and excretion – Germfree – Mouse – Sulfaguanidine*

## INTRODUCTION

LEVEILLE AND CHAKRABARTY[1] observed that sulfaguanidine depressed liver cholesterol and lipid levels in rats fed cholesterol-supplemented diets. In a preliminary communication[2] we reported that feeding of sulfaguanidine promoted the fecal excretion of cholesterol in mice and rats. The drug also inhibited the rise in the cho-

This work was supported by a grant of the "Fonds voor Kollektief Fundamenteel Onderzoek".

lesterol content of livers of mice fed a cholesterol-rich diet, whereas none of several other sulfonamides had the same effect. It thus appeared that the cholesterol-lowering effect of this product was not a property of sulfonamides in general.

The present report describes the effect of sulfaguanidine when fed under various experimental conditions affecting the absorption of cholesterol. An attempt to clarify the mode of action of sulfaguanidine was made by further investigation of its effect on fecal cholesterol, bile salt and fatty acid excretion and by following its influence on endogenous cholesterol.

## MATERIALS AND METHODS

Unless otherwise stated, female NMRI mice weighing between 20 and 22 g were used. Basal food consisted of 56% corn starch, 20% casein, 5% gelatin, 9% corn oil, 5% powdered cellulose, 3% mixed salts*, and 2% mixed vitamins**. Cholesterol (Merck, Darmstadt) was dissolved in ether and bile salts in ethanol before being mixed with the food. Crystalline sulfaguanidine (PCB, Brussels) was added as such. After the solvents were allowed to evaporate, enough water was added to obtain a kneadable dough. This was cut in cubes which were dried at room temperature.

Test meals were prepared by blending 125 g of lean white cheese and 60 g of sucrose with water to obtain a final volume of 300 ml. Radioactive tracers were added in a small volume of ethanol. By means of a syringe with a blunt needle, 0.4 ml was administered in the esophagus of the mice.

Germfree animals were kept in Trexler plastic isolators[3]. They were fed a formula consisting of 58.22% corn starch, 20% casein, 9% corn oil, 5% cellulose, 5% mixed salts, 0.5% mixed vitamines, 0.3% methionine, 0.2% chromium oxide and 0.2% choline hydrochloride. The composition of salt and vitamin mixtures was the same as that used in the Lobund-356 formula[4]. The food was sterilized in an autoclave at 121°C for 25 min.

Food consumption was measured by adding 0.2% chromium oxide to the diet, and assaying it in the feces by the method of EDWARDS AND GILLIS[5]. For these experiments the diets were also sterilized, since this reduced crumbling, so that contamination of feces by spilled food was kept to a minimum.

Cholic acid was purchased from Fluka AG (Switzerland), the other bile salts from Maybridge Chemical Co. (Cornwall, U.K.). [4-$^{14}$C]Cholesterol was obtained from CEN (Centre Energie Nucléaire), Mol, Belgium, and [14-$^{14}$C]cholic acid from NEN (New England Nuclear), Boston, U.S.A.

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* Composition of salt mixture: $CaCO_3$, 1.086 g; $MgCO_3$, 50 g; $MgSO_4$, 32 g; NaCl, 138 g; KCl, 224 g; $KH_2PO_4$, 42 g; $FePO_4 \cdot 4H_2O$, 41 g; KI, 0.160 g; $MnSO_4$, 0.7 g; NaF, 2 g; $Al_2(SO_4)_3 \cdot K_2SO_4$, 0.34 g; $CuSO_4$, 1.8 g.

** Composition of vitamin mixture: inositol, 200 g; vitamin $B_1$, 2 g; vitamin $B_2$, 2 g; pyridoxine, 2 g; nicotinamide, 10 g; calcium pantothenate, 10 g; menadione, 1 g; Rovimix E-25, 100 g; Rovimix (325-110) A + $D_3$, 73 g.

The powdered mixture is diluted with enough glucose to obtain a final weight of 2000 g. In addition to these vitamins, 2 mg of biotin, 40 mg of neutralized folic acid and 1000 U. of vitamin $B_{12}$ were added per 10 kg of food.

*Analyses*

*Cholesterol determinations.* Mice were decapitated and blood was collected on weighed filter paper disks. After drying at room temperature the disks were weighed again and snippered in glass-stoppered tubes. They were saponified in 3 ml of a mixture of equal volumes of toluene, isopropanol, and methanol, to which 100 mg stigmasterol per 300 ml had been added as internal standard and also 10% by volume of a 45% aqueous solution of KOH. After 90 min at 65°C in a water bath, 1 ml of water was added and the sterols extracted with 5 ml light petroleum (b.p. 60–80°C). This extract was analyzed in a gas–liquid chromatograph on a column of 3% JXR on Gas-chrom Q (Applied Sciences Lab., U.S.A.) at 290°C. The amount of cholesterol was computed from the relative peak heights of cholesterol and stigmasterol. For each series of determinations a set of cholesterol standards was processed along with the samples.

Livers were saponified in strong alcoholic kali. Suitable aliquots were extracted with light petroleum. This was evaporated and the residue further treated as the blood samples. Carcasses were homogenized in a meat grinder and skins fragmented with scissors before aliquots were saponified and further treated in the same way.

*Fecal bile salts* were assayed by the method of EVRARD AND JANSSEN[6], and fecal fatty acids according to VAN DE KAMER *et al.*[7].

*Radioactivity determinations* were made in a Nuclear Chicago liquid scintillation spectrometer. Cholesterol extracts in light petroleum were evaporated in counting vials and counted after addition of 15 ml toluene containing Omnifluor (NEN, U.S.A.). Bile acid extracts in either were treated in the same way but counted in a dioxane-based scintillator mixture. Quench corrections were done with internal standards.

*Synthesis of new compounds*

3,5-Dibromosulfaguanidine was prepared by treatment of sulfaguanidine with bromine in acetic acid. Acetylation of sulfaguanidine with acetic anhydride in pyridine gave $N^4$-acetylsulfaguanidine[8]. Reaction of guanidine nitrate with *p*-toluenesulfonyl chloride in aqueous acetone in the presence of alkali yielded *p*-tolylsulfonylguanidine[9].

## RESULTS

*Influence of sulfaguanidine on liver cholesterol levels of mice fed cholesterol and cholic acid added to different diets*

The absorption of cholesterol partly depends on the type of diet in which it is fed[10–14]. To ascertain whether the cholesterol-lowering effect of sulfaguanidine was influenced by dietary variables, we fed 1% of the drug to groups of 10 mice that received 1% cholesterol and 0.1% cholic acid in commercial food or in basal food with 56% starch or in the same with 56% sucrose. Control groups without sulfaguanidine and controls without the cholesterol and cholic acid supplements were also included in

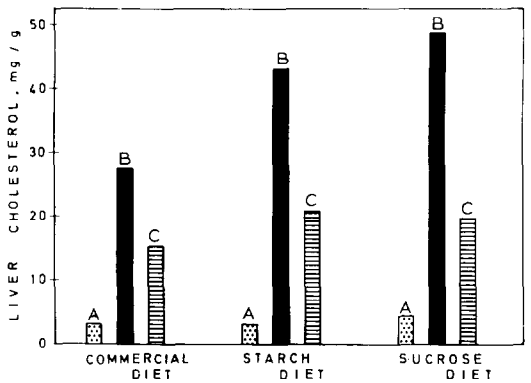


Fig. 1. Average concentration of cholesterol in livers of mice fed different types of diets, with added cholesterol and cholic acid, and sulfaguanidine. A: diet without additions; B: cholesterol and cholic acid added; C: idem plus sulfaguanidine.

the experiment. The animals were killed after 14 days and the cholesterol content of their livers determined.

The results are summarized in Fig. 1. The groups that received the purified diet developed significantly higher cholesterol concentrations than the one fed the commercial diet. Sulfaguanidine was active in either type of diet and inhibited the increase of liver cholesterol concentrations by 45–60%.

The low concentration of liver cholesterol obtained by adding sulfaguanidine to cholesterol-supplemented diets could be caused by a decrease of intestinal cholesterol absorption, an increase of sterol elimination, a change of body cholesterol distribution, or a combination of these factors.

Possible differences in cholesterol distribution were examined in an experiment in which 2 groups of 10 mice were fed 1% cholesterol and 0.1% cholic acid with or without 1% sulfaguanidine; a third group received the diet without these additions. After 2 weeks the animals were decapitated and skinned, and blood samples were taken. Cholesterol was determined in total blood, the livers, the skins, and the carcasses (including the intestines and their contents but excluding the heads). We conclude from the result (Table 1) that only the livers took up appreciable amounts of cholesterol during the feeding period, while the other tissues examined showed quantitatively unimportant changes. Sulfaguanidine caused no shift of cholesterol from the liver to other parts of the body and it reduced the total body cholesterol.

*Influence of sulfaguanidine on absorption of cholesterol from a test meal*

The effect of sulfaguanidine on cholesterol absorption was investigated by feeding various single doses of sulfaguanidine in a test meal containing 0.1 $\mu$Ci of [4-$^{14}$C]cholesterol to groups of 10 mice. The feces were quantitatively collected and pooled during 3 days, and the amount of radioactivity was determined in the neutral sterol extract. The results, shown in Fig. 2, indicate that the drug increased the

TABLE 1

INFLUENCE OF SULFAGUANIDINE ON CHOLESTEROL DISTRIBUTION IN TISSUES OF MICE FED A CHOLESTEROL–CHOLIC ACID DIET FOR 2 WEEKS

S = significantly different ($P < 0.01$) from group a ($S_a$), group b ($S_b$) or groups a and b ($S_{ab}$). NS = not significantly different.

| *Cholesterol* | *Controls (Group a)* | *Cholesterol + 0.1 % cholic acid diet (group b)* | *Cholesterol–cholic acid + 1 % sulfaguanidine (group c)* |
|---|---|---|---|
| Liver, total mg | 4.2 | 84.7 | 28.9 $S_{ab}$ |
| Skin, total mg | 10.0 | 10.9 | 9.9 NS |
| Carcass, total mg | 23.0 | 24.5 | 23.5 NS |
| Total | 37.2 | 120.1 | 62.3 $S_{ab}$ |
| Blood, mg/g dried blood | 5.6 | 7.3 | 6.2 $S_b$ |

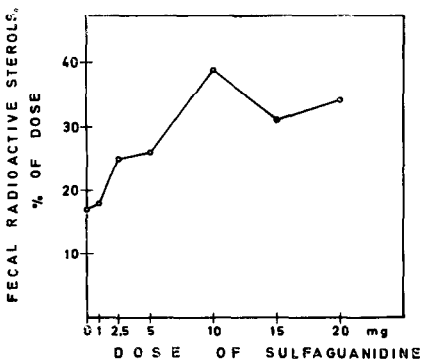


Fig. 2. Effect of single oral doses of sulfaguanidine on the fecal excretion of simultaneously administered [4-$^{14}$C]cholesterol.

excretion of the administered tracer from 17% in the controls to about 35%; the maximal effect was obtained from 10 mg on.

In the next experiment we investigated whether feeding of sulfaguanidine prior to the administration of cholesterol would also cause an increased sterol excretion. Mice were fed 1% of sulfaguanidine during 1 week. Thereafter the animals received a test meal containing 0.1 $\mu$Ci of radioactive cholesterol (specific activity 47 $\mu$Ci/m$M$) either with or without 10 mg of sulfaguanidine. Groups of mice not prefed with sulfaguanidine were treated in the same way. After administration of the test meal, all groups received the diet without sulfaguanidine.

The fecal radioactivity recovered after 3 days showed that the excretions of the animals prefed with the drug were not different from those of the others (Fig. 3). These results indicate that sulfaguanidine promoted the fecal output of cholesterol only when it was in the intestine at the same time as cholesterol, and that its effect disappeared immediately upon withdrawal of the drug.

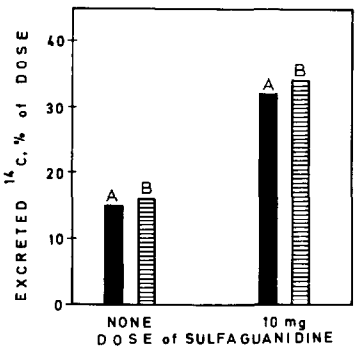


Fig. 3. Effect of sulfaguanidine on the fecal excretion of [4-$^{14}$C]cholesterol. A: controls; B: mice fed 1% sulfaguanidine during 1 week before test meals.

*Lack of effect of sulfaguanidine on the absorption of bile salts and fatty acids*

The absorption of cholesterol strongly depends on the presence of mixed micelles, fatty acids and monoglycerides, in which cholesterol is solubilized before it is taken up by the mucosa[15–18]. Therefore we studied the effect of sulfaguanidine on absorption and excretion of bile salts and fatty acids.

The effect of sulfaguanidine on absorption of cholic acid was followed by feeding a test meal containing 0.1 $\mu$Ci of [24-$^{14}$C]cholic acid to 3 groups of 15 mice. One group received 5 mg sulfaguanidine 4 h before the tracer, a second time together with it, and a third time 4 h after it. The second group received 3 doses of 10 mg, and the third was fed the test meals without the drug. Feces of each group were collected during 3 days and pooled; the radioactivity of the acid sterol fraction was determined. No effect of sulfaguanidine was found: the controls excreted 30.3% of the administered dose, and the groups treated with 3 times 5 or 10 mg of sulfaguanidine excreted, 31.9 and 31.5% respectively. In a second experiment we injected 0.25 $\mu$Ci of [24-$^{14}$C]cholic acid intraperitoneally in groups of 10 mice fed 1% sulfaguanidine or basal diet. The radioactivity of fecal bile acids was followed during 14 days. The cumulative excretion was 20% lower in the treated group than in the controls. This may be the result of the antibacterial effect of the drug.

Both experiments indicate that in contrast with the effect of sulfaguanidine on cholesterol, the absorption of cholic acid is not inhibited. The possible interference of sulfaguanidine with other types of bile salts was investigated by using diets with different types of bile salts. Replacement of 0.1% cholic acid in the experimental diets by an equimolar concentration of taurocholic, deoxycholic or taurodeoxycholic acid did not interfere with the activity of sulfaguanidine.

The influence on triglyceride absorption was measured by feeding 1% sulfaguanidine to groups of 8 male and 8 female C3H mice for 2 weeks. Their feces were collected, the fatty acid content was determined. Food consumption was measured, and the excretion of fatty acids per g food was calculated. No consistent effect of sulfaguanidine was found: male controls excreted 3.57 mg fatty acid per g food consumed, the treated ones 2.91 mg; for the females the figures were 2.46 and 2.63 mg for the controls

and the treated groups respectively. Since the food intake was the same in treated and untreated groups, we may conclude that the efficiency of digestion and absorption of fat was not disturbed by the drug.

The evidence provided by all the experiments of this paragraph warrants the conclusion that sulfaguanidine does not induce a general malabsorption of intestinal lipids. Its effect on cholesterol therefore seems to be highly specific.

*Influence of sulfaguanidine on mice prefed a cholesterol–cholic acid diet*

Although the data presented above showed that sulfaguanidine interfered with the intestinal absorption of cholesterol, an additional direct effect on liver cholesterol deposits was not excluded. The next two experiments explored this latter possibility.

In the first we fed 1% cholesterol and 0.1% cholic acid in the basal diet to 160 female mice for 2 weeks. The diet was then continued without cholesterol or cholic acid. After 2 days 20 mice were killed and their liver cholesterol contents determined. The remaining animals were divided in 2 groups, one of which was fed 1% sulfaguanidine, while the other continued to receive the control diet. Ten mice of each group were killed at different times, indicated in Fig. 4 which shows the evolution of liver cholesterol values in both series. The average value of all sulfaguanidine-treated mice was 24% lower than that of the controls. This rather small difference in disappearance of the excess of liver cholesterol is in contrast with the pronounced inhibition of cholesterol uptake in the experiments where the drug was fed together with the sterol. The cumulative output of fecal neutral sterols was nearly twice as high in the treated group as in the controls (Fig. 4). The difference between the fecal sterol outputs of treated and control mice was thus proportionally larger than the difference found in

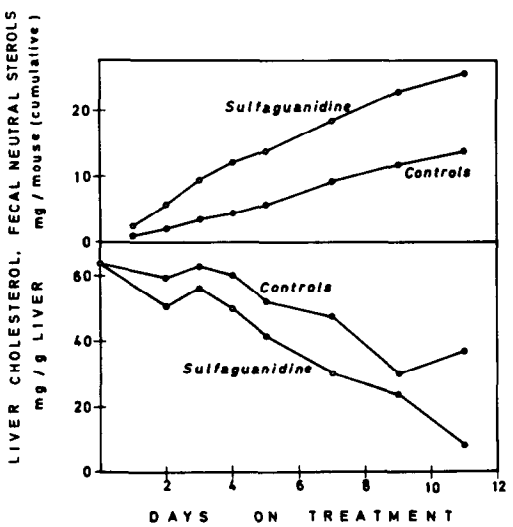


Fig. 4. Effect of dietary sulfaguanidine on liver cholesterol concentrations and fecal excretion of neutral sterols of mice. All animals had received a diet with 1% cholesterol and 0.1% cholic acid for 2 weeks before treatment with sulfaguanidine was started.

TABLE 2

EFFECT OF DIETARY SULFAGUANIDINE ON LIVER CHOLESTEROL OF GERMFREE C3H MICE FED 1 % CHOLESTEROL FOR 2 WEEKS

S = significantly different from untreated controls ($P < 0.01$). NS = not significantly different.

| *Treatment* | *Sex* | *No. of animals* | *Body weight (g)* | *Liver cholesterol (mg/g)* |
|---|---|---|---|---|
| 1 % Cholesterol | female | 13 | 30.0 | 21.32 |
| Idem + 1 % sulfaguanidine | female | 11 | 29.4 NS | 10.07 S |
| 1 % Cholesterol | male | 13 | 36.8 | 7.95 |
| Idem + 1 % sulfaguanidine | male | 13 | 36.2 NS | 4.36 S |

the livers. The fecal loss of bile salts was also determined, and amounted to 0.38 mg per day in the control mice and 0.28 mg per day in the treated ones.

*Influence of germfree state and sex*

Intestinal microflora interfere with absorption of cholesterol[11,19,20]. To investigate whether the inhibition of cholesterol absorption could be mediated by the antibacterial effect of the drug, sulfaguanidine was administered to germfree mice. Groups of 13 germfree C3H mice of both sexes were fed 1 % cholesterol (without cholic acid), and the treated groups the same with 1 % sulfaguanidine. This treatment lowered liver cholesterol concentrations in males as well as in females (Table 2). There is, however, a striking difference in liver cholesterol values between the sexes: female mice had almost three times as high levels as males. In other experiments, to be reported later, we found that a similar sexual difference exists in conventional mice and that it persists when cholic acid is included in the diet.

*Specificity of sulfaguanidine*

Our previous communication[2] reported that none of several sulfonamides shared the cholesterol-lowering effect with sulfaguanidine. However, biguanides, remotely resembling sulfaguanidine in that these molecules also possess a guanidino moiety, have been reported to lower cholesterol and reduce atherogenesis in rabbits[21,22]. We examined whether this effect was related to that of sulfaguanidine in mice by giving three feedings of 5, 10 or 20 mg of 1,1-dimethylbiguanidine to groups of 5 mice. The drug was given in test meals, at 4-h intervals between doses; the second test meal contained 0.1 $\mu$Ci of [4-$^{14}$C]cholesterol. Three groups receiving sulfaguanidine in the same way and in the same quantity and a control group receiving test meals without drugs were also included in the experiment. The effect on absorption of cholesterol was assessed by measuring the total output of radioactive neutral sterols in the feces of 3 days. No increase was obtained with biguanide, whereas sulfaguanidine had a clear and dose-dependent effect (Fig. 5). We conclude that the cholesterol-lowering activity of biguanides in rabbits depends on a mechanism different from that of sulfaguanidine in mice.

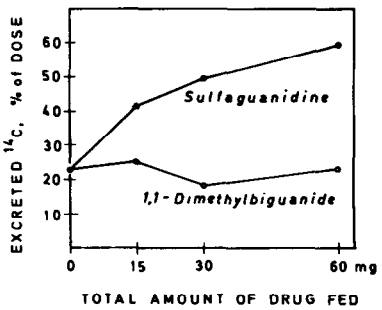


Fig. 5. Effect of sulfaguanidine and 1,1-dimethylbiguanide on the fecal excretion of labeled cholesterol. All mice received 3 test meals, spaced at 4-h intervals, containing the drugs; a tracer dose of [4-$^{14}C$]cholesterol was included in the second test meal.

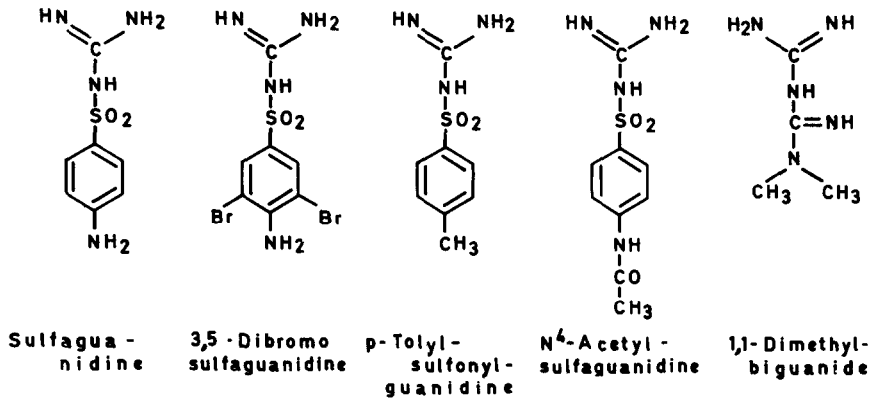


Fig. 6. Structural formulas of compounds related to sulfaguanidine which failed to inhibit cholesterol absorption.

In a further attempt to investigate the specificity of the sulfaguanidine molecule in this respect, we synthetized three new compounds with structural formulas closely resembling that of sulfaguanidine (Fig. 6). Their possible effect on cholesterol absorption was tested by feeding 0.5 or 1% in cholesterol–cholic acid-supplemented diets to mice for 2 weeks. None of these compounds reduced liver cholesterol concentrations. This result again emphasizes the uniqueness of sulfaguanidine.

## DISCUSSION

The present study confirms our previous observation that sulfaguanidine inhibits the rise of liver cholesterol concentrations of cholesterol-fed mice[2]. Sulfaguanidine belongs to the class of poorly absorbed sulfonamides. After prolonged administration of high doses, sulfaguanidine and certain other sulfonamides depress thyroid function[23,24]. The goitrogenic effect, however, cannot explain the cholesterol-lowering activity since depression of thyroid function is associated with a rise of the cholesterol

levels, not with a decrease[25]. Moreover, desiccated thyroid did not vitiate the effect of sulfaguanidine in cholesterol-fed rats[1].

The results of the present investigations point to the intestine as the site of action of sulfaguanidine. When single doses were given together with trace amounts of cholesterol, a two-fold increase of fecal excretion of the tracer was observed. Prior feeding of sulfaguanidine for 1 week did not affect cholesterol absorption from a test meal given 18 h after withdrawal of the drug, thus suggesting that the intestinal mucosa remained intact. Sulfaguanidine was most effective when the drug and the sterol were fed simultaneously. Mobilization of cholesterol from the livers of mice prefed a cholesterol–cholic acid diet was only slightly stimulated. However, the cumulative output of fecal neutral sterols was doubled. Although this experiment does not exclude the possibility that sulfaguanidine stimulated biliary excretion of cholesterol, it seems more likely that the effect of the drug was limited to a local action in the intestine resulting in increased removal of cholesterol from the enterohepatic circulation.

The question of the mechanism of action of sulfaguanidine as an inhibitor of cholesterol absorption remains unsolved. There are many conceivable ways by which the absorption of cholesterol could be reduced. The intricate organisation of intestinal lipids by which mixed micelles are formed can be disarranged by removing bile salts, either by surgical diversion of bile or by administering bile salt sequestrants such as cholestyramine[26,27]. This compound forms an insoluble complex with bile salts and increases their fecal excretion; when a large enough dose is given, the excretions of neutral sterols and fatty acids are also promoted[28]. A similar effect is found with neomycin, which precipitates certain bile salts from their solutions[29,30], and breaks up micellar complexes when they contain fatty acids[31]. Our data do not sustain the hypothesis that sulfaguanidine affects the absorption of cholesterol by a similar mechanism. The intestinal absorption of labeled cholic acid was not changed, and after parenteral injection of this bile acid the drug slightly reduced rather than increased its fecal output. Chemical assays of bile acids confirmed these results. We conclude that sulfaguanidine is not a bile salt sequestrant, and that, in view of its unaltered effect in diets with different bile salts, its mechanism of action is altogether independent of them.

Fatty acids and monoglycerides, the products of triglyceride hydrolysis, promote the absorption of cholesterol by increasing its solubility in the mixed micelles, and one could imagine that the low absorption of cholesterol would be a consequence of a shortage of micelle constituents. However, normal amounts of fatty acids were excreted when sulfaguanidine was fed. This indicates that the drug did not disturb the digestion of triglycerides, and that only cholesterol itself became unabsorbable in the presence of sulfaguanidine.

In this respect the action of sulfaguanidine resembles that of plant sterols, which also inhibit the absorption of cholesterol without affecting that of other intestinal lipids. At the present stage of our work, it is difficult to compare the mechanism of sulfaguanidine with that of plant sterols. The latter could act by blocking absorption

sites of cholesterol[32], by competing with the esterification and transport of cholesterol in the mucosal cell[33], or by displacing cholesterol from the mixed micelles. All these possibilities find reasonable ground in the close chemical and physical resemblance between cholesterol and the plant sterols. The chemical and physical properties of sulfaguanidine are totally unrelated to those of cholesterol. It seems improbable, therefore, that the drug would compete with cholesterol for the same absorption sites of the cell. Interference with cholesterol esterification in the mucosal cells should be considered as a possible mode of action. It should be mentioned, however, that experiments to be reported later have shown that cholesterol in the thoracic duct lymph of rats fed sulfaguanidine is equally well esterified as that in the lymph of control animals. Competition between sterols and sulfaguanidine for solubilization in micelles is an alternative hypothesis, but it is difficult to accept the proposition that such a mechanism would be specific for this drug alone.

So far as we know, sulfaguanidine is the only compound of its class to inhibit cholesterol absorption. It does not do so because it is an antibacterial drug, as evidenced by our results with germfree animals and by the observation that several other sulfonamides were inactive. The hypocholesterolemic effect of hypoglycemic biguanides[21,22] is different from that of sulfaguanidine, since 1,1-dimethylbiguanide did not promote cholesterol excretion under experimental conditions in which sulfaguanidine did. Newly synthetized derivatives that differed only slightly from sulfaguanidine completely lacked its effect on cholesterol absorption.

Finally, one could speculate that sulfaguanidine inhibits an enzyme involved in absorption or transport of cholesterol through the mucosal cells to the lymph. This would be compatible with the present observations and would also explain the high specificity of the drug as an inhibitor of cholesterol absorption. This hypothesis is now under investigation.

ACKNOWLEDGEMENTS

The skilful assistance of Misses A. Hendrickx, A. Heyvaert, and R. Massonet, and of Misters G. de Pauw, J. Mertens, L. Palmaerts, W. Put, and W. Wuyts, are gratefully acknowledged.

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