Biochemical Pharmacology, Vol. 24, pp. 1593–1598. Pergamon Press, 1975. Printed in Great Britain.

BILE ACID-BINDING ACTIVITY OF DODECA-*N*-METHYL NEOMYCIN HEXAMETHOCHLORIDE AND DIMETHYLAMINOPROPYL NEOMYCIN

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(*Received* 26 *November* 1974; *accepted* 17 *February* 1975)

Abstract—The effect of neomycin and polybasic neomycin derivatives on mixed micellar solutions of bile salt, cholesterol, oleic acid and glyceryl mono-oleate was studied *in vitro*. Neomycin and its polybasic derivatives caused precipitation of bile salt, fatty acid and cholesterol. Precipitation was more complete with taurochenodeoxycholate micelles than with taurocholate micelles. Dimethylaminopropyl neomycin was the most active compound, followed in order of decreasing activity by neomycin, dodeca-*N*-methyl neomycin hexamethochloride and *N*-methylated neomycin. Hexa-*N*-acetyl neomycin failed to precipitate any of the micellar components. The same order of activity was found when these compounds were tested for hypocholesterolemic activity in newborn chicks fed either a diet without added cholesterol or a diet with 0·25% exogenous cholesterol. The hypocholesterolemic effect in chicks correlated with a 2–5-fold increase of fecal excretion of bile salts, fatty acids and cholesterol. Dimethylaminopropyl neomycin and *N*-methylated neomycin significantly inhibited the rise of liver cholesterol levels in mice fed a 1% cholesterol–0·1% cholic acid diet. Neither compound was active when given to mice fed a diet without added cholesterol or a diet with 1% cholesterol but without cholic acid.

Oral administration of certain polybasic aminoglycosidic antibiotics such as neomycin [1] and paromomycin [2], lowers serum cholesterol levels in man. This reduction is accompanied by a decrease in the exchangeable pools of cholesterol [3], and by a significant increase in the fecal excretion of neutral sterols and bile acids [4, 5]. Kanamycin and neamine are less active than neomycin, whereas streptomycin is devoid of cholesterol-lowering activity [6, 7]. Neomycin also reduces serum- and liver cholesterol concentrations in chickens [8, 9]. Conversely, neomycin induces hypercholesterolemia in cholesterol-fed rats and rabbits [10, 11].

Since the aminoglycosidic antibiotics are poorly absorbed from the gut and since intramuscular injection of neomycin does not lower serum cholesterol [6], their hypocholesterolemic effect seems to be dependent upon their activity in the gastro-intestinal tract. Alteration of the microflora has been suggested as a possible mode of action [4]. However, previous studies in this laboratory demonstrated that the hypocholesterolemic effect of neomycin is independent of its antimicrobial activities since neomycin also reduces serum and liver cholesterol pools of germfree chicks [8], and since non-antibiotic derivatives of neomycin are hypocholesterolemic as long as their polybasic character is unaltered [8, 9]. Neomycin precipitates bile acids and fatty acids from micellar solutions *in vitro* [12, 13, 14], and promotes fecal excretion of bile acids and neutral sterols in man [4] and in chicks [8, 9]. These observations support the hypothesis that the cholesterol-lowering effect of neomycin is due to the ability of the drug to interact with bile acids and fatty acids in the intestine during the micellar phase of lipid absorption.

N-methylated neomycin, a polybasic non-antibiotic derivative of neomycin, has been shown to lower serum cholesterol concentrations in human patients [12]. However, the dose required for hypocholesterolemic activity was 3–6 g/day, as compared to 1·5–3 g for neomycin. The lower activity of *N*-methylated neomycin might be due to weakening of the basicity of the amino functions upon *N*-methylation. To investigate this hypothesis, we prepared dodeca-*N*-methyl-neomycin hexamethochloride*. This substance is the quaternary ammonium salt of dodeca-*N*-methyl neomycin B and bears six strongly basic functions. In addition, we investigated compound 1291 which is a *N*-dimethylaminopropyl derivative of neomycin† and bears up to 12 basic groups/molecule. Both substances have lost more than 99 per cent of the antibiotic activity of the parent neomycin molecule. In the present investigations we compared the cholesterol-lowering and bile acid-binding activity of these products to those of neomycin, *N*-methylated neomycin and hexa-*N*-acetyl neomycin.

* Dodeca-*N*-methyl neomycin hexamethochloride, the quaternary ammonium salt of dodeca-*N*-methyl neomycin B, is referred to as *N*-methylated neomycin-Q.A.S.

† Dimethylaminopropyl neomycin was prepared by P. Crooy, R.I.T., Genval, Belgium, and is referred to as compound 1291.

MATERIALS AND METHODS

Materials. [4-^{14}C]Cholesterol and [1-^{14}C]oleic acid were obtained from CEN (Centre de l'Energie Nucléaire, Mol, Belgium) and had a specific activity

of 42·4 mCi/m-mole and 53·4 mCi/m-mole, respectively. Unlabeled cholesterol and oleic acid (Merck, Darmstadt, Germany) were at least 99% pure by thin-layer and gas-liquid chromatography (t.l.c. and g.l.c.). Glyceryl-mono-oleate (Calbiochem, Los Angeles, U.S.A.) was only 90% pure by t.l.c. Sodium taurocholate and sodium taurochenodeoxycholate were obtained from Maybridge (Tintagel, Cornwall, U.K.) and were shown to contain no other bile salts by g.l.c.

N-methylated neomycin was prepared as described previously [9]. Hexa-*N*-acetyl neomycin was prepared according to Rinehart *et al.* [15]. Compound 1291 was prepared by reacting neomycin with dimethylaminopropyl chloride, with resultant mono- and di-substitution of the primary aminofunctions of the neomycin molecule. Dodeca-*N*-methyl neomycin hexamethochloride was obtained by reaction of dodeca-*N*-methyl neomycin B with methyl iodide in a mixture of ethanol and acetonitrile followed by conversion of the iodide to the chloride by means of an ion-exchange resin.

Micellar solutions. Double-strength mixed micellar solutions of bile salts, glyceryl-1-mono-oleate, oleic acid and cholesterol were prepared according to Thompson *et al.* [13, 16] by mixing on a magnetic stirrer appropriate amounts of the ingredients in 0·15 M NaCl–0·1 M phosphate buffer (5:1, v/v) at pH 6·5 for 2 hr at 37°. Two solutions were prepared: one in which oleic acid was labelled by adding tracer amounts (6000 cpm/ml) of [1-^{14}C]oleic acid, and one in which cholesterol was labelled by adding [4-^{14}C]cholesterol (15,000 cpm/ml).

Appropriate amounts of the sequestrants were dissolved in 0·15 M NaCl–0·1 M phosphate buffer (5:1, v/v) at pH 6·5 and 1 ml portions of these solutions were added to 1 ml of the double strength micellar solutions in 16 × 100 mm test tubes. The final concentrations of the micellar components were: 20 μmoles bile salt, 4·8 μmoles glyceryl-1-mono-oleate, 9·6 μmoles oleic acid and 0·1 μmole cholesterol per ml. The final concentrations of sequestrant ranged from 0·07 mg to 15 mg/ml.

After gentle shaking, the mixtures were incubated in stoppered tubes for 1·5 hr at 37° on a rotary shaker (100 rev/min). After incubation, the precipitates were centrifuged at 3,000 *g* for 15 min at 37°. Samples of the supernatants were removed for determination of the unprecipitated micellar constituents. The bile acids were determined by g.l.c. according to Evrard and Janssen [17]. The percentages of unprecipitated radioactive cholesterol and oleic acid were determined by dissolving 0·1 ml aliquots of the supernatants in 15 ml of a scintillation fluid consisting of 750 ml dioxane, 175 ml ethylene glycol mono-methyl ether, 45 g naphthalene and 8 g Omnifluor® (New England Nuclear, Boston, U.S.A.). Radioactivity was measured in a Nuclear Chicago system 727 liquid scintillation spectrometer, and corrections for quenching were done by addition of an internal standard. Glyceryl-1-mono-oleate was not determined.

Animals and diets. Newborn Hyline broiler chicks of both sexes were obtained from a commercial poultry farm. They were fed a purified casein–sucrose–starch diet as described previously [9].

Mice were 8-week-old females of the NMRI strain obtained from the Proefdierencentrum, Heverlee, Belgium. The animals were fed a purified diet the composition of which has been described elsewhere [18].

Feces were collected and pooled for each group

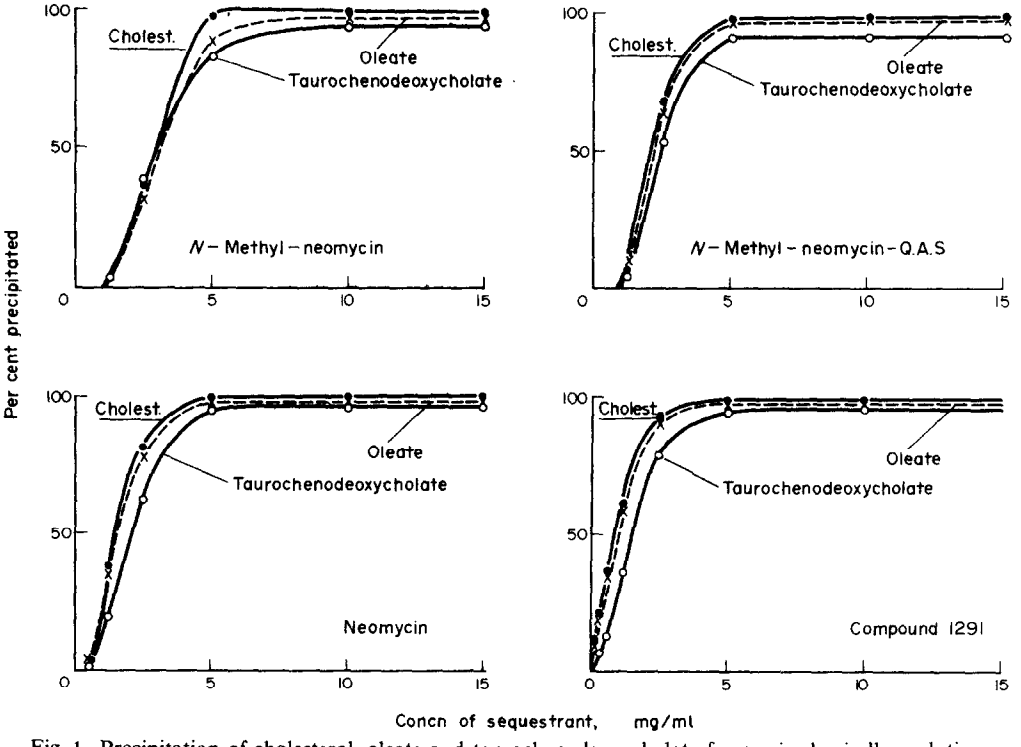

Fig. 1. Precipitation of cholesterol, oleate and taurochenodeoxycholate from mixed micellar solutions of cholesterol (0·1 μmole/ml), oleic acid (9·6 μmoles/ml), glyceryl-mono-oleate (4·8 μmoles/ml) and taurochenodeoxycholate (20 μmoles/ml) phosphate buffer at pH 6·5.

during four periods of 3 days. After homogenisation in an equal volume of water, two samples of each pool were freeze-dried and analyzed for fecal fatty acids, bile acids and cholesterol by g.l.c. [18]. At the end of the experiment the animals were fasted overnight and bled by cardiac puncture. Cholesterol was determined in serum and liver by g.l.c. as described elsewhere [18].

RESULTS

Precipitation of bile acids, fatty acids and cholesterol from mixed micellar solutions. The precipitating activities of *N*-methylated neomycin-Q.A.S. and compound 1291 were compared to those of neomycin, *N*-methylated neomycin and hexa-*N*-acetyl neomycin. The data in Figs. 1 and 2 show that all polybasic substances precipitated cholesterol, oleate and bile salts, albeit to different degrees. *N*-methylated neomycin was only weakly active. The concentration of sequestrant required for precipitation of 50% of taurocholate was 11·5 mg/ml for *N*-methylated neomycin-Q.A.S., 7 mg/ml for neomycin and 2·7 mg/ml for compound 1291. The same order of activity was noted for precipitation of oleate and cholesterol. The maximal amounts of taurocholate precipitated by sequestrants at a concentration of 15 mg/ml were 38% for *N*-methylated neomycin, 52% for *N*-methylated neomycin-Q.A.S., 58% for neomycin and 76% for compound 1291. At the same concentration of sequestrant, the maximal amounts of oleate and cholesterol precipitated were, respectively, 68 and 71% for *N*-methylated neomycin, 82 and 83% for *N*-methylated neomycin-Q.A.S., 89 and 92% for neomycin, and almost 100% for compound 1291. Hexa-*N*-acetyl neomycin, which is a neutral substance, failed to precipitate any of the components of the mixed micellar solutions.

Taurochenodeoxycholate micelles were more susceptible to the precipitating activity of polybasic neomycin derivatives than were taurocholate micelles. Precipitation of 50% of taurochenodeoxylate required no more than 3·2 mg/ml *N*-methylated neomycin, 2·4 mg/ml *N*-methylated neomycin-Q.A.S., 2·1 mg/ml neomycin and 1·6 mg/ml compound 1291. The data in Figs. 1 and 2 also show that, irrespective of the type of sequestrant, oleate and cholesterol were more easily and more completely precipitated from taurochenodeoxycholate micelles than from taurocholate micelles. This indicated that their incomplete precipitation from the taurocholate micelles was related to the type of bile acid.

Effect on cholesterol and bile acids of chicks. The cholesterol-lowering and bile acid-precipitating activity of the neomycin derivatives were studied in groups of 20 newborn chicks fed the experimental diets for 2 weeks.

None of the compounds affected the cholesterol concentrations in the liver when fed at the 0·2% level to chicks given a diet without added cholesterol. However, all but hexa-*N*-acetyl neomycin significantly reduced the serum cholesterol levels (Table 1). Chicks fed the basal diet lost 22 mg fecal bile acids per animal during the 12 days of the experiment. As could be expected from its neutral character, hexa-*N*-acetyl neomycin had no effect on the fecal excretion of bile acids, fatty acids or neutral sterols. Chicks fed 0·2% *N*-methylated neomycin, *N*-methylated neomycin-Q.A.S. or compound 1291, lost 39, 62 and

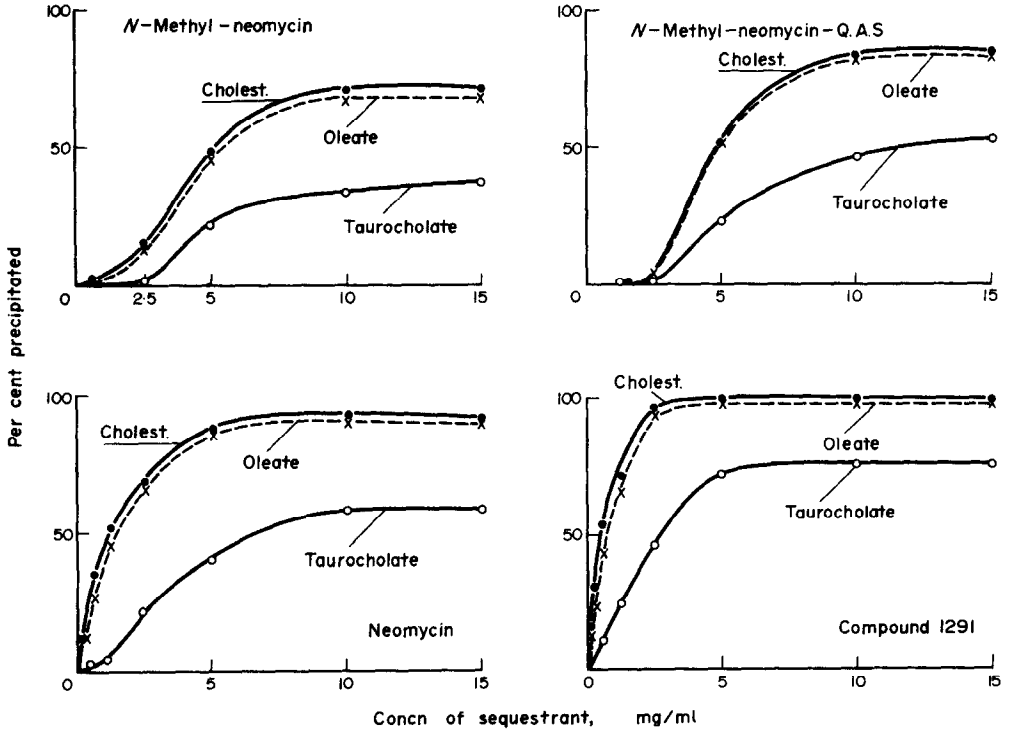

Fig. 2. Precipitation of cholesterol, oleate and taurocholate from mixed micellar solutions of cholesterol (0·1 μmole/ml), oleic acid (9·6 μmoles/ml), glyceryl-mono-oleate (4·8 μmoles/ml) and taurocholate (20 μmoles/ml) in phosphate buffer at pH 6·5.

Table 1. Effect of neomycin-derivatives on cholesterol and bile acids of newborn chicks fed a diet without added cholesterol for 2 weeks

Treatment	Body wt at 2 weeks (g)	Cholesterol levels: Serum (mg/100 ml)	Cholesterol levels: Liver (mg/g)	Fecal excretion: Bile acids	Fecal excretion: Cholesterol (mg animal per 289 hr)	Fecal excretion: Fatty acids
(1) Basal diet	173 ± 6*	206 ± 6·2	2·04 ± 0·04	22·0	36·6	300
(2) +0·2% *N*-methylated neomycin	167 ± 7	183 ± 5·6†	2·4 ± 0·06	39·4	52·5	686
(3) +0·4% *N*-methylated neomycin	163 ± 7	167 ± 8·7†	2·4 ± 0·09	56·4	64·5	1092
(4) +0·2% dodeca-*N*-methyl neomycin hexamethochloride	176 ± 8	182 ± 5·8†	2·6 ± 0·04	62·4	46·5	625
(5) +0·4% dodeca-*N*-methyl neomycin hexamethochloride	168 ± 6	173 ± 5·4†	2·7 ± 0·08	104·8	67·9	1208
(6) +0·2% compound 1291	178 ± 7	171 ± 10·8†	2·8 ± 0·20	134·2	61·8	1405
(7) +0·2% hexa-*N*-acetyl neomycin	165 ± 8	215 ± 6·9	2·7 ± 0·07	22·0	42·6	368

* Av. ± S.E.M. Groups of 18–20 animals.
† Significantly different ($P < 0·01$) from the animals fed the basal diet.

134 mg bile acids, respectively. Fecal excretion of cholesterol was increased to a lesser extent whereas the excretion of fatty acids followed the pattern of the bile acid excretion.

The effect of these substances on serum- and liver cholesterol concentrations was also studied in newborn chicks fed a 0·25% cholesterol diet for 2 weeks. The data presented in Table 2 show that addition to the basal diet of 0·25% cholesterol resulted in a rise of the serum cholesterol levels from 231 mg/100 ml in the controls, to 392 mg/100 ml in the cholesterol-fed group. Liver cholesterol increased from 2·6 to 17 mg/g. Feeding of 0·2% hexa-*N*-acetylneomycin did not alter the increase of serum- or liver cholesterol concentrations. Feeding of 0·2% *N*-methylated neomycin reduced the serum cholesterol concentrations by 22% and liver cholesterol by 48%. The same concentration of *N*-methylated neomycin-Q.A.S. reduced the serum and liver cholesterol concentrations by 36 and 44% respectively. Compound 1291 was, by far, the most active substance; feeding of 0·2% of 1291 reduced the concentration of serum cholesterol to values below those of the animals fed the diet without cholesterol, and the increase of cholesterol in the liver was inhibited more than 75%.

Effect of compound 1291 on liver cholesterol of mice. Nine groups of 12 female mice each were adapted to the basal diet for 1 week. Thereafter, three types of diet were fed. Three groups were continued on the basal diet; three groups received the basal diet supplemented with 1% cholesterol; three other groups were fed the basal diet supplemented with 1% cholesterol plus 0·1% cholic acid. In each dietary regimen, two groups were treated with 0·25 or 0·5% of compound 1291 mixed in the diet.

The data in Table 3 show the liver cholesterol concentrations after 2 weeks of treatment. Feeding of compound 1291 had no influence on the liver cholesterol levels of mice fed the basal diet or the 1% cholesterol diet. However, in mice fed a diet with 1% cholesterol plus 0·1% cholic acid, feeding of 0·25 or 0·5% of compound 1291 inhibited the rise of serum- and liver cholesterol concentrations by 10 and 50% respectively. In a similar experiment, *N*-methylated neomycin failed to inhibit the rise of liver cholesterol in mice fed a 1% cholesterol diet without addition of cholic acid. In mice given a 1% cholesterol–0·1% cholic acid diet, feeding of 1% *N*-methylated neomycin reduced the liver cholesterol by 22 ± 1%, whereas concentrations below 1% were ineffective. In this type of test, neomycin was inactive at a level of 0·25% and could not be administered at higher levels because the drug caused diarrhea and a decrease in body wt.

DISCUSSION

It is now well recognized that neomycin has a dual potential with regard to absorption and excretion of

Table 2. Effect of neomycin-derivatives on serum- and liver-cholesterol levels of newborn chicks fed a cholesterol diet for 2 weeks

Treatment	Body wt at 2 weeks (g)	Cholesterol levels: Serum cholesterol (mg/100 ml)	Cholesterol levels: Liver cholesterol (mg/g)	Cholesterol levels: Liver cholesterol (mg/liver)
(1) Basal diet without cholesterol	165 ± 4*	231 ± 9†	2·6 ± 0·1†	16·2 ± 0·8†
(2) Basal diet with 0·25% cholesterol	160 ± 3	392 ± 17	17·0 ± 1·7	105·2 ± 5·3
(3) +0·2% *N*-methylated neomycin	159 ± 4	307 ± 27†	9·6 ± 1·2†	55·8 ± 3·7†
(4) +0·4% *N*-methylated neomycin	153 ± 4	201 ± 24†	6·6 ± 1·5†	37·4 ± 2·8†
(5) +0·2% dodeca-*N*-methyl neomycin hexamethochloride	165 ± 3	251 ± 23†	7·5 ± 1·0†	41·6 ± 2·6†
(6) +0·4% dodeca-*N*-methyl neomycin hexamethochloride	146 ± 6	178 ± 10†	3·3 ± 0·4†	19·0 ± 1·4†
(7) +0·2% compound 1291	152 ± 5	161 ± 9†	3·8 ± 0·2†	27·7 ± 2·0†
(8) +0·2% hexa-*N*-acetyl neomycin	171 ± 6	407 ± 25	15·3 ± 1·0	106·9 ± 4·8

* Av. ± S.E.M. Groups of 18–20 animals.
† Significantly different ($P < 0·01$) from group 2 (basal diet + 0·25% cholesterol).

Table 3. Effect of compound 1291 on liver cholesterol of female NMRI mice

Diet*	Body wt (g)	Liver cholesterol (mg/liver)	(mg/g)
(1) (a) Basal	23·9 ± 1	3·3 ± 0·2	3·1 ± 0·1
(b) idem + compound 1291 0·25%	24·1 ± 1·2	3·3 ± 0·2	3·0 ± 0·1
(c) idem + compound 1291 0·5%	24·2 ± 0·8	3·0 ± 0·1	2·8 ± 0·1
(2) (a) Basal + 1% cholesterol	23·7 ± 0·7	27·6 ± 1·6	22·5 ± 0·9
(b) idem + compound 1291 0·25%	23·2 ± 1·4	21·0 ± 2·4	18·6 ± 1·8
(c) idem + compound 1291 0·5%	24·2 ± 0·9	25·6 ± 1·8	20·3 ± 1·2
(3) (a) Basal + 1% cholesterol + 0·1% cholic acid	22·9 ± 0·9	56·1 ± 1·2	45·3 ± 2·1
(b) idem + compound 1291 0·25%	24·9 ± 1·2	50·4 ± 1·3†	37·5 ± 1·8†
(c) idem + compound 1291 0·5%	24·0 ± 1·1	28·1 ± 0·9†	23·3 ± 0·9†

* The experimental diets were fed for 2 weeks to groups of 12 female mice.
† Significantly different ($P < 0.01$) from group (3(a)) (basal diet + 1% cholesterol + 0·1% cholic acid).

cholesterol and bile acids. By its antimicrobial activity against certain components of the intestinal microflora, neomycin tends to improve the absorption of cholesterol and bile acids. This mechanism seems to be predominant in rats and in rabbits. By its polybasic character, neomycin could precipitate bile acids, fatty acids and cholesterol from micellar solutions. This mechanism, which seems to operate in chicks and in humans, would result in increased fecal excretion of cholesterol and bile acids.

So far, no final explanation can be given for the species difference in response to neomycin. However, studies *in vitro* have shown that the bile acid- and fatty acid-binding activity of the neomycin class of compounds is influenced by several variables, e.g. the type of bile acid, the pH of the micellar solution and the number and character of the basic groups on the molecule of the sequestrant [12–14].

The observation that conjugated dihydroxy bile acids are precipitated more easily and over a broader pH range than are trihydroxy bile acids could at least partly explain the species differences in response to neomycin and its derivatives. Neomycin and its polybasic derivatives are potent bile salt-binding agents in chicks; yet, taurochenodeoxycholate accounts for more than 90% of the bile acids in chicken bile [9]. Conversely, neomycin does not promote the excretion of bile acids in rats [19]. The bile acids in rat bile almost exclusively consist of trihydroxy bile acids such as taurocholic acid and the tauromuricholic acids [20]. In germfree piglets, neomycin fails to increase the fecal output of hyocholic acid which is a trihydroxy bile acid accounting for more than 80% of the total bile acid pool in germfree pigs [21]. Approximately 60% of the bile acids in human bile are dihydroxy bile acids, mainly conjugated chenodeoxy- and deoxycholic acid [22]. Yet, neomycin and *N*-methylated neomycin promote fecal excretion of bile acids in human patients [4, 12].

Previous studies have shown that the effect of pH of the micellar solution on the bile acid- and fatty acid-binding activity of the sequestrant could be related to the number and the nature of the basic functions on the molecule [13]. The taurocholate-binding effect of compound 1291—with 12 basic groups—is stable up to pH 10, whereas the binding effect of neomycin—with 6 amino groups—falls off sharply above pH 6·5–6·7. Although *N*-methylated neomycin also bears six basic functions, its taurocholate-binding activity already declines at a pH above five. This might be related to weakening of the basicity of one of the amino groups of neomycin upon *N*-methylation since the titration curves show that *N*-methylation shifts the pK_a of one of the amino groups from 5·1 to 4·0. A similar weakening of basicity was observed upon *N*-methylation of other polyamines [23].

The present observations demonstrate that, in addition to the number and the basicity of the amino functions, other factors could control the bile acid-binding activity of the neomycin class of compounds. The six quaternary ammonium groups of *N*-methylated neomycin-Q.A.S. are significantly more basic than are the six amino functions of neomycin. In spite of this, *N*-methylated neomycin-Q.A.S. was consistently less active in precipitating bile acids than was neomycin. Conceivably, the presence of three methyl groups per amino function could result in a weakening of the binding affinity, e.g. by steric interference.

The results of studies on the hypocholesterolemic activity of neomycin and its derivatives in cholesterol-fed chicks correlate well with the results of the studies *in vitro* with mixed micellar solutions. Both *in vitro* and *in vivo*, the same order of activity was found. Compound 1291 was, by far, the most active substance, followed in order of decreasing activity by neomycin, *N*-methylated neomycin-Q.A.S. and *N*-methylated neomycin. In addition, compound 1291 was definitely active in the mouse.

Our previous studies in human patients showed that a daily dose of 3–6 g of *N*-methylated neomycin was required to obtain a hypocholesterolemic response of the same order as that observed by other investigators with a daily dose of 1·5–3 g of neomycin [12]. This observation is in agreement with the present data on the effects of neomycin and *N*-methylated neomycin on mixed micellar solutions. *N*-methylated neomycin-Q.A.S. and compound 1291 could not yet be tested in man. In contrast to *N*-methylated neomycin which is a non-toxic compound, the strongly basic neomycin derivatives are definitely nephrotoxic when injected into experimental animals. Although the available evidence indicates that *N*-methylated neomycin-Q.A.S. and compound 1291 are not absorbed after oral administration, extensive chronic toxicity studies should be conducted before clinical trials with these compounds can be performed.

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