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EFFECTS OF SINGLE OR REPEATED TREATMENT WITH SEVERAL ANTICHOLESTEROLEMIC COMPOUNDS ON BILIARY EXCRETION OF CHOLESTEROL

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Abstract—The effects of single or repeated treatments with nicotinic acid, 3-pyridine-acetic acid, inositol hexanicotinate, Na-phenylbutyrate, betaine phenylbutyrate, 1 : 4-dicaffeylquinic acid and sodium dehydrocholate on biliary excretion of cholesterol were studied in the rat. After a single intravenous administration, among the drugs experimented only 1 : 4-dicaffeylquinic acid at the highests doses examined and phenylbutyrates, these at the lowest doses, increased the biliary excretion of cholesterol. After repeated administrations by the intraperitoneal route none of the compounds examined succeeded in modifying in a statistically significant manner the biliary excretion of cholesterol.

THE cholesterol (CHOL) in the bile is nowadays considered to be a quota of the sterol synthetized by the hepatic parenchyma. In fact most authors consider the biliary and ematic rates of CHOL to be absolutely independent. It is usually maintained that the biliary concentrations of the sterol cannot be modified in any appreciable measure by variations of the content of sterol in the diet, by eventual administration of such by means of injections, or by chronic hypercholesterolemic conditions.

According to Friedman and Byers[1] "the biliary cholesterol appears to represent a rather fixed function of that cholesterol synthetized or discharged from the hepatic cell itself".

On the other hand, research carried out with radio-active isotopes seems to permit the assumption that at least a part of the biliary CHOL is derived from the fraction of the free plasmatic CHOL. In 1943 Bloch *et al.*[2] affirmed that "after injection of deuterium CHOL into a dog, the activity of biliary cholesterol was considerably less than that of hepatic cholesterol, but similar to that of blood, suggesting the probability that bile cholesterol represented a moiety of plasma cholesterol".

Very recently Rosenfeld and Hellman[3] administered CHOL-^{14}C orally to human subjects with complete biliary fistula. They found CHOL-^{14}C in the bile after too short a time to exclude an effective passage of the sterol directly from the blood, after absorption, into the bile.

According to Friedman and Byers[1], since the biliary CHOL represents a quota of the hepatic synthesis of the sterol, its dosage in the bile of rats treated for a certain period with a specific compound can be utilized to estimate eventual pharmacological effects on such synthesis. Without going into the merits of the origins of biliary

cholesterol, which are not yet completely elucidated, we have studied, the effects of several cholesterol-acting drugs employed in a human clinic as cholesterol-lowering substances on the biliary excretion of the sterol in the rat.

METHODS AND MATERIAL

For all the experiments, 350 albino rats of the Morini strain, weighing 250–300 g, were used. In our experiments we used the technique of temporary biliary fistula, as described by Preziosi *et al.*[4–6]

The compounds examined, in the doses marked at the side of each and expressed in mg/kg are: nicotinic acid (NA: 30—85·5—171); 3-pyridineacetic acid (3-PAA : 30—85·5—171); inositol hexanicotinate (IHN : 30—85·5—171); Na-phenylbutyrate (Na-PHB : 25—50—100); betaine phenylbutyrate (betaine—PHB : 25—50—100); 1 : 4-dicaffeylquinic acid or cynarine, true active principle of the artichoke (CYN : 16·6—83—166), and Na-dehydrocholate (Na-DHC : 13·11—65·57—131·14). Reagent-grade compounds were employed. The lowest dose of each compound used by us corresponds, per kilogram, to that advised for human therapy per kilogram, daily. The doses of NA, 3-PAA and IHN are expressed as nicotinic acid, those of Na-PHB and of betaine-PHB as phenylbutyric acid, those of Na-DHC are equimolecular to those of CYN.

All drugs were dissolved in distilled H_2O, with the exception of IHN and CYN, dissolved as described by Donatelli *et al.*[7] and Preziosi *et al.*[6] respectively. The strength of all solutions was calculated in order to inject the required amount of drugs in a total volume of 5 ml/kg.

In a first group of experiments (experiment A) the drugs were administered by intravenous injection. The biliary flow was controlled and measured 1 hr before and 4 hr after the administration of NA, 3-PAA, IHN, Na-PHB, betaine-PHB, CYN and Na-DHC. In the controls, without any treatment, the flow was recorded for 5 hr. In a second group of experiments (experiment B) the method suggested by Byers and Friedman[8] was adopted. This consists in assaying the amount of CHOL emitted in the bile over a period of 24 hr, in rats pre-treated for some days with the drug under test, and comparing the results with those of the controls (untreated animals). The rats were injected by the intraperitoneal route with NA, 3-PAA, IHN, Na-PHB, betaine-PHB, CYN and Na-BHC, for 10 days. On the eleventh day, 14–16 hr after the last injection of the different substances, all rats (untreated controls and treated animals) underwent a temporary biliary fistula, as described above. The bile issuing from the fistula was collected over 24 hr and assayed for CHOL content. During all the experiments, the rats were kept at a temperature of 20–22 °C.

On the samples of bile of each group of animals, collected every hour for 5 hr in experiment A, and for 24 hr in experiment B, the dosage of the total CHOL was carried out by the method of Sperry and Webb[9] with the modifications described by us elsewhere.[4, 5] For the extraction of the CHOL, 1·5–2 ml of bile were used (due to the very small quantity of CHOL present in the bile), which compelled us, in the experiment A, to pool the samples of each group relative to the different times of collection, the quantity of each single sample being insufficient to establish an exact estimation. In order to eliminate biliary chromogens, which we found were capable of remaining adsorbed to the cholesterol digitonide precipitate and thus disturbing the final colour reaction, we proceeded to a first washing of the precipitate with alcohol

acetone, then carried out the subsequent washings, with acetone-ether and ether alone respectively, as described in the original method by Sperry and Webb.[9]

In each case the rate of CHOL was established in milligrams per 100 millilitres (mg/100 ml) of bile. Taking into consideration the volumes of bile obtained, the calculation was carried out of the absolute figures and of the concentrations (mg per cent) of CHOL excreted in the basal hour and in the four successive hours (hours of observation for the controls, post-treatment hours for the rats injected with the drugs), and finally the hourly percentage variations in the quantities of CHOL excreted after the basal hour.

Whenever necessary, the numerical data obtained from the tests were statistically evaluated according to methods suggested by Burn.[10] Values of $P<0{\cdot}05$ were considered to be significant.

RESULTS

1. *Experiment A, single intravenous administration of drugs*

As shown in Figs. 1 and 2 (controls), subsequent to the basal hour a progressive decrease occurs in the biliary flow as well as in the biliary concentrations of CHOL, and of the absolute figures of this excreted with the bile. Regarding the compounds examined, we shall observe the results obtained with NA, 3-PAA and IHN lacking in choleretic effects and those registered with Na-PHB, betaine-PHB, CYN and Na-DHC, all these latter drugs being endowed with intense choleretic action, some very lasting.

NA, 3-PAA and IHN: The comportment of the excretion of bile, expressed in percentage variations in respect to figures of basal hour, proved to be similar in the control rats and in those injected with 3-PAA at all doses tested and with NA and IHN at the highest dose. However, with the lowest doses of the latter two products used, the biliary flow is found to diminish with time more than in the controls. With the exception of the rats treated with NA and the highest dose of those used, there did not appear to be any significant variation in the progressive decreases with time either of the biliary concentration of CHOL or of the excretion of CHOL in absolute figures. In fact the decrease itself appeared, for the lowest doses, slightly accentuated with respect to the controls.

Na-*PHB, betaine-PHB, CYN and* Na-*DHC*. In the rats treated with Na-PHB and in particular, betaine-PHB in the dose of 25–50 mg/kg, a strong choleretic effect is to be observed and there is, as in the controls, a decrease in the concentrations of CHOL in the bile. However, the treated rats differ from the controls by registering an increase in absolute figures of the sterol excreted, due to the larger quantities of bile eliminated for the choleretic effect of the two products. In the rats treated with the two compounds with a dose of 100 mg/kg, however, the controls showed a much more marked decrease in the concentrations of CHOL in the bile emitted following injection of the drugs, and consequently also a greater decrease in the absolute figures of CHOL excreted.

CYN partly opposes (especially at the highest doses and notwithstanding the increased biliary flow determined by such doses) the decrease in concentrations of CHOL in the bile excreted during the 4 hr following the intravenous administration and determines, therefore, at the highest doses, an increased excretion of CHOL in absolute figures.

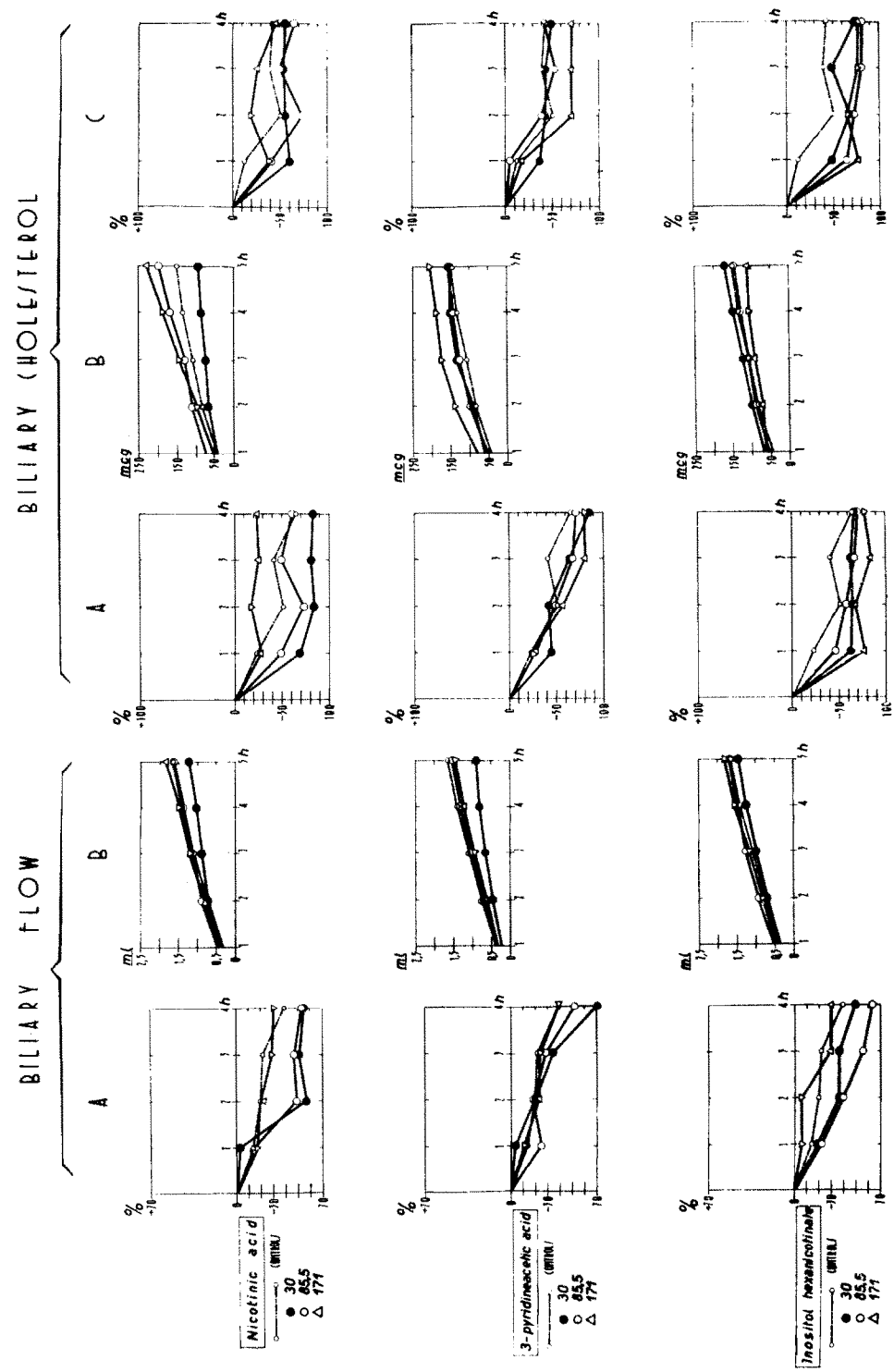
BILIARY FLOW
BILIARY CHOLESTEROL
A
B
C
Nicotinic acid
3-pyridineacetic acid
Inositol hexanicotinate
30
85.5
171

FIG. 1. Effects of nicotinic acid, 3-pyridineacetic acid and inositol hexanicotinate on biliary flow and on biliary excretion of cholesterol.

In the graphs indicated with *biliary flow*, A and B, there are respectively reported, for the three substances examined, the percentage variations of the biliary flow in respect to the figures of the basal hour, that is before treatment, taken as 100 and the biliary flow (expressed in ml) in respect to time obtained adding to the figures of the first hour those of the second hour; to those of the second hour those of the third hour and so on.

In the graphs indicated with *biliary cholesterol* there are respectively reported, for the three substances experimented: in the graph A the percentage variations of the absolute quantities of CHOL excreted by means of the bile in the hours following the administration of the drugs, in respect to the values of the basal hour, taken as 100 (graph A); in graph B the absolute figures of CHOL excreted with the bile in respect to time obtained adding to the figures in the first hour those of the second; to these, those of the third; and so on; in graph C the percentage variations of the biliary concentrations of CHOL in the hours following the basal one, taken as 100, being the figures recorded in this latter.

The doses of the three compounds are expressed as nicotinic acid.

Controls = untreated animals.

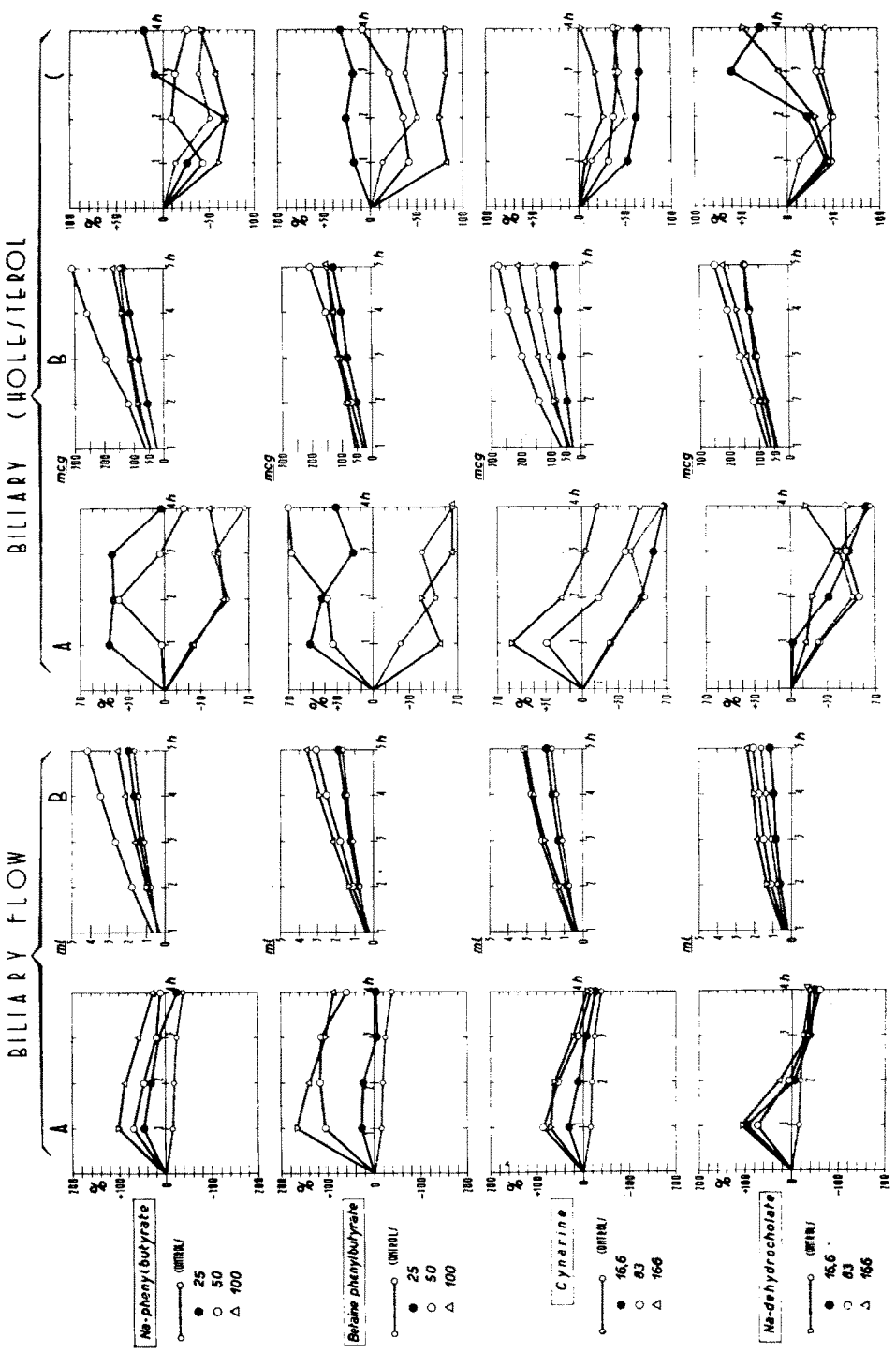
BILIARY FLOW
BILIARY CHOLESTEROL
A
B
C
ml
mcg
%
h
Na-phenylbutyrate
Betaine phenylbutyrate
Cynarine
Na-dehydrocholate
CONTROL
25
50
100
16.6
83
166

FIG. 2. Effects of Na-phenylbutyrate, betaine phenylbutyrate, cynarine and Na-dehydrocholate on biliary flow and on biliary excretion of cholesterol.

In the graphs indicated with *biliary flow*, A and B, there are respectively reported, for the four substances examined, the percentage variations of the biliary flow in respect to the figures of the basal hour, that is before treatment, taken as 100 and the biliary flow (expressed in ml) in respect to the time obtained adding to the figures of the first hour, those of the second; to those of the second, those of the third; and so on.

In the graphs indicated with *biliary cholesterol* there are respectively reported, for the four substances experimented: in graph A the percentage variations of the absolute quantities of CHOL excreted by means of the bile in the hours following the administration of the drugs, in respect to the basal hour, taken as 100 (graph A); in graph B, the absolute figures of CHOL excreted with the bile in respect to the time obtained adding to the figures of the first hour those of the second, to those of the third, and so on; in graph C the percentage variations of the biliary concentrations of CHOL in the hours following the basal one, taken as 100 being the figures of concentration recorded in this.

The doses of Na-phenylbutyrate and of betaine phenylbutyrate are expressed as phenylbutyric acid; the Na-dehydrocholate was administered at doses equimolecular to cynarine ones.

Controls = untreated animals.

In Na-DHC-treated animals, the biliary concentration of CHOL presents a comportment, in the first 2 hr after treatment, not very different from that of the controls. In the first hour, moreover, a positively marked decrease in respect to the figures mentioned in the controls is shown. Since the decrease in biliary concentrations of CHOL takes place during the period of choleretic effect of the product, which lasts nearly 2 hr, there is, in consequence of such a phenomenon and in spite of the reduction of the biliary concentration of the sterol, a reduction (even though slight) of the progressive decrease in the absolute quantity of CHOL excreted by means of the bile. In the third and fourth hours following the administration of the compound, there can be noted, in proportion to a reduction in the biliary flow (even more intense than that noted in the controls at corresponding times), a progressive increase in the biliary concentration of CHOL for which, finally, the excretion of the sterol in absolute figures is comparable, at those times, to those noted in the controls or slightly less.

2. *Experiment B, administration of drugs by the intraperitoneal route for ten consecutive days*

The results of this group of tests are reported in Table 1. Significant variations are not found between differently treated animals (with respect to the control rats) in the quantity of bile emitted for 24 hr and per kg as well as the percentage of CHOL present in the bile and the total quantities excreted of the sterol. Let us mention, however, that only the CYN, at the highest dose of the three examined, reduces the biliary concentration of CHOL, thus confirming what was already noted by us in former research.[4, 5]

DISCUSSION

Among the drugs studied in experiment A, only CYN, at the highest doses examined, and Na-PHB and betaine-PHB, at the lowest doses, increased the biliary excretion of CHOL. The increased excretion of CHOL provoked by CYN may represent a useful factor in clarifying the mechanism of the hypercholesterolemic effects of the product also observed by us in certain experimental hypercholesterolemias[4, 5] and also observed for prolonged treatments at high doses in a human clinic[11, 12] in conditions of deranged cholesterol metabolism and atherosclerosis.

We consider it worthwhile noting that the effect of Na-PHB and betaine-PHB on the biliary excretion of CHOL can differ in the acute experiments, in relation to the dose. In fact, for the lower doses (25–50 mg/kg) practically corresponding per kilogram to those of human therapy per kilogram and daily, there is an increased excretion of CHOL in absolute figures (in the percentage sense a decrease can always be observed, strongly counteracted, however, by the increased biliary flow). At the highest dose, 100 mg/kg, there is a decrease even in absolute figures, there being the strong reduction for such doses of biliary cholesterol concentrations, this time of such entity as not to be compensated by the nevertheless very high increase in the biliary flow. In all probability, for high doses of phenylbutyrates, with the intensification of the phenomenon of inhibition of the hepatic synthesis of the sterol, this phenomenon prevails in respect to the increased biliary excretion, with the result that the bile, even though abundant, contains lesser amounts of CHOL.[13] In spite of the fact that these are also powerful choleretics, this phenomenon does not occur with CYN and Na-DHC even at the highest doses. This variation in action according to the dose of

TABLE 1. BILIARY FLOW AND BILIARY EXCRETION OF TOTAL CHOLESTEROL IN TEN DAYS TREATED RATS WITH SEVERAL ANTI-CHOLESTEROLEMIC COMPOUNDS

Rats no.	Average weight	Treatment (10 days)		Volume of biliary flow		Total cholesterol content		
		Substance	mg/kg per day i.p.	ml 0–24 hr (± s.e.)	ml/100 g body wt.	mg % (± s.e.)	mg	mg/100 g body wt.
25	230	—	—	6·00 ± 0·935	2·68	15·29 ± 0·709	0·917	0·398
7	254	Nicotinic acid	30	6·93 ± 0·930	2·74	16·91 ± 1·829 $P = 0·2$–$0·1$	1·221	0·459
7	266	Nicotinic acid	85·5	6·92 ± 0·709	2·60	17·78 ± 0·525 $P = 0·4$–$0·3$	1·220	0·458
9	280	Nicotinic acid	171	7·52 ± 1·910	2·68	16·46 ± 0·922 $P = 0·3$–$0·2$	1·237	0·441
7	272	3-Pyridine acetic acid	30*	7·00 ± 0·455	2·57	15·87 ± 0·792 $P = 0·6$–$0·5$	1·110	0·408
8	250	3-Pyridine acetic acid	85·5	5·66 ± 0·473	2·26	15·93 ± 0·856 $P = 0·6$–$0·5$	0·901	0·360
6	260	3-Pyridine acetic acid	171	6·15 ± 0·534	2·32	15·23 ± 0·657 $P = 0·7$–$0·6$	0·936	0·360
5	234	Inositol hexanicotinate	30*	5·50 ± 0·944	2·35	15·89 ± 0·590 $P = 0·9$–$0·8$	0·873	0·373
5	265	Inositol hexanicotinate	85·5	6·00 ± 0·850	2·64	15·88 ± 0·789 $P = 0·6$–$0·5$	0·952	0·359
5	248	Inositol hexanicotinate	171	6·00 ± 0·790	2·41	16·15 ± 1·310 $P = 0·6$–$0·5$	0·969	0·390
10	278	Na phenylbutyrate	25*	7·24 ± 0·663	2·60	18·41 ± 1·600 $P = 0·02$–$0·01$	1·334	0·479
7	238	Na phenylbutyrate	50	8·00 + 1·021	3·36	18·46 ± 1·191 $P = 0·02$–$0·01$	1·476	0·620

TABLE 1—*continued.*

Rats no.	Average weight	Treatment (10 days)		Volume of biliary flow		Total cholesterol content		
		Substance	mg/kg per day i.p.	ml 0–24 hr (± s.e.)	ml/100 g body wt.	mg % (± s.e.)	mg	mg/100 g body wt.
8	236	Na phenylbutyrate	100	6·50 ± 0·037	2·75	18·32 ± 1·259 $P = 0·02–0·01$	1·190	0·504
8	272	Betaine phenylbutyrate	25*	8·40 ± 1·042	3·09	18·85 ± 2·382 $P = 0·05–0·02$	1·583	0·582
6	208	Betaine phenylbutyrate	50	7·50 ± 0·707	3·60	16·06 = 1·410 $P = 0·6–0·5$	1·204	0·578
9	266	Betaine phenylbutyrate	100	8·00 ± 0·670	3·07	16·23 ± 0·870 $P = 0·3–0·2$	1·290	0·484
8	287	Cynarine	16·6	7·00 ± 0·740	2·43	15·01 ± 1·350 $P > 0·9$	1·050	0·356
6	237	Cynarine	83	7·00 ± 0·630	2·99	15·32 ± 1·415 $P > 0·9$	1·072	0·452
6	288	Cynarine	166	6·50 ± 1·340	2·25	13·56 ± 0·660 $P = 0·1–0·05$	0·881	0·305
7	283	Na dehydrocholate	13·11*	7·20 ± 0·420	2·54	16·87 ± 0·629 $P = 0·2–0·1$	1·214	0·428
6	297	Na dehydrocholate	65·7	8·00 ± 0·836	2·69	14·22 ± 0·447 $P = 0·3–0·2$	1·137	0·382
14	288	Na dehydrocholate	131·14	8·60 ± 0·876	2·98	14·46 ± 0·541 $P = 0·4–0·3$	1·243	0·431

i.p. = intraperitoneally.
* The doses of NA, 3-PAA and IHN are expressed as nicotinic acid, those of Na-PHB and of betaine-PHB as phenylbutyric acid, those of Na-DHC are equimolecular to those of CYN.
s.e. = standard error.
P with respect to the controls.

phenyl-ethylbutyrates may explain the different or even opposite effects noted by different authors with phenylethylbutyric acid relative to the biliary excretion of CHOL.[14, 16]

The inability of Na-DHC, among the compounds endowed with a choleretic action examined by us, to determine an increased excretion of CHOL, even if for some of the doses it behaved in the way we observed, confirms the observations of Turba and Piccinini.[17] It is also worthwhile noting that NA is like 3-PAA and IHN, i.e. drugs endowed with marked cholesterol-lowering properties (NA[18]; 3-PAA;[14, 19, 20] IHN[21]), have proved themselves to be incapable of increasing the biliary excretion of CHOL and the biliary flow. The observations made by us confirm the findings of Mainardi[22] and Gunther[23] regarding the inability of NA to modify the biliary flow, and of Friedman and Byers[24] regarding the inability of the acid recorded to favour the excretion of CHOL with the bile; our results, on the other hand, do not agree with those of Parson and Flinn[25], who appear to have found a remarkable capacity of NA of favouring an increased biliary excretion of CHOL.

It must be emphasized again that in the chronic experiments (experiment B), carried out according to the advice of Byers and Friedman,[8] none of the compounds examined, even at much higher daily doses per kilogram than those of human therapy, succeeded in modifying in a statistically significant manner the excretion of CHOL via the biliary route. With the exception of 3-PAA[26] and CYN,[4, 5] the other compounds were never tested by Byers and Friedman.[8] We must therefore conclude, according to Byers and Friedman, that none of the compounds examined (including the phenylthylbutyrates) is able to inhibit the hepatic synthesis of CHOL, with perhaps the exception of CYN ($0{\cdot}1 > P > 0{\cdot}05$) at the highest dose examined, this fact having already been observed by us in former experiments.[4, 5] We postpone, however, such conclusions, since at present a series of investigations is in hand, with the aim of ascertaining the real significance and possibilities of the test advised by the American authors.

Finally we think it worthwhile making note of the possibility (clearly established by the tests carried out by us) that the excretion of CHOL by means of the bile presents variations even in a relatively short time (experiment A) and that such variations, in the sense of an increase in the quota of sterol excreted, can be determined by certain compounds (e.g. phenylethylbutyrates, CYN) active as cholesterol-lowering substances in humans and possessing also marked choleretic action, whilst other powerful choleretics, even though endowed with antiaterosclerotic action[27] even if questionable do not prove to be as active in this sense.

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