REDUCTION OF EXCESS CHOLESTEROL IN THE RABBIT AORTA BY INHIBITION OF ENDOGENOUS CHOLESTEROL SYNTHESIS*

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The study of factors affecting cholesterol synthesis has led to the finding that salts of vanadium in low concentrations exert a marked inhibitory effect on hepatic cholesterol synthesis *in vitro* and *in vivo* (1) in the rat. It seemed indicated, therefore, to determine whether vanadium, as vanadyl sulfate, would inhibit cholesterol synthesis sufficiently in the intact animal to induce mobilization of excess arterial cholesterol. The following representative experiments demonstrate that, in the rabbit, this hypothesis is valid.

Materials and Methods

Preparation of Sodium Acetate-1-C^{14}.—Methylmagnesium bromide was carbonated with $C^{14}O_2$ (2).

Diets.—The control ration consisted of Purina rabbit chow. The cholesterol diet was prepared by dissolving 3 gm. of cholesterol in an excess of ethyl ether and then adding the solution dropwise, a few milliliters at a time, to 100 gm. of control diet. The addition of the ethereal solution was performed slowly enough as to allow complete absorption of the ether by the pellets and evaporation of the excess ether before addition of more solution. When addition of the cholesterol solution was completed, the diet was placed in a shallow pan and allowed to stand at room temperature for 1 week to ensure evaporation of all the ether. The vanadium diet was prepared in a similar fashion by adding 500 mg. of $VOSO_4 \cdot 2H_2O$ in aqueous solution to 1 kilogram of diet. When dry, the vanadium diet was well mixed to ensure equal distribution of the vanadium.

Cholesterol Determination.—Serum cholesterols were determined by the method of Sperry and Webb (3). Tissue samples were dried to constant weight at 80°C. and then saponified overnight at 37°C. in 70 per cent alcohol with a slight excess of KOH. After addition of 1:1 acetone-alcohol to known volume and filtering, cholesterol was determined on an aliquot of the filtrate (3).

Vanadium Determination.—24 hour urine samples were evaporated to dryness at 110°C. in small pyrex beakers. The residues were then ashed in a muffle furnace at 600°C. overnight. In the case of tissues, a weighed amount of dried tissue was ashed in a similar manner

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in a platinum crucible. The vanadium content of the ash was determined by the method of Sandell (4).

Feeding Experiment.—Male white rabbits weighing about 2 kilograms each were fed the control ration to which were added on Monday, Wednesday, and Friday of each week 3 gm. of cholesterol per rabbit. The cholesterol feeding was discontinued after 4 weeks at which time 10 ml. of blood was removed by cardiac puncture. The serum cholesterol was determined and the rabbits paired according to the level of their serum cholesterol (Table I, columns 2 and 3). For the next 6 weeks one animal of each pair received the control ration while the other received the same diet containing 0.05 per cent $VOSO_4 \cdot 2H_2O$. The rabbits were allowed to eat *ad libitum*. The presence of the vanadium salt did not make the diet unpalatable to the rabbits and the weekly consumption of diet was identical at 900 gm. for both groups. At the end of 6 weeks the animals were sacrificed and blood and tissue samples removed. The aorta sample consisted of that vessel from its origin at the aortic valve to the origin of the right renal artery. The adventitia and adherent fat were carefully stripped from the aorta before drying.

TABLE I

*Cholesterol Levels of Sera and Tissue from Cholesterol-Prefed Rabbits before and after 6 Weeks on Vanadium-Supplemented Diet**

Rabbit	Initial serum cholesterol‡		6 wk. aortic cholesterol§		6 wk. serum cholesterol‡		6 wk. liver cholesterol‖	
	Control diet	Vanadium diet	Control diet	Vanadium diet	Control diet	Vanadium diet	Control diet	Vanadium diet
	mg. per cent	*mg per cent*	*mg./gm.*	*mg./gm.*	*mg. per cent*	*mg. per cent*	*mg./gm.*	*mg./gm.*
1	1202	1119	27	9	72	103	8.5	29.0
2	1270	1035	49	15	152	31	9.4	5.4
3	823	898	25	12	64	29	8.0	6.5
4	702	705	24	32	46	17	4.3	6.3
5	1369	1508	28	24	175	61	21.0	7.9
6	884	1018	30	17	16	26	4.8	5.6
7	1053	1021	27	6	57	37	9.8	3.4
8	872	843	16	6	41	14	7.7	6.9
9	697	667	26	10	16	19	5.1	3.3
10	684	558	14	7	13	30	3.8	5.6
Mean.....	955	937	26.6	13.8	65.2	36.7	8.24	7.99
S.E.....	±79.9	±86.4	±2.97	±2.69¶	±17.7	±8.4	±1.5	±2.4

* Control diet consisted of Purina rabbit chow. Vanadium diet consisted of Purina rabbit chow plus 0.05 per cent $VOSO_4 \cdot 2H_2O$.

‡ Normal serum cholesterol range, 20 to 50 mg per cent.

§ Normal aorta cholesterol range, 2.5 to 4.5 mg. per gm. of dry aorta.

‖ Normal liver cholesterol range, 4 to 6 mg. per gm. of dry liver.

¶ Statistical analysis of differences in aortic cholesterol content between control and vanadium diet animals: t, 3.192; P, <0.01.

Isotopic Experiments.—Rabbits from the control and vanadium diet groups were paired according to final weight and preceding 2 week weight gain. Liver cell clusters (5) were pre-

pared from each liver; the incubation with C^{14}-carboxyl-labelled acetate, isolation of cholesterol, and analysis of its radioactivity were performed as described previously (6).

RESULTS

Table I shows the cholesterol contents of the sera, aortas and livers from the rabbits fed a control diet and those given the vanadium-supplemented diet. The elevated mean serum cholesterol values after the cholesterol feeding period (Table I, columns 2 and 3) are not significantly different in the two groups and the groups are homogeneous with respect to variance. It will be seen on comparison of columns 4 and 5 that there was considerably less cholesterol remaining in the aortas of the vanadium-fed rabbits than in the aortas of the control animals. Statistical analysis of mean aortic cholesterol values at 6 weeks shows these differences to be highly significant. A gross difference was apparent at the time of autopsy when the control group generally showed more and heavier atheromatous plaque formation.

TABLE II

Urinary Excretion of Vanadium by Rabbits Fed a 0.05 per cent $VOSO_4 \cdot 2H_2O$ Diet

Rabbit	Amount of vanadium excreted in 24 hr. urine				
	7th day	14th day	22nd day	29th day	36th day
	mg.	*mg.*	*mg.*	*mg.*	*mg.*
Controls 1–10	<0.002	<0.002	<0.002	<0.002	<0.002
Vanadium 1	0.008	0.016	0.075	0.057	0.056
" 2	0.046	0.040	0.062	0.053	0.095
" 3	0.035	0.064	0.086	0.056	0.059
" 4	0.031	0.076	0.085	0.034	0.099
" 5	0.048	0.066	0.060	0.033	0.079
" 6	0.037	0.035	0.053	0.037	0.082
" 7	0.044			0.049	0.034
" 8	0.047	0.055	0.100	0.056	0.094
" 9	0.015	0.045	0.159	0.079	0.110
" 10		0.185	0.094		0.090

In the cases of the 6 week serum cholesterol levels and the liver cholesterol contents there is no statistically significant difference between the control and vanadium groups. The sera and liver, as well as the skeletal and cardiac muscles (not included in chart) showed in most cases cholesterol levels which approached normal values. The normal range of rabbit serum cholesterol values in this laboratory is 20 to 50 mg. per cent. It can be seen that the sera of both control and vanadium diet animals had mean cholesterol levels after 6 weeks which approximated this range. Likewise, the mean liver cholesterol

concentrations of both groups had fallen to values not greatly beyond the normal range of 4 to 6 mg. cholesterol per gm. of dry liver.

Only in the aorta was there consistent elevation of the cholesterol content above the normal range (2.5 to 4.5 mg. cholesterol per gm. of dry aorta). And the elevation is far greater in the animals fed the control diet than in the rabbits receiving the vanadium diet. The aortas of five rabbits which were sacrificed at the end of the 4 week cholesterol feeding period had a mean cholesterol concentration of 27.6 mg. cholesterol per gm. of dry aorta. It is thus apparent that the control animals mobilized only a small amount of aortic cholesterol during the ensuing 6 weeks while the vanadium-fed animals

TABLE III

*Incorporation of C^{14} from Sodium Acetate-1-C^{14} into Cholesterol by the Excised Livers of Cholesterol Prefed Rabbits after 6 Weeks on a 0.05 per cent Vanadyl Sulfate Diet**

Rabbit	Specific activity of liver cholesterol‡	Liver cholesterol
		mg./gm. (dry weight)
Control 6	1451	4.8
Vanadium 1	389	5.5
Control 7	131	9.8
Vanadium 2	28	29.0
Control 20	1501	5.5
Vanadium 5	87	6.3

* Each vessel contained 3 to 4 gm. (wet weight) of liver cell clusters (5), 40 ml. of buffer (6), and 5 mg. of $CH_3C^{14}OONa \cdot 3H_2O$ (5.1 mc./mM).

‡ Counts per minute per milligram of cholesterol.

mobilized far more. This finding in the control diet rabbits is compatible with earlier work which showed that, after ingestion of cholesterol was discontinued, the atheromatous lesions persisted for more than 800 days (7).

It is known that about 61 per cent of a parenterally administered dose of vanadium (as $NaVO_3 \cdot H_2O$) is excreted by the kidneys within 24 hours (8). By determining the 24 hour urinary excretion of vanadium by the animals receiving vanadyl sulfate, it was possible to estimate that at least 1 per cent of the vanadium administered orally as vanadyl sulfate was retained. In Table II, it can be seen that the urine of the control rabbits did not contain amounts of vanadium measurable by the method used, while the ten rabbits on the vanadium diet all had vanadium excretions of the same order of magnitude. Owing to this similarity of vanadium absorption a comparison of the vanadium excretions in Table II with the cholesterol remaining in the aorta of the same rabbit in Table I fails to show any correlation. Each rabbit in the

vanadium group received 115 mg. of vanadium weekly of which an average of 1 mg. (range, 0.5 to 1.5 mg.) was absorbed and an average of 0.7 mg. (range, 0.35 to 1.00 mg.) was excreted in the urine.

It was, of course, highly desirable that there be no toxicity from the vanadium. The studies of Talvitie and Wagner (8) show that larger parenteral doses of vanadium for 1 week are not toxic. At the dosage used in these rabbits there were no physical evidences of toxicity during a 6 week period. Weight gains were similar for control and vanadium-fed animals. Routine urinalyses were normal. Vanadium determinations on the bones of the vanadium diet rabbits at the end of the 6 week feeding period showed a maximum concentration of only 0.009 mg. V per gm. of bone (dry weight). Maximum vanadium content per 10 gm. of dried liver was 0.005 mg. and all 2 ml. samples of blood contained less than 0.002 mg. of vanadium. At this dosage and for this length of time the rapid renal excretion of the vanadium is sufficient to prevent toxicity due to accumulation.

It is apparent that this low concentration of vanadium in the rabbit is sufficient to inhibit cholesterol synthesis as is indicated in Table III which shows the abilities of the excised livers of some of these rabbits to incorporate C^{14}-labelled acetate into cholesterol. In each instance, the radioactivity of the cholesterol isolated from the control livers is greater than that of the cholesterol from the vanadium diet animals, indicating a higher rate of synthesis. It is evident, therefore, that the small amount of vanadium absorbed by these animals was sufficient to inhibit cholesterol synthesis.

Aside from the demonstrated effect on hepatic cholesterol synthesis, it was of interest to exclude the possibility that the vanadium was inducing greater intestinal loss of cholesterol, even though the vanadium-fed rabbits showed no evidences of diarrhea. Feces from the rabbits were collected for 72 hours during the 1st and 3rd week of the feeding period. Total digitonin-precipitable sterols (9) and cholesterol contents were determined and found to be essentially identical for control and vanadium diet animals.

DISCUSSION

In evaluating the data in this paper there are several important points to be considered. The first of these is the use of the amount of cholesterol remaining in the aorta as the criterion for assessing the value of an anti-atherosclerotic regimen. The 6 week serum cholesterol values in Table I show no significant difference between control and vanadium groups, yet there is a very significant difference between the amount of cholesterol remaining in the aortas of the two groups. It is once again evident that the serum concentration of a substance, in this case cholesterol, is by no means an accurate index of its tissue concentration. Further, in man, as in these rabbits, it is the cholesterol deposited in the blood vessel—not that which circulates in the

plasma—which is of primary importance. The demonstration of atheromatous material in the arteries of infants (10) underlines the necessity for orienting the approach of studies in experimental atherosclerosis towards investigation of mobilization of predeposited arterial cholesterol rather than the prevention of deposition of cholesterol from high cholesterol diets.

One reason for the lack of investigative emphasis on removal of tissue cholesterol has been the failure to appreciate completely the implications of the demonstration some 20 years ago of endogenous cholesterol synthesis (11, 12). The mammalian body is capable of maintaining its necessary level of tissue cholesterol by endogenous synthesis without any dietary cholesterol whatsoever. This endogenous synthesis of cholesterol will decrease when large amounts of dietary cholesterol are fed (13) so as to maintain in so far as possible the normal internal concentration of cholesterol. It is obvious that the animal fed cholesterol in amounts which exceed the normal rate of endogenous synthesis and catabolism will go into positive cholesterol balance and cholesterol will be deposited in the tissues. However, we have seen from Table I that within a short time after cessation of cholesterol feeding the serum and tissue values of cholesterol, excepting the aorta, rapidly return towards normal. Reference to Table III, at this point, gives an insight into the mechanism at work.

As noted earlier it is seen that in each instance the rate of synthesis of cholesterol by the control rabbit liver is higher than the synthesis by the vanadium-fed rabbit liver. It will be noted that the liver of control rabbit 7 contained about twice the normal concentration of cholesterol. The specific radioactivity of cholesterol from this liver is markedly lower than that from controls 6 and 20. Here, undoubtedly, is an example of an inhibition of cholesterol synthesis due to excessive exogenous cholesterol in the liver as described by Gould (13). It is of great interest that controls 6 and 20 show a normal cholesterol liver concentration but a rate of synthesis markedly higher than that of vanadium rabbits 1 and 5 whose hepatic cholesterol concentrations are essentially identical with those of the controls. The depression of incorporation of labelled acetate into cholesterol found in these animals fed vanadium is of the same order of magnitude as the depression of hepatic cholesterol synthesis produced by vanadyl sulfate *in vitro* and after a single parenteral injection *in vivo* (1).

From these data one may suggest that the following events have occurred. In both control and vanadium animals, following the cholesterol prefeeding period, there is an almost complete mobilization of excess cholesterol from the various tissues except the aorta. During this period of time a homeostatic effect (13) is causing decreased hepatic cholesterol synthesis. Once, however, the concentration of hepatic cholesterol has been reduced towards normal in the control animals there is a resumption of normal hepatic synthesis. Conse-

quently the degree of negative cholesterol balance is markedly lessened and mobilization of excess aortic cholesterol proceeds very slowly. The analogy between the situation in these control rabbits and the normocholesteremic man with atherosclerosis is evident. In the vanadium-fed animals, however, this resumption of normal cholesterol synthesis is inhibited by the vanadium with maintenance of a negative cholesterol balance and resultant mobilization of more aortic cholesterol.

It can be concluded that these experiments demonstrate a reduction in excess aortic cholesterol produced within a period of 6 weeks by inhibition of endogenous cholesterol synthesis. The data further suggest that to be successful any regimen for reducing arterial cholesterol content should include inhibition of endogenous cholesterol synthesis. If this were not true, the control rabbits in Table I would have mobilized aortic cholesterol at a rate comparable to the vanadium-fed animals.

Since the perfused aorta (14), aorta slices (15), and aorta minces (16) synthesize cholesterol from C^{14}-labelled acetate under conditions identical for its synthesis by similar liver preparations, it is probable that the same intermediary metabolic pathways are utilized by both tissues for cholesterol synthesis. If this is true, then an inhibitor of hepatic cholesterol synthesis may inhibit aortic cholesterol synthesis as well. Thus it is possible that inhibition of aortic cholesterol synthesis in the vanadium-fed rabbits may contribute to the reduction in excess aortic cholesterol found in these animals.

SUMMARY

Hepatic cholesterol synthesis in rabbits, as measured by the incorporation of C^{14}-labelled acetate, was inhibited by addition of non-toxic amounts of vanadyl sulfate (0.05 per cent $VOSO_4 \cdot 2H_2O$) to the diet. This diet reduced excess aortic cholesterol in cholesterol prefed rabbits when fed during the 6 weeks immediately following a 4 week period of cholesterol feeding.

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