Effect of Ingested Vanadium on Cholesterol and Phospholipid Metabolism in the Rabbit.* (22551)

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The metabolic effects of vanadium have been investigated as part of a study of the toxicology of vanadium relative to industrial exposures. Lipid metabolism was given particular scrutiny because the following isolated reports strongly indicated involvement of this metabolic component: (a) Congestion and fine droplets of fat in the liver have been produced by administration of vanadium compounds to rats(1). (b) Reduction in fat and lipid content of adrenal cortex occurred at the same time. (c) Addition of μg quantities of vanadium acetate or metavanadate to minced liver slices caused increased oxygen uptake: the fatty acid moiety was the reactive part of the substrate molecule(2). (d) In man, intramuscular injection of sodium tetravanadate resulted in increased catabolism as indicated by increased output of all nitrogen, sulfur, and phosphorous constituents determined in the urine(3). (e) Ingestion of vanadium pentoxide at dietary levels beginning at 100 ppm vanadium caused lessening of cystine content of rat hair(4). (f) While the present work was in progress, a report appeared stating that vanadium inhibited *in vitro* synthesis of cholesterol by rat liver cells(5).

Methods. 2.5 kg albino rabbits were used, 5 to a group, for each diet. The stock diet for control groups consisted of Purina Rabbit Chow Checkers. Test diets consisted of stock diet pellets coated with dextrin and the desired additives. Test diets were prepared by sprinkling 2.5 kg lots of stock diet with a blended corn dextrin suspension containing vanadium pentoxide and cholesterol additives. About 500 ml of distilled water containing 25-50 g of dextrin and the required amounts of additives were homogenized in a Waring Blendor. The creamy suspension was applied from a separatory funnel and stirred into the pellets. The coated pellets were air-dried 1 or 2 days. Vanadium was used as vanadium pentoxide which is of particular interest in industrial hygiene. Cholesterol was first converted to the amorphous form as recommended by Popjak(6). Coated pellets lost some vanadium, but not cholesterol, during handling, and rabbits would not eat the fine material. In practice 20-30% excess of vanadium over the calculated amount produced the desired vanadium content of large food pellets which the rabbits would eat. Spectroscopic analysis was used in checking diets for proper vanadium content(7). Food was given *ad libitum*: the amount consumed by each rabbit was recorded twice weekly.

Sampling and analysis. Blood was drawn from the marginal ear vein into tubes containing 1-2 mg Manuronate® anti-coagulant; specimens were taken at weekly or biweekly intervals. Food was withheld 16 hours prior to bleeding. Duplicate analyses were carried out: where only total cholesterol was determined, the method of Abell was used(8). Free and total cholesterol were determined by the digitonin-anthrone method (9, a, b); Zak's procedure(12) was used to obtain data in Tables II and III. Phospholipid analyses were carried out using a modified Youngburg method(10); the phosphomolybdenum blue was developed by Allen's method(11). The animals were killed by cardiac injection of air after final blood samples were drawn. At autopsy, tissue specimens were taken for analysis and pathologic examination. Liver samples were ground with sand and 12 parts anhydrous sodium sulfate and extracted with chloroform in a Soxhlet extractor. The chloroform extracts were evaporated at low heat on a steam bath, taken up in petroleum ether, filtered and the lipid content of the extracts determined by weighing after warm water bath evaporation. The lipid residue dissolved in 100 ml petrol-

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TABLE I. Effects of 100 ppm Vanadium, as V_2O_5, with and without 1% Cholesterol on Livers and Liver Lipids of Rabbits in 4 Groups.

Diet	Lipid in liver, %	Total cholesterol, mg	Free cholesterol, mg	Phospholipid, mg	Vanadium in liver, μg
1: Control			*	†	
Mean	2.6	323	155	1840	2.0
Range	(2.0–3.1)	(168–826)	(128–176)	(1410–2152)	(.9–2.5)
2: 100 ppm V			*	†	
Mean	4.5	230	52	1100	54
Range	(2.4–8.9)	(175–286)	(20–89)	(495–1553)	(39–82)
3: 1% cholesterol					
Mean	9.3	3945	657	2170	2.3
Range	(8.1–10.9)	(2726–5425)	(397–1310)	(1540–3205)	(.9–3.3)
4: 1% chol + 100 ppm V					
Mean	8.9	2668	269	1634	66
Range	(3.3–14.1)	(651–3575)	(165–427)	(985–2579)	(13–112)

* Differing significantly, $P < .005$ by "F" test.
† *Idem* $P < .05$

eum ether was aliquoted for determinations of free and total cholesterol and phospholipid.

Diets. Composition of diets and duration of feeding are given in the Tables. Fig. 1 and Table I give results of the preliminary experiment to determine if vanadium affects plasma and liver cholesterol content. Fig. 2 shows data obtained in determining minimal levels of dietary cholesterol and vanadium necessary to produce notable differences among the groups compared. Tables II and III show effects of vanadium on plasma and liver cholesterol and phospholipid levels, both on continued cholesterol feeding with and without vanadium, and on these components when cholesterol was withdrawn from the diet, but vanadium intake continued after high plasma cholesterol levels had been attained. Some exceptions to the general procedure occurred: the rabbits used to obtain the data in Fig. 1 and Table I were not from the same source as

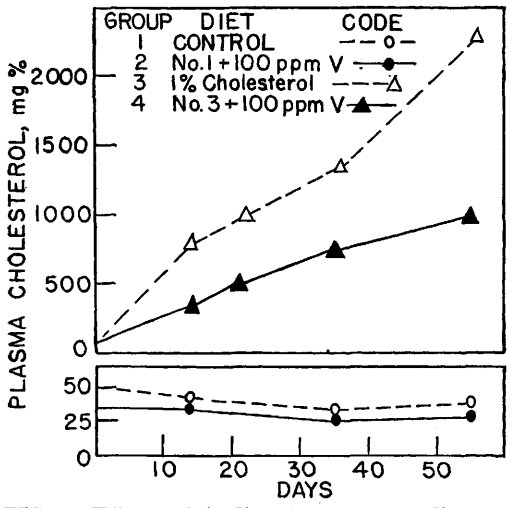

FIG. 1. Effects of feeding 100 ppm vanadium, as V_2O_5, with and without 1% cholesterol, on rabbit plasma cholesterol levels. Points represent averages of 5 animals.

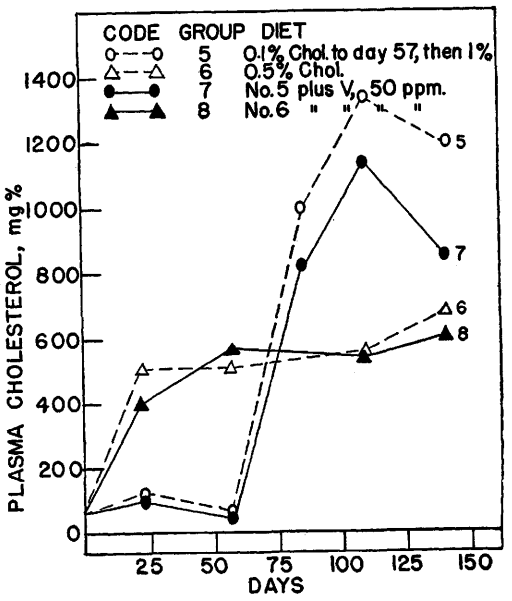

FIG. 2. Effects of feeding 50 ppm vanadium, as V_2O_5, with various amounts of cholesterol, on cholesterol content of rabbit plasma. Points represent averages of 5 animals.

TABLE II. Cholesterol and Phospholipid Content of Rabbit Plasma when 1% Cholesterol Was Fed with and without 100 ppm Vanadium as V_2O_5. Cholesterol omitted from diet of half the animals in each group after 50 days.

Diet group	Total cholesterol, mg %					Free cholesterol, mg %					Phospholipid, mg %				
↓ Days fed→	0	27	41	55	68	0	27	41	55	68	0	27	41	55	68
9: 1% chol															
Mean	51	*1100	*1688	§2198	*1634	13	*268	†412	511	*350	98	*432	*525	‡617	§545
Range	31–70	846–1387	1249–2147	1654–3168	1471–1843	9–21	173–377	323–521	387–662	277–400	88–110	342–545	435–672	445–807	390–685
10: No. 9 + 100 ppm V															
Mean	49	*818	*1204	§1443	*1123	13	*183	†325	442	*213	98	*315	*393	‡396	§375
Range	34–73	708–974	915–1690	1320–1584	936–1258	8–22	165–215	299–362	345–493	152–255	68–130	255–400	345–505	325–468	283–512
9-0: Chol omitted															
Mean				622	339				116	†106				258	§214
Range				528–781	183–464				92–136	77–128				218–288	158–255
10-0: Chol omitted															
Mean				636	286				110	†70				220	§167
Range				581–739	196–345				92–127	51–78				175–260	133–207

Corresponding pairs of means differing significantly by "F" test: * $P < .005$. † $P < .01$. ‡ $P < .025$. § $P < .05$.
Note: The groups compared are 9 *vs* 10, and 9-0 *vs* 10-0; although 9 and 10 are selectively reduced in number after day 50, they still represent equivalent samples with respect to each other, referred to the start of the experiment.

TABLE III. Effects on Growth, Liver and Liver Lipids of Rabbits when 1% Cholesterol Was Fed with and without 100 ppm Vanadium, as V_2O_5. Cholesterol omitted from diet of half the animals in each group after 50 days.

Diet group		Food consumed, g: All–68 days	Food consumed, g: Final 18 days	Body wt change, g	Liver wt, g	100 × Liver wt / Body wt	% lipid in liver	Total cholesterol, mg/100 g	Free cholesterol, mg/100 g	Phospholipid, mg/100 g	Vanadium in liver, μg/100 g
9: 1% Chol	Mean	8004	1524	949	144	4.3	9.7	*4627	1066	3849	
	Range	7677–8578	1355–1728	764–1202	132–152	4.1–4.4	7.4–13.6	2982–6687	794–1312	2485–5962	
10: No. 9 + 100 ppm V	Mean	8216	1616	1014	156	4.5	9.8	*4217	846	2687	38
	Range	7383–8762	1033–1965	841–1163	132–176	3.8–5.4	8.1–10.7	3470–5643	712–1100	1330–4062	33–54
9-0: Chol omitted	Mean	8103	1619	1019	136	4.1	7.5	3046	759	1884	
	Range	7727–8578	1442–1895	802–1161	115–142	3.4–5.4	5.8–8.4	2410–3540	455–1008	1425–2697	
10-0: Chol omitted	Mean	8112	1617	1084	118	3.5	7.7	2635	653	1746	45
	Range	7732–8657	1475–1840	848–1257	104–156	3.2–4.3	5.4–9.0	1471–3072	378–914	1320–2212	22–84

* Corresponding pairs of means differing significantly by "t" test, $P < .05$.
Comparisons are made between corresponding groups, *i.e.* 9 *vs* 10, and 9-0 *vs* 10-0.

the others; Tables II and III include data from animals on diets partially restricted to maintain uniform food consumption.

Results. Fig. 1 shows total cholesterol levels in the plasma of rabbits receiving vanadium with the stock diet were slightly, but not significantly, lower than those of the control group. However, when 1% cholesterol was fed, the addition of vanadium produced highly significant reductions in the total cholesterol of the plasma. Thus on day 55, Group 3, fed 1% cholesterol, averaged 2267 mg% total cholesterol in the plasma, while Group 4, which received the same diet with vanadium added, had an average level of but 896 mg%. When cholesterol was fed, the free cholesterol of the plasma also showed differences attributable to vanadium: an average of 659 mg% free cholesterol in the plasma of Group 3 rabbits (no vanadium) compared to 320 mg% for Group 4 (vanadium-fed). Table I shows that the addition of vanadium to the stock diet resulted in reduction of the average free and total cholesterol and phospholipid content of the liver of this group (Group 2) compared to the stock diet controls (Group 1). Similarly, the average quantities of these 3 substances in the livers of the animals fed 1% cholesterol along with vanadium (Group 4) are lower than the amounts found in the comparable group (Group 3) which received no vanadium. Food consumption, weight gains, liver weights and liver weight/body weight ratios were not significantly different for comparable groups.

In Fig. 2, it should be noted that Groups 5 and 7 are compared, so far as the plasma is concerned, at 2 levels of dietary cholesterol. It is evident that 50 ppm V had but little, if any, effect for the first 57 days when these groups were receiving 0.1% cholesterol. Subsequently, raising the cholesterol to 1% of the diet, definitely brings out differences in the plasma levels in these groups. On day 110 (53 days on 1% cholesterol), total cholesterol averaged 1333 mg% for Group 5, 848 mg% for Group 7, ($P<.05$). Correspondingly, free cholesterol was 507 mg% against 354 mg%: phospholipid levels followed this pattern to a lesser degree (390 *vs* 353 mg%). Groups 6 and 8 appear to show little, if any, real differences associated with the presence or absence of 50 ppm vanadium when cholesterol is fed as 0.5% of the diet. The livers of comparable groups showed slight differences associated with feeding vanadium under the conditions of this experiment.

Table II shows that by the 27th day 100 ppm dietary vanadium produces significant effects on all the plasma lipids in the table. These effects persisted for the duration of the experiment where the regimen was constant throughout. The peak followed by a drop (eventually leveling off) of plasma levels in groups 9 and 10 at about 55-68 days follows the pattern noted by Popjak(6). In the case of the animals from whose diet cholesterol was omitted on day 50, *i.e.* groups 9-0 and 10-0, the table shows the plasma lipids returned to near normal levels when vanadium was in the diet, whereas the rate of decline of the plasma lipids following withdrawal of cholesterol proceeds at a definitely slower pace when vanadium is not fed. The more rapid disappearance of pre-formed cholesterol in the presence of vanadium supports the oxidation-catalyst mechanism. Table III shows that food consumption of comparable groups was virtually identical: the vanadium groups consumed slightly more food, consequently more cholesterol. This tends to rule out lower food consumption as a reason for lower cholesterol levels in the vanadium-fed animals. Average concentrations of liver lipids are lower for vanadium animals: in most cases they are nearly great enough to be significant at the 5% level.

Pathologic examination of the tissues of the rabbits revealed that all animals which ingested cholesterol had atheromas of the coronary arteries and aortas, as well as lipid deposition in the heart. The liver sections exhibited foamy infiltration. Adrenals contained abundant lipid material and some animals had considerable lipid in the intestinal mucosa. Of possible significance was the absence of intracellular lipid deposition in the kidney tubules of vanadium treated rabbits, while 50% of the animals not given vanadium showed lipid deposition in the tubular epithelium. Marked

differences in appearance of vanadium and nonvanadium livers were evident. The color of the vanadium livers was similar to normal livers, while livers of rabbits fed cholesterol without vanadium were much lighter. The aortas of groups in Tables II and III were photographed in color and the color slides were examined and graded as to extent of plaque formation. Without knowledge of the diet the particular animal received, grouping the aortas according to severity of plaque formation served to separate them in relation to diets: the vanadium-fed animals invariably had less extensive areas of aortal plaques than did those receiving cholesterol alone. It may be noted here, that Curran has recently reported a reduction in aortal cholesterol following administration of vanadium to rabbits (15).

Discussion. The lower plasma cholesterol and phospholipid accompanied by lower free cholesterol and phospholipid in the liver of vanadium animals may result from vanadium either accelerating metabolism by the liver or by inhibiting cholesterol synthesis. Inhibition of synthesis has already been demonstrated *in vitro*(5): accelerated metabolism is suggested by the fact that with low vanadium (50 ppm, Fig. 2), it was found that no appreciable effects on plasma lipids were observed when cholesterol levels were low (0.1, 0.5%). This implies interaction and could be explained on the basis that vanadium is reacting chemically with cholesterol, perhaps as an oxidative catalyst. The minute amounts of vanadium (16-68 μg) in the liver support this concept. Vanadium is rapidly excreted after absorption(13): it is probable that less than 50 ppm of vanadium in the diet does not maintain enough vanadium in the liver to have measurable effects on the cholesterol passing through. Some evidence as to the form of cholesterol acted upon by vanadium appears in Table I: when no extra cholesterol was fed, the free cholesterol of the liver averaged only one-third that of nonvanadium controls. However, this could be explained on the basis of inhibition of cholesterol synthesis. The significant reduction in phospholipid paralleling the low free cholesterol in this case is consistent with the observations of Popjak(6) that free cholesterol controls phospholipid mobilization. Inhibition of protein synthesis resulting in lowered lipoprotein formation(14) does not appear to be a factor in the lowering of the plasma cholesterol levels; had this occurred, cholesterol would have accumulated in the liver: on the contrary, both plasma and liver cholesterol levels were reduced by vanadium. Finally, as judged by weight gains, liver weight-body weight ratios and examination of tissues and organs, vanadium, at the levels fed in this experiment, had no adverse effects on the animals.

Summary. 1. Vanadium, in the form of vanadium pentoxide, added to a standard diet, at a level of 100 ppm of the element lowered free cholesterol and phospholipid content of the liver of rabbits, while plasma cholesterol showed no significant changes. 2. Elevation of free and total cholesterol levels of plasma was restricted significantly by the addition of 50 ppm vanadium to a 1% cholesterol diet. Phospholipid levels also were maintained at lower levels, on the average, although not uniformly. 3. When rabbits were fed cholesterol to raise the plasma levels of cholesterol and phospholipid, and then the cholesterol was omitted from the diet, it was found that the presence of vanadium in the diet produced a faster rate of return to normal than occurred when no vanadium was furnished. 4. The mechanism by which vanadium restricts elevation of plasma cholesterol appears to involve both inhibition of cholesterol synthesis and accelerated catabolism of cholesterol. The latter may occur through a catalytic process.

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1. Daniel, E., and Lillie, R. D., *Public Health Rep.,* 1938, v53, 765.

2. Bernheim, F., and Bernheim, M. L. C., *J. Biol. Chem.,* 1939, v127, 353.

3. Proescher, F., Seil, H. A., and Stillians, A. W., *Am. J. Syph.,* 1917, v1, 347.

4. Mountain, J. T., Delker, L. L., and Stokinger, H. E., *Arch. Ind. Hyg. and Occup. Med.*, 1953, v8, 406.

5. Curran, G. L., *J. Biol. Chem.*, 1954, v210, 765.

6. Popjak, G., *Biochem. J.*, 1946, v40, 608.

7. Daniel, E. P., Hewston, E. M., and Kies, M. W., *Ind. Eng. Chem. Anal. Ed.*, 1942, v14, 921.

8. Abell, L. L., Levy, B. B, Brodie, B. B., and Kendall, F. E., *J. Biol. Chem.*, 1952, v195, 357.

9. (a) Feichtmeier, T. V., and Bergerman, J., *Am. J. Clin. Path.*, 1953, v23, 599.

(b) Loewus, F. A., *Anal. Chem.*, 1952, v24, 219.

10. *Practical Physiological Chemistry*, Hawk, Oser and Summerson, 12th ed., The Blakiston Co., Phila., 1947, p541.

11. Allen, R. J. L., *Biochem. J.*, 1950, v34, 858.

12. Zak, B., Dickenman, R. C., White, E. G., Burnet, H., and Cherney, P. J., *Am. J. Clin. Path.*, 1954, v24, 1307.

13. Talvitie, N. A., and Wagner, W. D., *Arch. Ind. Hyg. and Occup. Med.*, 1954, v9, 414.

14. Feinberg, H., Rubin, L., Hill, R., Entenman, C., and Chaikoff, I. L., *Science*, 1954, v120, 317.

15. Curran, G. L., and Costello, R. L., *J. Exp. Med.*, 1956, v103, 1.

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Quantitative Lipoprotein Studies in Normal and Abnormal Subjects Using Combined Electrophoretic and Chemical Technics.* (22552)

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The general problem of atherosclerosis may be considered to have 3 major facets: 1) Elucidation of pathogenesis of atherosclerosis; 2) establishment of diagnostic laboratory procedures; 3) evaluation of methods to prevent or treat the disease. The relationship of disturbances in serum lipids to pathogenesis of atherosclerosis has been studied extensively. Interest has centered on distribution of cholesterol, phospholipids, and neutral fat between the two major lipoprotein complexes, the so-called alpha and beta lipoproteins. Barr *et al.*(1) demonstrated a definite correlation between percent distribution of cholesterol in alpha and beta lipoprotein complexes and the presence of clinically evident atherosclerosis and diseases predisposing to atherosclerosis. Cohn fractionation, the method used by these investigators for separation of alpha and beta lipoprotein complexes, is technically difficult, expensive and time consuming.

Several reports have dealt with separation of alpha and beta lipoproteins by paper strip electrophoresis and subsequent semiquantitation of total lipid staining material in the alpha and beta fractions, by means of scanning devices(2). Determination of total lipids in lipoprotein complexes by staining, elution, and colorimetric determination has been reported(3). Kunkel and Slater(4a) and Nikkila(4b) have attempted chemical quantitation of serum cholesterol and phospholipid after paper electrophoretic separation, and subsequent elution of alpha and beta lipoprotein entities.

The present report deals with a method for the separation of alpha and beta lipoprotein complexes by paper strip electrophoresis, and subsequent quantitation of total lipid, total cholesterol, and phospholipid in each of these complexes.

Method. Two 6.5 cm wide Whatman No. 3MM filter paper strips are moistened in a barbital buffer solution (pH 8.6, ionic strength 0.05%). They are then placed in each of 3 plastic boxes having the dimensions of 24 x 18 x 6.5 cm. Each end of the paper strips is immersed in a 1.5 liter buffer reservoir placed at the end of the boxes. 0.20 ml

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