INTERRELATIONSHIPS BETWEEN EXPERIMENTAL HYPERCHOLESTEREMIA, MAGNESIUM REQUIREMENT, AND EXPERIMENTAL ATHEROSCLEROSIS*

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(Received for publication, May 20, 1957)

There is considerable evidence that thyroxine administration decreases the development of atherosclerosis in experimental animals fed various "atherogenic" diets and that thiouracil administration promotes its development (1). There is also suggestive evidence that patients with hypothyroidism may have more atherosclerosis than those with hyperthyroidism or with euthyroidism (2).

Recently, Vitale *et al.* (3) demonstrated that the feeding of thyroxine to young growing animals increased the magnesium requirement. The oxidative phosphorylation efficiency of heart mitochondria was decreased and typical signs of magnesium deficiency were observed. These changes were prevented by large doses of dietary magnesium. The metabolism of cardiac tissue seems to be peculiarly susceptible to magnesium deprivation and to thyroxine. Vitale *et al.* (4) observed changes in the oxidative phosphorylation of heart mitochondria in young rats after only 4 days upon a magnesium-deficient diet. At this time no change in the oxidative phosphorylation of mitochondria prepared from liver or kidney tissue was found. Furthermore, the administration of thyroxine to control, non-deficient animals resulted in decreased P/O ratios of heart mitochondria within 10 minutes. This was prevented by magnesium administration prior to the thyroxine injection.

The similarity of the effects of magnesium deficiency and thyroxine administration, as well as the interrelations between the two, suggested a possible

* Supported in part by grants-in-aid from the National Cancer Institute (Gr. No. C-1323 (C4)), the National Institute of Arthritis and Metabolism (Gr. No. 194), National Institutes of Health, Public Health Service, Bethesda; the John A. Hartford Memorial Fund; the Albert and Mary Lasker Foundation, New York; and the Fund for Research and Teaching, Department of Nutrition, Harvard School of Public Health, Boston.

role of magnesium in the development of atherosclerosis. The experimental work reported in this paper demonstrates that experimental hypercholesteremia does indeed have a profound effect upon magnesium metabolism.

Methods

Young albino rats weighing 50 to 55 gm. obtained from the Charles River Breeding Laboratories, Boston, were fed one of the diets listed in Table I, with and without thyroxine at a level of 2 mg. per cent. Various levels of magnesium in the form of MgO ranging in concentration from 12 to 768 mg. per 100 gm. of diet were added to these diets. The animals were fed *ad libitum* and housed in individual cages. At the end of 24 to 26 days, six animals from each group were sacrificed and serum and liver cholesterol concentrations determined according to the method of Carpenter *et al.* (5). The serum magnesium level was determined according to the method of Orange and Rhein (6). Sections of liver, kidney, and heart were fixed in 10 per cent formalin for microscopic examination. After the heart and aorta had been opened to expose aortic and mitral valves, and aortic intima, they were fixed in formalin, stained with Sudan IV, and graded as to the extent of intimal sudanophilia (heart score). The microscopic findings of these lesions as well as those seen in the liver and kidney are reported by Hellerstein *et al.* (7).

Oxidative phosphorylation by heart mitochondria was studied with methods described elsewhere (3).

TABLE I

Composition of Diets

Diet	C	D	E	F	G	K
Constituents						
gm. per 100 gm. of diet						
Casein (purified)	10.0	10.0	20.0	10.0	20.0	20.0
Glucose	58.1	54.1	44.1	56.8	46.8	61.8
Salt mixture*	5.0	5.0	5.0	5.0	5.0	5.0
Celluflour	5.0	5.0	5.0	5.0	5.0	5.0
Choline chloride	0.3	0.3	0.3	0.3	0.3	0.3
A, D, E mixture‡	0.1	0.1	0.1	0.1	0.1	0.1
Fat (Spry)	20.0	20.0	20.0	20.0	20.0	5.0
Cholesterol	0.0	3.0	3.0	1.0	1.0	1.0
Cholic acid	0.0	1.0	1.0	0.3	0.3	0.3
$CaCO_3$	1.5	1.5	1.5	1.5	1.5	1.5
	100.0	100.0	100.0	100.0	100.0	100.0

* Salt mixture of Jones, J. H., and Foster, C., *J. Nutrition*, 1942, **24**, 245, with the $CaCO_3$ and $MgSO_4$ removed.

‡ A mixture of vitamins A, D, and E were prepared by adding 5 gm. of halibut oil and 1 gm. of alpha tocopherol acetate to 44 gm. of corn oil (Mazola). The following vitamins were added per kilo of diet: 4 mg. thiamine hydrochloride, 8 mg. riboflavin, 4 mg. of pyridoxine hydrochloride, 25 mg. of calcium pantothenate, and 40 mg. of niacin.

RESULTS

Animals fed diet C, a 10 per cent protein diet without cholesterol of cholate added, underwent greatest growth when fed the diet containing 24 mg. per cent magnesium (Table II). After 24 days, the animals fed this diet had gained approximately 50 gm. and had a serum magnesium level of 1.33 mg. per cent. Animals fed twice and four times this amount of magnesium grew no faster, but had somewhat higher levels of serum magnesium; 1.66 and 1.75 mg. per cent, respectively. Serum cholesterol levels

TABLE II

Effect of Feeding Diet C (10 per Cent Protein) on Growth, Heart Score, and Serum Cholesterol and Magnesium Levels

Dietary magnesium	Weight gained	Control		Heart score
		Serum		
		Magnesium	Cholesterol	
mg./100 gm.	*gm.*	*mg. per cent*	*mg. per cent*	
12	39	0.46	100	0
24	52	1.33	104	0
48	48	1.66	99	0
96	54	1.75	108	0

TABLE III

Effect of Feeding a 10 (D) and 20 (E) per Cent Protein Diet (3 per Cent Cholesterol and 1 per Cent Cholate) on Growth, Heart Score, and Serum Cholesterol and Magnesium Levels

	Weight gained		Serum				Heart score	
			Magnesium		Cholesterol			
Diet	D	E	D	E	D	E	D	E
Dietary Magnesium								
mg./100 gm.	*gm.*	*gm.*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*		
12	11	32	0.71	0.64	1360	636	5	7
24	24	52	0.37	0.33	1162	603	3	6
48	29	54	1.30	0.91	1693	756	6	6
96	33	60	1.40	1.21	1550	520	5	4
192	30	80	1.65	1.34	1539	777	4	3
384	25	71	1.86	1.87	1866	650	5	4

of animals fed diet C (Table II) averaged approximately 100 mg. per cent and were not influenced by the level of dietary magnesium. These animals had no gross sudanophilia of the heart valves and aorta, and no observable pathological lesions.

Table III demonstrates the effect of adding cholesterol (3 per cent) and cholate (1 per cent) to diets containing 10 and 20 per cent protein and 20 per cent fat (diets D and E) on growth, serum magnesium, and cholesterol levels and on fat deposition

of the heart valves and aorta. Animals fed the 10 per cent protein diet grew maximally when the diet contained 96 mg. per cent magnesium whereas those fed the 20 per cent protein diet grew maximally only when the dietary magnesium was increased to 192 mg. per cent. As much as 192 mg. per cent of magnesium was required to produce serum magnesium levels comparable to those of the control animals. This is approximately an eightfold increase in the normal magnesium content of the diet. Animals fed the 10 per cent protein diet (D) had serum cholesterol levels ranging from 1200 to 1900 mg. per cent while animals fed the 20 per cent protein diet (E) had serum cholesterol levels ranging from 500 to 800 mg. per cent. The intake of magnesium had no apparent effect on the levels of serum cholesterol.

Magnesium intake, however, had an effect on the amount of fat (sudanophilia) deposited in the heart and aorta of the animals fed the 20 per cent protein diet (E). Animals fed the 24 mg. per cent magnesium diet had a heart score of 6.0 while those fed a diet containing eight times as much magnesium had a heart score of only 3.0.

TABLE IV

Effect of Feeding a 10 per Cent Protein Diet (F) (1 per Cent Cholesterol and 0.3 per Cent Cholate) on Growth, Heart Score, and Serum Cholesterol and Magnesium Levels

Dietary magnesium	Weight gained	Serum		Heart score
		Magnesium	Cholesterol	
mg./100 gm.	*gm.*	*mg. per cent*	*mg. per cent*	
12	29	0.13	639	7
24	34	0.26	672	6
48	48	0.68	742	5
96	44	1.35	720	3
192	48	1.62	808	3

Magnesium intake had no effect on the heart score of animals fed the 10 per cent protein diets. Fig. 1 illustrates a gross section of heart and aorta whose score is approximately 7. When the level of dietary cholesterol and cholate was reduced to 1 and 0.3 per cent respectively (diet F) with a consequent reduction in the serum cholesterol level, the effect of magnesium on the heart score of the animals receiving a 10 per cent protein diet could be demonstrated (Table IV). Animals fed the diet containing only 12 mg. per cent of magnesium had a heart score of 7.0 while those animals fed the diet containing 192 mg. per cent magnesium had a heart score of 3.

Although the serum cholesterol values did not change markedly, there is a tendency for an inverse relationship between serum cholesterol levels and heart score ($p > 0.05$). The serum magnesium levels were low at the lower levels of magnesium intake and did not approach normal values until the dietary magnesium was raised eightfold or to 192 mg. per cent.

Tables V and VI record the results of another experiment in which similar diets were fed, with and without thyroxine (diets F and G). It is evident from Table V, as was shown in Table IV, that on 10 per cent protein diets containing 1 per cent cholesterol and 0.3 per cent cholate, the production of atherosclerosis can be delayed quite

markedly by increasing the level of dietary magnesium. Again, there was an inverse relationship between serum cholesterol and heart score. As the dietary magnesium was increased, the serum cholesterol levels rose from 575 to 1364 and the heart score

TABLE V

Effect of Feeding a 10 per Cent Protein Diet (F) (1 per Cent Cholesterol and 0.3 per Cent Cholate) with and without Thyroxine

	Weight gained		Serum				Liver cholesterol		Heart score	
			Magnesium		Cholesterol					
Thyroxine*.....	−	+	−	+	−	+	−	+	−	+
Dietary magnesium										
mg./100 gm.	*gm.*	*gm.*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*gm.*	*gm.*		
24	43	31	0.75	0.61	575	299	6.8	5.2	6	1
48	52	23	0.67	0.75	585	357	5.6	5.1	4	2
192	48	29	1.97	1.68	997	323	8.8	5.6	2	2
384	40	31	2.00	1.75	825	535	7.2	6.0	2	1
768	38	29	2.08	2.08	1364	406	8.9	7.4	2	2

* 2 mg./100 gm. of diet.

TABLE VI

Effect of Feeding a 20 per Cent Protein Diet (G) with and without Thyroxine

	Weight gained		Serum				Liver cholesterol		Heart score	
			Magnesium		Cholesterol					
Thyroxine*.....	−	+	−	+	−	+	−	+	−	+
Dietary magnesium										
mg./100 gm.	*gm.*	*gm.*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*gm.*	*gm.*		
24	95	72	0.65	0.72	560	271	5.2	6.9	3	2
48	105	94	1.35	1.32	436	228	6.5	5.2	3	1
192	110	86	2.18	2.91	417	241	5.3	5.5	4	1
384	100	81	2.02	2.06	352	244	7.1	7.2	4	1
768	105	79	2.03	2.06	478	213	6.7	6.3	3	2

* 2 mg./100 gm. of diet.

decreased from 6 to 2. However, the level of dietary magnesium did not influence the heart score when the diet fed contained 20 per cent protein at these lower levels of cholesterol and cholate (Table VI). Regardless of the amount of magnesium fed, an over-all average heart score of 3.5 was observed which was greater than the lowest heart score observed when the 10 per cent protein and high magnesium diet was fed (Table V).

The effect of cholesterol and cholate feeding on the concentration of serum magnesium was again observed. Regardless of the amount of protein fed, (10 or 20 per cent protein) control serum magnesium values were not attained until approximately 192 mg. per cent was added to the diet. Thyroxine, when added to the diets at a level of 2 mg. per cent, caused a decrease in the serum cholesterol levels, protected against the development of atherosclerosis, had little or no effect on liver cholesterol levels and decreased the rate of growth.

The effect of protein on reducing serum cholesterol levels was again observed in this experiment (Table V *versus* VI). At each of the concentrations of cholesterol and cholate fed, 3 and 1 per cent or 1 and 0.3 per cent, respectively, the addition of more protein to the diet resulted in lowered serum cholesterol levels (Tables III, V, and VI), but did not have any effect on liver cholesterol deposition.

TABLE VII

Effect of Feeding a 20 per Cent Protein, 5 per Cent Fat Diet (K) with and without Thyroxine

	Weight gained		Serum				Liver Cholesterol		Heart score	
			Magnesium		Cholesterol					
Thyroxine*.....	−	+	−	+	−	+	−	+	−	+
Dietary magnesium										
mg./100 gm.	*gm.*	*gm.*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*gm.*	*gm.*		
20	80	41	0.56	0.51	192	132	3.1	3.6	0	0
40	91	67	1.15	0.58	142	138	2.5	3.2	0	0
80	113	69	2.33	1.32	231	132	3.6	3.7	0	0
160	109	65	2.00	1.76	181	133	2.4	3.8	0	0

* 2 mg./100 gm. of diet.

The feeding of a 20 per cent protein diet (K) with a reduction in dietary fat from 20 to 5 per cent resulted in cholesterol levels ranging from 142 to 231 regardless of the amount of magnesium fed (Table VII). These values are significantly lower than those found when the same diet was fed but with 20 per cent fat (Table VI). Similarly, the serum magnesium levels were normal at magnesium intakes between 40 and 80 mg. per cent rather than at intakes of 192 to 384 mg. per cent. The heart scores were zero at this level of fat intake.

Table VIII demonstrates the results from a preliminary study in which the effect of cholesterol and cholate feeding on oxidative phosphorylation of heart mitochondria was measured. There is a decrease in oxidative phosphorylation by heart mitochondria when cholesterol and cholate were added to the control diet containing 24 mg. per cent magnesium. However, this effect of cholesterol and cholate was completely prevented by increasing the level of dietary magnesium to 192 mg. per cent. The P/O ratio was restored to the control value of 2.0.

On gross examination of the animals fed the cholesterol and cholate diet, fatty livers were always encountered. Gross pathological lesions were usually seen in the

kidneys of the low magnesium-fed animals. The obstructive internal hydronephrosis seen microscopically in these animals could be completely prevented by feeding eight to sixteen times the normal magnesium requirement. Similarly, these kidney lesions could be prevented by the addition of thyroxine to the diet or by increasing the protein of the diet from 10 to 20 per cent (7).

TABLE VIII

Effect of Dietary Cholesterol and Cholic Acid on Oxidative Phosphorylation of Heart Mitochondria

Group	No. of animals	Diet fed	Dietary magnesium	Oxidative phosphorylation
			mg./100 gm.	*P/O*
Control	3	C	24	2.0
Treated*	3	F	24	1.4
Treated	3	F	192	1.9

* 10 per cent protein, 20 per cent fat diet containing cholesterol (1 per cent) and cholic acid (0.3 per cent).

DISCUSSION

We have previously shown (3, 8) that animals fed a usual purified diet containing 20 per cent casein require approximately 24 mg. of magnesium per 100 gm. of diet to grow maximally on that diet. Higher levels of magnesium produced no more rapid gain. The serum magnesium levels of the animals fed adequate amounts of magnesium averaged approximately 1.8 mg. per cent. In this study it has been shown that the addition of cholesterol and cholic acid to such a diet produces typical magnesium deficiency. The symptoms included hyperemia of the ears, low serum magnesium levels, hyperexcitability, decreased oxidative phosphorylation of heart mitochondria, and kidney lesions (9–11) similar to those described in animals fed magnesium-deficient diets. Higher magnesium intakes, from four to eight times the normal requirement of 24 mg. per cent, completely prevented these changes. The low serum magnesium levels were particularly resistant to change and usually eight times the normal dietary magnesium was required to bring them into the normal range of 1.3 to 1.8 mg. per cent. In addition, cholesterol and cholate feeding resulted in poorer growth. This was partially overcome in most experiments by higher levels of dietary magnesium.

The mechanism involved in this induced deficiency is unknown. It would not appear to be related to decreased absorption since the urinary excretion of magnesium is high and the deficiency is induced more rapidly by cholesterol-cholate feeding than by simple magnesium restriction (12).

Of particular interest was the observation that the early lesions of atherosclerosis induced by cholesterol and cholate feeding were diminished or retarded by increasing the magnesium intake. This occurred despite the fact that serum

cholesterol levels were not diminished by magnesium feeding. When the diet contained 10 per cent protein, 1 per cent cholesterol, and 0.3 per cent cholic acid, there was actually an increase in the serum cholesterol levels with higher levels of magnesium feeding. This effect of magnesium apparently provides one of the first demonstrations of a disassociation between degree of hypercholesteremia and the extent of vascular sudanophilia. However, in studies dealing with the effect of different dietary fats upon vascular sudanophilia and serum cholesterol levels using rats fed diets similar to those used here, Hegsted *et al.* (13) concluded that the extent of atherosclerosis was proportional to and probably dependent upon the serum cholesterol levels. Magnesium levels were not varied in these experiments on fats.

Previous studies by Fillios *et al.* (14, 15) have adequately demonstrated that other conditions being held constant, the degree of hypercholesteremia produced by cholesterol-cholate feeding is influenced by the amount of protein in the diet. This was also apparent in the present studies. However, the situation is obviously very complex. The interrelations of the level of dietary magnesium, protein, cholesterol, and cholate and the resultant poor growth of the animals, the low serum magnesium levels, the degree of hypercholesteremia, the heart score and kidney lesions all require definition. When extremely high serum cholesterol levels were produced with the 10 per cent protein, 3 per cent cholesterol, 1 per cent cholate diet (Table III), no effect of magnesium upon heart score was evident. This was, perhaps, to be expected since the hypercholesteremia may have been sufficiently severe to overshadow any other effects. However, when the degree of hypercholesteremia was decreased by either feeding the 20 per cent protein diet at the same level of dietary cholesterol (3 per cent) and cholate (1 per cent), or by lowering the dietary level of cholesterol (1 per cent) and cholate (0.3 per cent) with the 10 per cent protein diet, then the effect of magnesium on heart score was usually evident (Tables III, IV, and V), but not in one experiment (Table VI). Whether the effect of protein is entirely related to its influence upon the serum cholesterol level remains to be demonstrated. In any event, it is clear that the experimental conditions have to be such that the variable under study is critical.

The degree of hypercholesteremia was dependent upon the fat intake when cholesterol and cholate were held constant (Tables VI and VII). The fact that the animals fed the lower fat diets did not have gross sudanophilia of the vascular system or kidney lesions as severe suggests that high serum cholesterol levels are required for the pathogenesis of such lesions. The rate of absorption of cholesterol and cholate or both is undoubtedly influenced by dietary fat.

The effect of thyroxine under differing conditions is also complex and difficult to interpret. Under conditions not complicated by hypercholesteremia, thyroxine clearly raises the magnesium requirement as measured by the growth of the animals, serum magnesium, and oxidative phosphorylation of heart

mitochondria (3). The latter two effects are completely reversible by high levels of dietary magnesium and the growth effect is partially reversible. In the hypercholesteremic animals, thyroxine administration greatly lowered the serum cholesterol values and the heart scores and prevented the development of kidney lesions. Serum magnesium levels, however, were not decreased below the levels found in the hypercholesteremic animals not receiving thyroxine. Thus, the effect of hypercholesteremia and thyroxine on serum magnesium levels were not additive. It should be noted that the experiments recorded in the various tables of this paper were done at different times. Although the general trends are similar in repeat experiments, quantitative reproducibility of serum magnesium and cholesterol levels in different groups of animals fed the same diet at different times are not obtained (see Tables IV and V). Thus, comparisons of different experiments are open to criticism and we prefer to present them as separate experiments.

Hartroft (16) has pointed out that perhaps the "most promising lead to the problem of atheroma in man may lie in experimental and clinical studies designed to elucidate the interrelationships between dietary fat, cholesterol, lipotropic factors, and renal function." The hyperlipemia and hypercholesteremia associated with the nephrotic syndrome are well known (16, 17). The production of a hydronephrosis in these animals by calcium deposition in the tubules may thus be of particular interest. These lesions were completely prevented under the experimental conditions by raising the dietary magnesium or protein, or by thyroxine administration. The protective effect of thyroxine and protein occurred even though the serum magnesium levels remained low. The mechanism and interrelationships involved are not clear.

Many adult rats made hypercholesteremic by diets similar to those used in the present experiments have been studied in this laboratory (14), but such kidney lesions have not been described. This is presumably explained by the levels of magnesium used in the diets, and the greater resistance of adult animals to magnesium deficiency. Adult animals have greater body stores of magnesium and presumably their requirement is smaller than the growing animals.

SUMMARY

The addition of cholesterol and cholic acid to a diet containing 24 mg. per cent of magnesium, an amount normally required for young rats, resulted in magnesium deficiency in rats. This was characterized by hyperexcitability, hyperemia of the ears, calcium deposition in the kidney tubules, low serum magnesium levels, and decreased oxidative phosphorylation of heart mitochondria. All these lesions were prevented by raising the dietary magnesium level four to eight times.

Feeding the atherogenic diet produced the deposition of lipide in the aorta and in the heart valves. The extent of this intimal sudanophilia was reduced by

EXPLANATION OF PLATES

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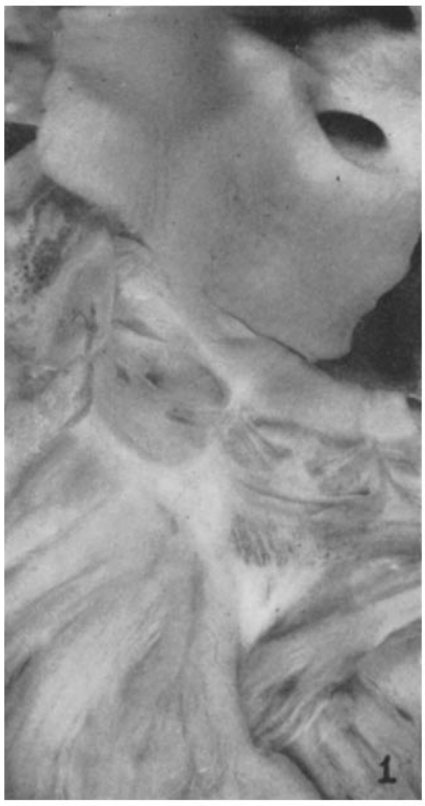
FIG. 1. Kidney. Note calcium deposition (black spots) within the tubules of the outer zona intermedia of the medulla. Within the cortex there is tubular dilatation. This kidney was scored as showing 3+ calcium deposition. Hematoxylin and eosin. × 9.

FIG. 2. Kidney. There is calcium deposition with and without giant cell response. Tubular dilatation is also present. Hematoxylin and eosin. × 440.



(Vitale *et al.*: Hypercholesteremia, magnesium, and atherosclerosis)