FURTHER STUDY ON PREVENTIVE EFFECT OF s-METHYL METHIONINE SULFONIUM IODIDE ON HYPERCHOLESTEREMIA CAUSED IN CHOLESTEROL FED RABBITS

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By

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In the previous papers (1, 2), the authors reported that a sulfonium salt of methionine, S-methyl methionine sulfonium iodide (MMSI) has a potentiality to prevent the elevation of serum cholesterol level, caused by cholesterol feeding in rats and rabbits.

In the last paper, the authors vertified that this sulfonium salt of methionine has a preventive effect of dietary hypercholesteremia and atheromic denaturation of the rabbits, when the sulfoinum salt is injected in parallel with cholesterol feeding.

Recently, the authors recognized these effects on serum cholesterol level and arterial tissue of atheroma, in cholesterol feeding, and the results are detailed below.

Experimental

The experimental materials and the procedures are mostly common to that of the previous paper (2).

Animals. Male albino rabbits, weighing approximately 1.9kg (*Min.* 1670 g to *Max.* 2100 g) were fed on the basal diet, mainly composed of *Okara* (Waste product of *Tofu*, or soy curd), for 100 days, *ad libitum.*

Test groups. Twelve rabbits were allotted into four lots, *i.e.*, the none cholesterol fed, none injected lot (No Ch-No inj) ; the cholesterol fed, methionine administrated lot (Ch-Met) ; the cholesterol fed, MMSI administrated lot (Ch-MMSI) ; the cholesterol fed, none injected lot (Ch-No inj). The last named was sacrificed at the 70th day, because of the detection of atheromic alternation of aortae. All animals, except in the first lot, were administrated with a dose of 0.4g cholesterol, suspending in a 1:1 mixture of lard and soy bean oil, every

day using the stomach tube, after preliminary feeding of 10 days, without cholesterol supply. The animals of the Ch-Met lot received an intravenous injection of aqueous solution of DL-methionine 10 mg in a dose, and the Ch-MMSI lot were of 20 mg MMSI into the veins of their ears, after the 71st experimental day to the final day.

These animals in the No Ch-No inj lot and the Ch-No inj lot were free from any injection.

The constitution of the test groups is shown in Table 1.

Table 1. Constitution of the test groups.

—Male rabbits—

Lot	Number of animals	Administration of Ch *per os*	Injection#	Days fed
No Ch-No inj	3	None*	None	100
Ch-Met	3	0.4g/day after 10th day	DL-Methionine 10 mg/day, after 71st day.	100
Ch-MMSI	3	〃	MMSI 20mg/day after 71st day	100
Ch-No inj	3	〃	None	70

Ch : Cholesterol. # Intravenonous injection. * Administrated the suspending oil free from cholesterol.

Substances tested. MMSI was prepared by methylation of DL-methionine with methyl iodide (3). DL-Methionine was obtained as a commercial supply of guaranteed degree.

Analytical procedures. Serum cholesterol of the total and free types were determined following the method of Zak *et al.* (4), every 10 days. Cholesterol in viscera and aortae were extracted from the minced tissue with acetone-ethanol mixture in equal volume, at 37°C for 48 hr and determined by the same method (1). Levels of serum protein every 10 days were determined refractmetrically.

Histological procedure. Livers, kidneys and aortae (*Aorta descendans thoracia* and *A. abdominals*) of all animals were fixed in ten-times diluted formalin (*ca.* 3.5%), embedded in paraffin, sectioned 8μ slices, and stained with Van Gieson stain and haematoxylin-eosin double stain. Besides these two stains, aortae were sectioned into 10μ slices in freezing condition and stained with Sudan III.

Results

Growth. All animals of the four lots had grown without showing any injurious effect of cholesterol administration and injections, though there were some deviations in their growing pattern. The growth curves of each animal are shown in Fig. 1.

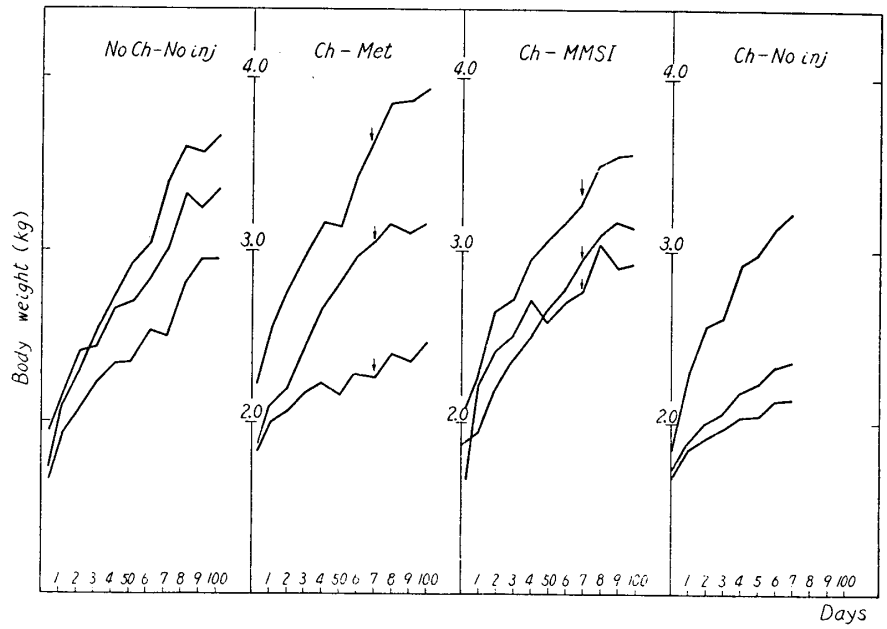

Fig. 1. Growth of the rabbits.

—Male rabbits: Fed for 100 days, except Ch-No inj lot, feeding for 70 days.— The arrows in the figure mean the beginning of administration of methionine or MMSI.

Weights of viscera and carcasses. After the feeding of 70 or 100 days, the animals were sacrificed by carotidal amputation without anaesthesia, and dissected into individual organs. These viscera (Kidney, spleen, gall bladder, digestive tracts, heart, lung and testis) showed no remarkable deviation in their net weights or the ratios to the body weights.

However, there were declination of hypertrophic changes of the adrenals in the cholesterol fed animals, and this seemed to be emphasized by both injections. The net weights of the adrenals and the ratios to the right kidney weights are shown in Table 2.

In the livers from these aninals of the cholesterol fed lots were also detectable very slight hypertrophic alternations. The net weight of them and the ratios to the body weights are shown in Table 3.

The carcasses and pelts from the animals showed no significant differences among them, in the ratios to the whole body weights.

Serum cholesterol. The cholesterol levels in the sera of total and free types, determined at 10 days' intervals, are presented in Table 4, and illustrated

Table 2. Adrenal weight.

Lot	Right adrenal weight (g)	Ratio to the kidney* weight (%)
No Ch-No inj	0.05 .10 .10	1.11 0.87 1.33 1.10±0.14**
Ch-Met	0.15 .20 .15	2.45 1.75 1.65 1.94±0.27
Ch-MMSI	0.40 .20 .40	2.70 3.08 2.55 2.77±0.16
Ch-No inj	0.20 .10 .10	1.36 1.76 1.67 1.56±0.17

* Right kidney
** Mean ± standard error of the mean.

Table 3. Liver weight

Lot	Liver weight (g)	Ratio to the body weight (%)
No Ch-No inj	62 78 75	1.85 2.13 2.54 2.17±0.02*
Ch-Met	85 63 90	2.79 2.71 2.31 2.60±0.15
Ch-MMSI	63 75 95	2.16 2.40 2.68 2.41±0.15
Ch-No inj	80 70 70	2.48 3.30 2.98 2.92±0.03

* Mean±standard error of the mean.

Table 4. Cholesterol levels in the sera determined every 10 day (mg/*dl*).

Lot \ Days		10	20	30	40	50	60	70	80	90	100
No Ch-No inj	Total	33 45 55 47±7*	62 47 47 52±5	57 36 63 51±8	45 55 59 53±4	42 63 58 54±6	54 66 58 59±4	42 43 44 43±1	53 57 62 57±3	42 52 41 45±4	56 64 61 60±2
	Free	22 18 23 21±1	47 34 36 39±4	23 31 50 35±8	39 49 42 43±2	35 37 24 32±4	42 58 42 47±5	33 24 32 29±3	40 42 29 37±4	31 45 38 38±5	39 48 38 42±3
Ch-Met	Total	55 33 37 42±7	74 97 93 88±7	91 111 75 93±11	143 125 150 139±7	235 208 213 219±8	156 208 218 194±19	200 246 229 225±13	242 231 195 223±14	219 246 237 234±8	322 250 266 283±10
	Free	33 28 33 32±1	44 57 59 53±5	77 64 63 68±5	137 117 136 130±7	198 118 102 139±30	134 116 113 128±5	179 161 177 172±5	144 183 118 148±19	138 167 188 164±15	190 197 207 198±5
Ch.MMSI	Total	46 35 49 44±4	84 71 119 91±15	99 87 103 97±5	169 177 187 177±7	151 219 226 199±24	231 185 175 197±17	228 218 231 226±4	178 205 229 204±20	199 187 243 210±14	210 178 254 214±22
	Free	35 22 31 29±4	51 47 81 59±11	42 41 84 55±14	83 146 146 125±21	125 171 167 155±11	116 108 177 134±22	167 179 146 164±14	56 131 125 104±24	92 150 139 127±16	154 129 164 149±10
Ch-No inj	Total	33 44 37 37±3	111 111 125 115±5	104 115 125 115±9	146 139 195 160±17	166 195 161 174±11	169 164 161 165±3	213 182 208 201±23	—	—	—
	Free	22 20 21 21±1	73 45 50 56±9	95 94 69 86±9	107 112 127 115±6	134 144 155 143±5	102 85 125 104±13	198 108 100 135±31	—	—	—

* Mean±standard error of the mean. Methionine and MMSI were administrated after 71st day. See the text.

in Figs. 2 and 3.

There were remarkable and abrupt increase of cholesterol of both types in the cholesterol fed lots, for successive administration of 60 days, without exception, and no deviation among them were detectable.

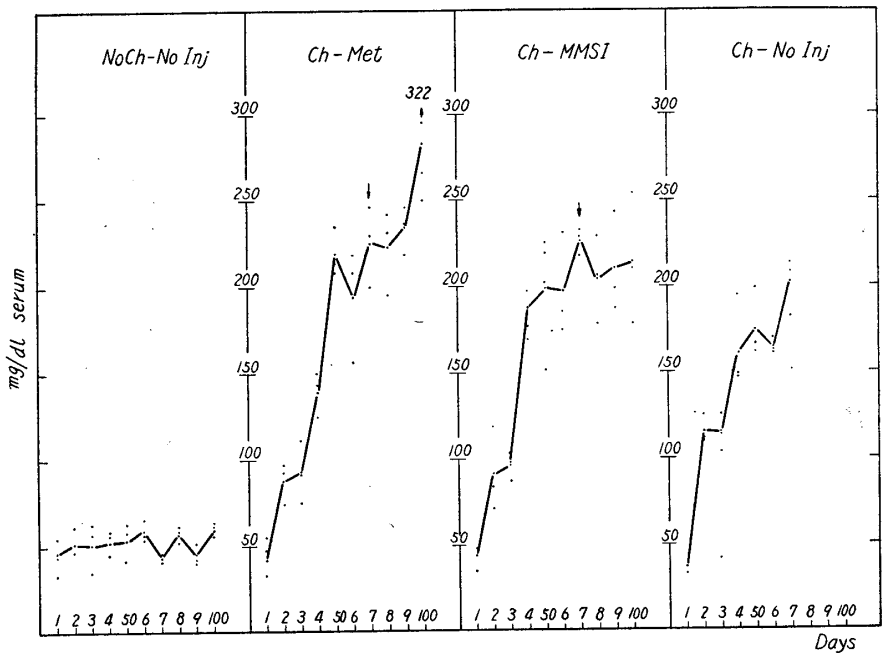

Fig. 2. Changes of total cholesterol levels in the sera.
The downward arrows show the beginning of the administration of methionine or MMSI.
· Determined -·- Mean

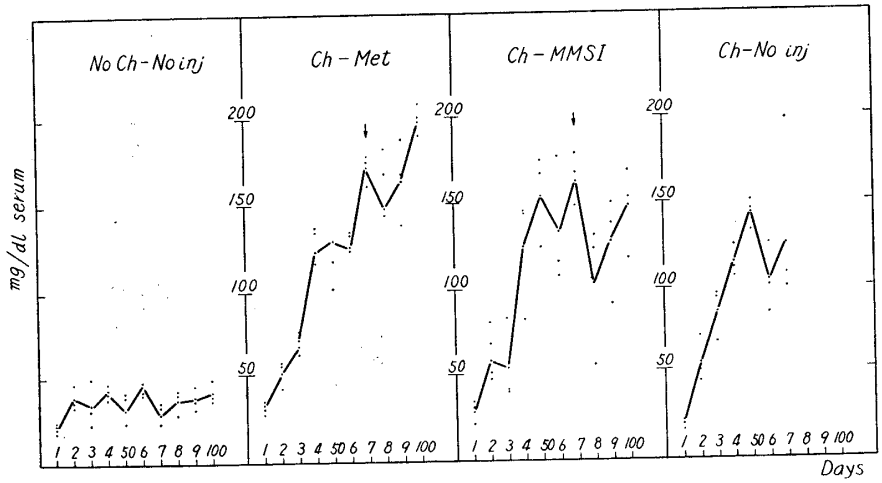

Fig. 3. Changes of free cholesterol levels in the sera.
The arrows show the beginning of the administration of methionine or MMSI.
· Determined -·- Mean

The No Ch-No inj lot had no elevation of serum cholesterol levels of both types, though there were very little changes through the experimental period. The arrows, shown at the 71st day in Figs. 2 and 3, mean the beginning of the administration of methionine or MMSI. The serum cholesterol of the Ch-Met lot seemed to decrease at the initial stage of methionine administration, but soon recovered the increasing declination rapidly. Contrasting to the Ch-Met lot, elevating serum cholesterol level of the Ch-MMSI lot changed to be stopped at the determination on the 81st day, and the declination of the serum cholesterol level had preserved in plateau to the final day, even though successive administration of cholesterol had continued. Although the data determined at the final day had some deviations, the difference of the serum cholesterol of total type between the Ch-Met lot and the Ch-MMSI lot is significant of somewhat strength ($F_{0.2}^{3}=4.84$, P:20-5%; $t_4=1.64$, P:20-10%). This difference between them is distinct in free type more than that of the total type ($F_{0.1}^{3}=4.00$, P:20%; $t_4=8.33$, P:1-0.1%).

Lipids in liver. The contents of total lipid and unsaponifiable lipid in the livers are shown in Fig. 4. Much increases of both lipids, which had been noticed in the previous paper (2), are detectable in cholesterol fed lots, but the differences among them are significant in weakness.

Cholesterol in the viscera. Liver: Both types of cholesterol in the livers are shown in Fig. 5. The cholesterol fed lots were of higher content than that

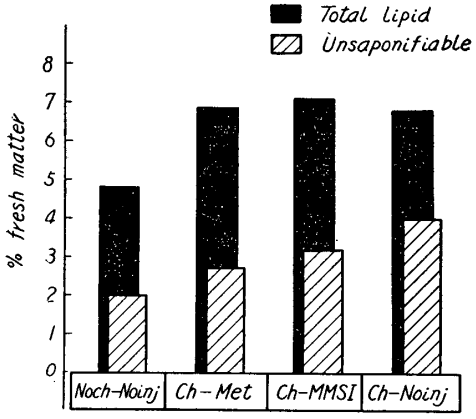

Fig. 4. Total and unsaponifiable lipids in the liver.
The mean values of each lot are represented in the figure.

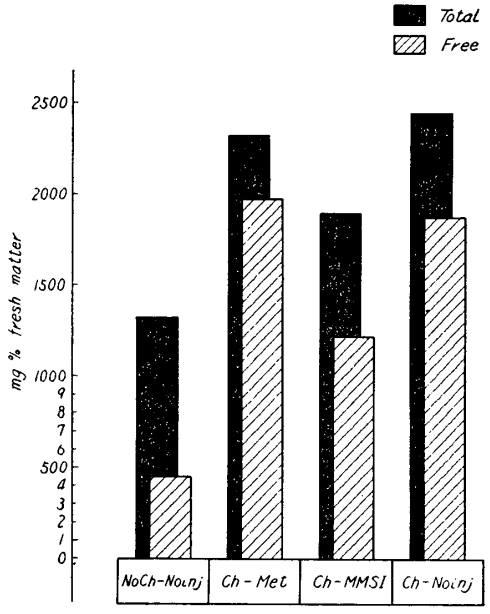

Fig. 5. Cholesterol in the liver.
The mean values of each lot are represented in the figure.

of the cholesterol free lot, but the content of the Ch-MMSI lot was less than other two lots of cholesterol fed.

Kidney, spleen, and adrenal: Cholesterol levels in these organs are illustrated in Figs. 6 to 8. At a glance, it seems that there are some declination of increasing of both cholesterol contents in the cholesterol fed lots, but the differences among them in each organ are of little significant. Although the increase of cholesterol contents in the adrenals in these lots are not so considerable, the net cholesterol contents of the whole tissues should be much in amount, because of their hypertrophic alternation. Extremely high contents of cholesterol of conjugated, but very less of the free type in the adrenal are worthy of notice, though this strange fact has been reported by other investigators (5).

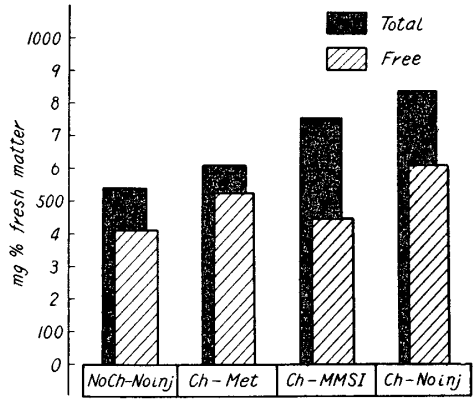

Fig. 6. Cholesterol in the kidney.
The mean values of each lot are represented in the figure.

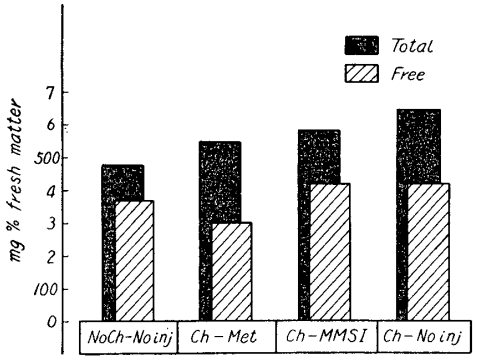

Fig. 7. Cholesterol in the spleen.
The mean values of each lot are represented in the figure.

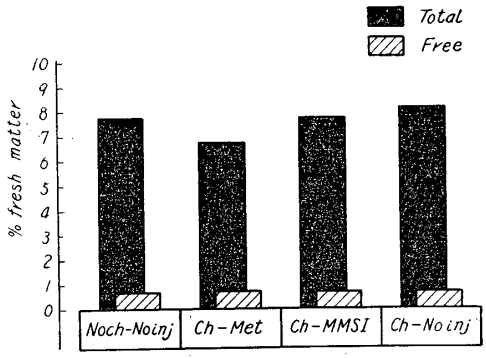

Fig. 8. Cholesterol in the adrenal.
The mean values of each lot are represented in the figure.

Aorta: The cholesterol contents of aortae are presented in Fig. 9.

It is apparent that cholesterol feeding causes a high accumulation in the tissue (See the content of the Ch-No inj lot in Fig. 9).

But the accumulated cholesterol in the tissue may be swept by MMSI

administration, *i.e.*, the level in the Ch-MMSI lot was much less than that of the Ch-No inj lot, and moreover, lower than that of the No Ch-No inj lot. This may be a fact of interest, and this declination corresponded to the histological inspection of these arterial tissue. Cholesterol content of the Ch-Met lot is rather higher than that of the No Ch-No inj lot, but the difference between these two lots is little significance.

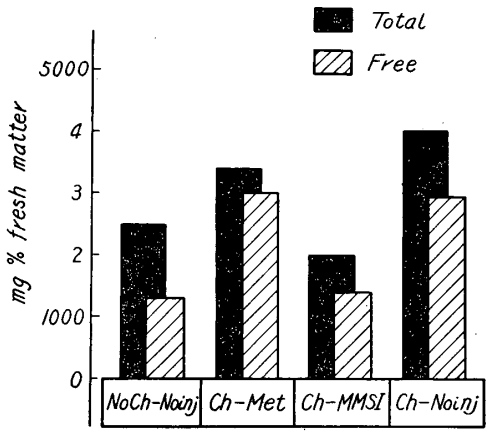

Fig. 9. Cholesterol in the aorta. The mean values of each lot are represented in the figure.

Serum protein levels. Protein levels of the sera every 10 days are shown in Fig. 10. There are no significant changes and deviations in each lot and *inter se.*

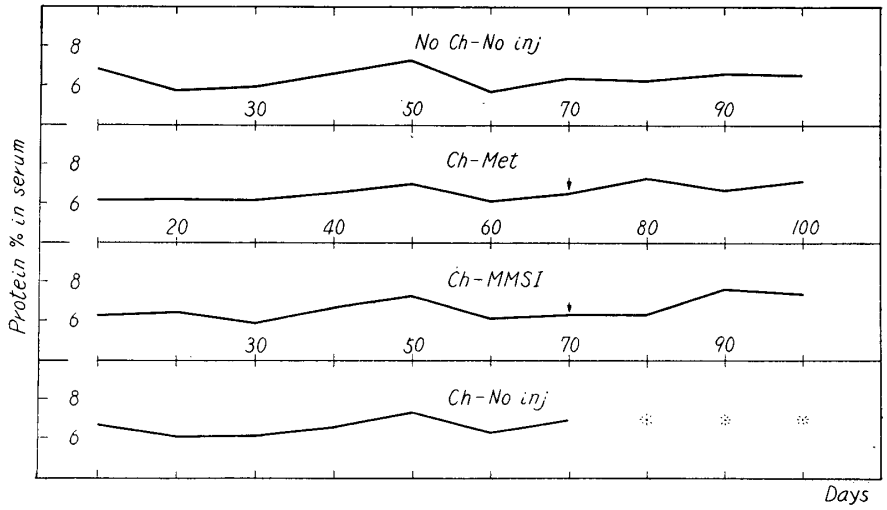

Fig. 10. Serum protein levels determined every 10 days. The mean values are shown. The arrows in the figure mean the beginning of the injection of methionine or MMSI.

Histological inspection. Livers, kidneys and aortae of all animals were inspected histologically, under 150× and 600× fields. There are no pathological alternations in any of these hepatic and renal tissues. All aortae from the cholesterol fed lots were detectable to be severe or of slight hypertrophic alternations in these two strains. These hypertrophic changes of the aortae from the animals in the Ch-MMSI lot were less severe than that of the Ch-Met lot.

In the field of fat stain with Sudan III, there were severe deposition of sudanophilic substance on the inner sides of the aortae, dissected from the Ch-No inj lot, but very less in the Ch-MMSI lot. Sudanophilic depositions on *Tunica intimae* from the Ch-No inj lot are compact and thick all around, but the depositions of the Ch-MMSI lot were perfectly free or partial and very thin. In the Ch-Met lot, the aortae from two animals were clean from the deposition, but one case of them was very severe in the deposition of thick sudanophilic matter. And so, the cleaning ability of methionine to remove the deposit is doubtful.

This will be traced in another experiment, designed on a larger scale.

In Table 5, these histological inspections are summarized, and the tissues of aortae in the fat stain are illustrated in the associated Plate.

Table 5. Histological examination of liver, Kidney and Aorta.

Lot	Animal No.	Aorta			Liver		Kidney	
		Sudan III	H-E	V.G.	H-E	V.G.	H-E	V.G.
No Ch-No inj	1	None	None	None	None	None	None	None
	2	〃	〃	〃	〃	〃	〃	〃
	3	〃	〃	〃	〃	〃	〃	〃
Ch-Met	4	Fat+	Atm±	Atm±	None	None	None	None
	5	++	+	++	〃	〃	〃	〃
	6	+	+	++	〃	〃	〃	〃
Ch-MMSI	7	Fat±	Atm+	Atm+	None	None	None	None
	8	±	+	+	〃	〃	〃	〃
	9	None	+	+	〃	〃	〃	〃
Ch-No inj	10	Fat+	Atm+	Atm+	None	None	None	None
	11	+	++	++	〃	〃	〃	〃
	12	+	++	+	〃	〃	〃	〃

Sudan III : Sudan III stain. H-E : Haematoxylin-eosin stain.
V.G. : Van Gieson stain.
None : No histological alternation is detectable, under 150× and 600× fields.
Fat : Sudanophilic deposition on *Tunica intima.*
Atm : Atheromic hypertrophy of *Tunica intima.*
+ : Number of the crosses represents the approximate degree of sudanophilic denaturation or hypertrophic alternation of the tissues (*Vid.* the photographes).

Conclusion and Discussion

It may be an interesting fact from both nutritional and pharmacological points of view, that MMSI has an ability to clear the sudanophilic scale on the insides of the artery, and decrease or no increase, at least, the serum cholesterol, even successive cholesterol feeding has been continued.

The mechanism of the effect to depress the serum cholesterol and to sweep the sudanophilic deposition of the aortae has not yet been elucidated.

The authors, however, provide a working hypothesis that MMSI may be able to facilitate the degradation and/or the dissimilation of cholesterol in the body, and to be less effective to diminish the synthetic site, because of that exogeneous cholesterol, supplied as a constituent of diet, regulates the synthesis of endogenous cholesterol and preliminally blocks the synthesic pathway in negative feedback control (6). The exact mechanism, by which MMSI regulates the excess cholesterol in serum, however, remains to be elucidated in the future.

Summary

1) Male albino rabbits were fed for 90 days, and laden 0.4g cholesterol every day *per os*. An experimental lot of the animals was administrated with intravenous injection of *S*-Methyl methionine sulfonium iodide (MMSI) at a dose of 20 mg every day, from the 61st experimental day to the final day. Another lot was administrated with 10 mg DL-Methionine in the same fashion.

2) At the 60th day, a lot of animals, fed with cholesterol and free from any injection, was sacrificed and detected the severe deposition of sudanophilic matter and hypertrophic alternation on their *Tunica intimae* of *Aorta descendans thoracica* and *A. abdominalis*. The cholesterol amounts in the sera of these three lots had rapidly increased by 60 days' cholesterol feeding, commonly.

3) By the administration of MMSI, the serum cholesterol ceased to increase during the last 30 days. Contrasting to the MMSI injected lot, administration of methionine could not stop the increasing elevation of the cholesterol level.

4) At the 90th day (MMSI or methionine had injected for 30 days), the methionine lot was severely atherosclerosic and hypertrophic in one case of aorta, but the MMSI lot was free or very scarcely atheromic in all of their aortae.

5) These results by chemical assay and histological inspection support the view that MMSI has a potential activity to prevent and to heal the dietary hypercholesteremia and atherosclerosis.

Acknowledgement

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Plate 1

Explanation of Figures

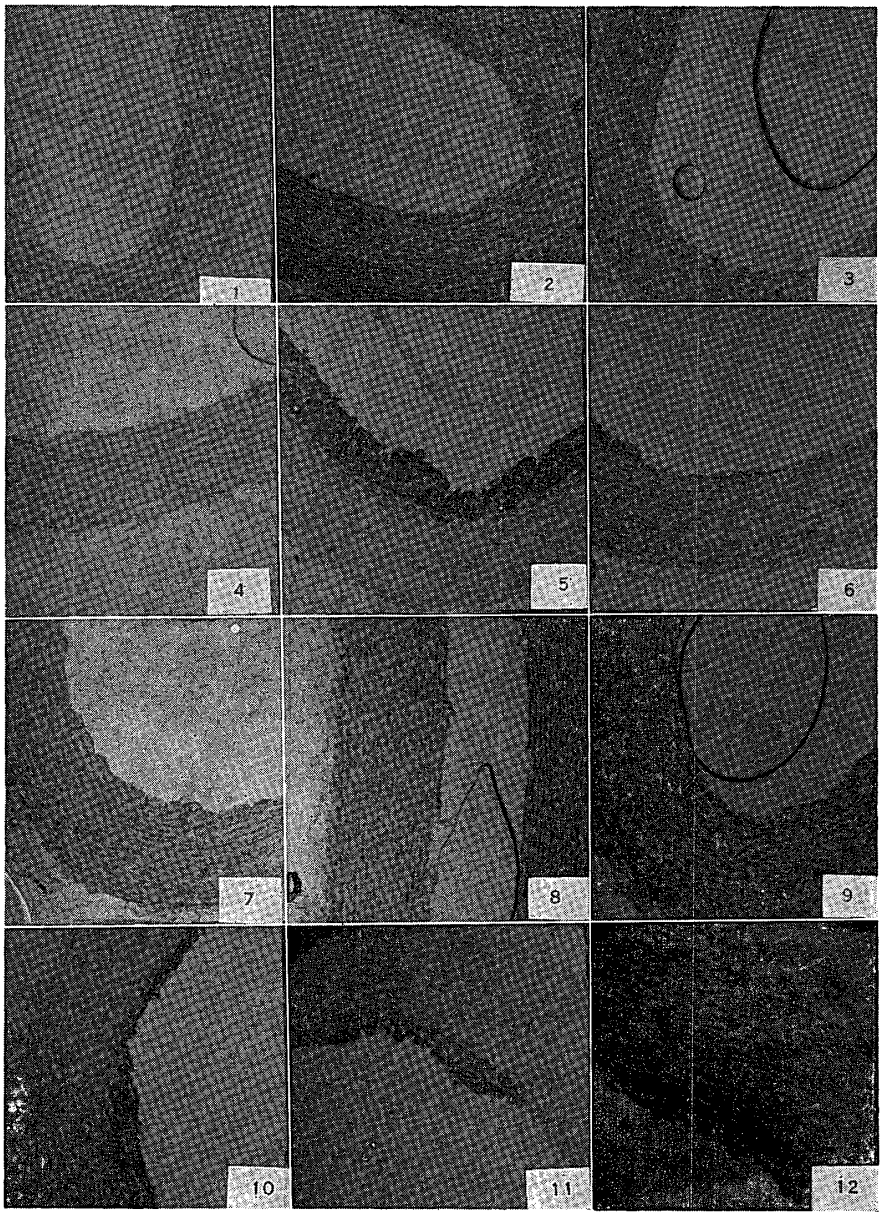
Histological sections of the aortae (*aorta descendans thoracica* and *A. abdominalis*) from the rabbits. Stained with Sudan III. 10μ slices of freezed. 150×.

1-3. No Ch-No inj lot ; Fed for 90 days, after 10 days' preliminary feeding. No sudanophilic deposition on *Tunica intima* is detectable.

4-6. Ch-Met lot ; Fed cholesterol 0.4g/day for 90 days, after 10 days' preliminary feeding, and injected with *DL*-Methionine 10 mg/day, intravenously, for the last 30 days.
Severe sudanophlic deposition on *Tunica intima* is detectable in one case, and two others are scarcely as to the deposition.

7-9. Ch-MMSI lot ; Fed cholesterol 0.4g/day for 90 days after 10 days' preliminary feeding, and injected intravenously with MMSI 20 mg/day for the last 30 days.
None or very less deposition of sudanophilic matter are detectable in all cases.

10-12. Ch-No inj lot ; Fed cholesterol 0.4 g/day for 60 days, without any injection, after 10 days' preliminary feeding.
Representative atherosclerosis with thick and compact accumulation of sudanophilic matter are noticed.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