PHYSIOLOGICAL SIGNIFICANCE OF s-METHYL METHIONINE SULFONIUM IODIDE AND γ-DIMETHYL BUTHYROTHETINE CHLORIDE IN EXPERIMENTAL HYPER-CHOLESTEREMIA AND ATHEROSCLEROSIS IN RABBITS

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By

Kikunoshin NAKAMURA and Hisashi ARIYAMA

Department of Food Chemistry, Faculty of Agriculture, Tohoku University. Sendai, Japan.

(*Received, November 6, 1961*)

In the previous papers (1-3), the authors reported that *s*-methyl methionine sulfonium iodide (MMSI) had a potentiality to prevent hypercholesteremia and atherosclerosis in rats and rabbits, caused by oral administration of cholesterol.

The present study was undertaken to confirm whether MMSI had a preventive and therapeutic effect to adjust the serum cholesterol level and to reduce the atheromous change of the aorta, produced by cholesterol feeding.

Associating this effect of MMSI, a similar study was performed on γ- dimethyl butyrothetine chloride (DMBTC), *i.e.*, the analogous substance of MMSI, and on methionine (Met) along the same line.

Experimental

Animals: Twenty-six male albino rabbits, weighing approximately 1.0 kg (0.92—1.8kg) at initial body weight, were kept in individual metabolic boxes, and fed on the basal diet composed of fresh grass, wheat bran and *Okara* (waste product of *Tôfu*, or soy-curd) for 100 or 70 days, *ad libitum*. The ingradients and the composition of the diet are listed in Table 1.

Test groups: These animals were classified into six groups as follows:

Group I. Normal control, *i.e.*, the animals fed without cholesterol. (No Ch-No inj)

Group II. Negative control, fed on cholesterol and injected with isotonic saline solution. (Ch-NaCl)

Group III. The test group, administrated with cholesterol and injected with MMSI from the 71st experimental day until the last day, to examine the therapeutic effect of MMSI on hypercholesteremia and atherosclerosis. (Ch-MMSI-A)

Group IV. The test group, administrated with cholesterol and injected

with MMSI, to examine the preventive effect of MMSI. (Ch-MMSI-B)

Group V. The group, administrated with cholesterol and injected with DMBTC. (Ch-DMBTC)

Group VI. The group, administrated with cholesterol and injected with Met. (Ch-Met)

Table 1. Ingredients and composition of the diet.

Ingredients (g/kg)		Composition (%)	
Fresh grass*	450	Moisture	74.84
		Crude protein	3.56
Okara**	400	Crude fat	0.95
		Soluble non-nitrogen	13.47
Wheat bran	150	Crude fiber	5.81
		Crude ash	1.37

* Mainly of white clover.

** Waste product of *Tôfu*, or soy-curd.

N.B. Calcium lactate and sodium chloride were supplied with an amount of 2 and 1g/week for each animal, respectively.

At the intial stage of the feeding, a compressed diet for rabbit and guinea pig, *Oriental RC5*, manufactured by the Oriental Yeast Co., Ltd., Tokyo, was rationed in liue of the fresh grass.

The animals belonging to the groups IV-VI were sacrificed on the 70th experimental day, but the others were sacrificed on the 100th day. All animals, except in the No Ch-No inj group, were administrated, *per os*, with a dose of 0.4 g cholesterol of extra pure grade, very fine crystal, suspended in 2 *ml* of 1:1 mixture of lard and soy-bean oil, every day by means of a 20*ml*-syringe, after preliminary feeding for 10 days without cholesterol supply. The No Ch-No inj group was supplied with the suspendig oil free from cholesterol, in equal volume and by the same procedure.

The members, which did not belong to the No Ch-No inj group and the Ch-MMSI-A group received intravenous injections of the testing substances or isotonic saline (0.9% NaCl aq. sol.), every two days throughout the experimental period. The Ch-MMSI-A group, however, were injected with MMSI every day from the 71st day until the day of sacrifice.

The No Ch-No inj group was free from any injection. The dosages of the injections were adjusted in equimolecular amount to 10 mg Met in 0.2 *ml* aquous solution at one dose.

The constitution of the test groups is listed in Table 2.

Substances tested: MMSI was synthesized by methylation of DL-Met with methyl iodide (4). DMBTC was also synthesized from γ-butyrolactone and dimethyl sulfide, *via* the iodide (5). DL-Met of guranteed quality was supplied

Table 2. Constitution of the test groups.

Group	Number of animals	Administration of cholesterol *per os*	Injection*	Days fed
I No Ch-No inj	3	None	None	100
II Ch-NaCl	3	0.4g/day, after 11th day	Isotonic saline	〃
III Ch-MMSI-A	5	〃	MMSI 20 mg/day, after 71 st day	〃
IV Ch-MMSI-B	6	〃	MMSI 20 mg/dose, every other day	70
V Ch-DMBTC	6	〃	DMBTC 12 mg/dose, every other day	〃
VI Ch-Met	3	〃	Met 10 mg/dose, every other day	〃

* Intravenous injection.

by the Nihon-Kayaku Co., Ltd, Tokyo.

Analytical procedures: Serum cholesterol of the total and the free form were determined following the method of Zak *et al.* (6), at ten days interval. Cholesterol in the aorta was extracted from the minced tissue with acetone-ethanol mixture in equal volume, at 37° for 48 hr. and determined by the same method.

The total lipid in the liver was determined gravinometrically, after refluxing with boiling ethanol for three hr. and re-extracted with ether. The serum protein levels were determined refractmetrically, every ten days.

Histological procedures: The aortae (*Aorta descendans thoracica* and *A. abdominalis*), dissected from each animal were fixed in 10-times diluted formalin (*ca.* 3.5%), sectioned into 20 μ slices in freezing condition, and stained with Sudan III and haematoxyline.

Results

Growth: All animals of the six groups had normal growth and showed no decrease in their body weights, though there were some deviations in their growing pattern.

The growth curves of each animal are shown in Fig. 1.

Weight of viscera and carcasses: On the final day of the experimental feeding, the animals were sacrificed by amputation of the carotid artery, without anaesthesis, and the aorta, the liver and other organs were taken out carefully.

There were no remarkable differences in net weights of viscera (Heart, lungs and bronchi, kidney, spleen, stomach, intestinal ducts and testis) in each group and *inter se*.

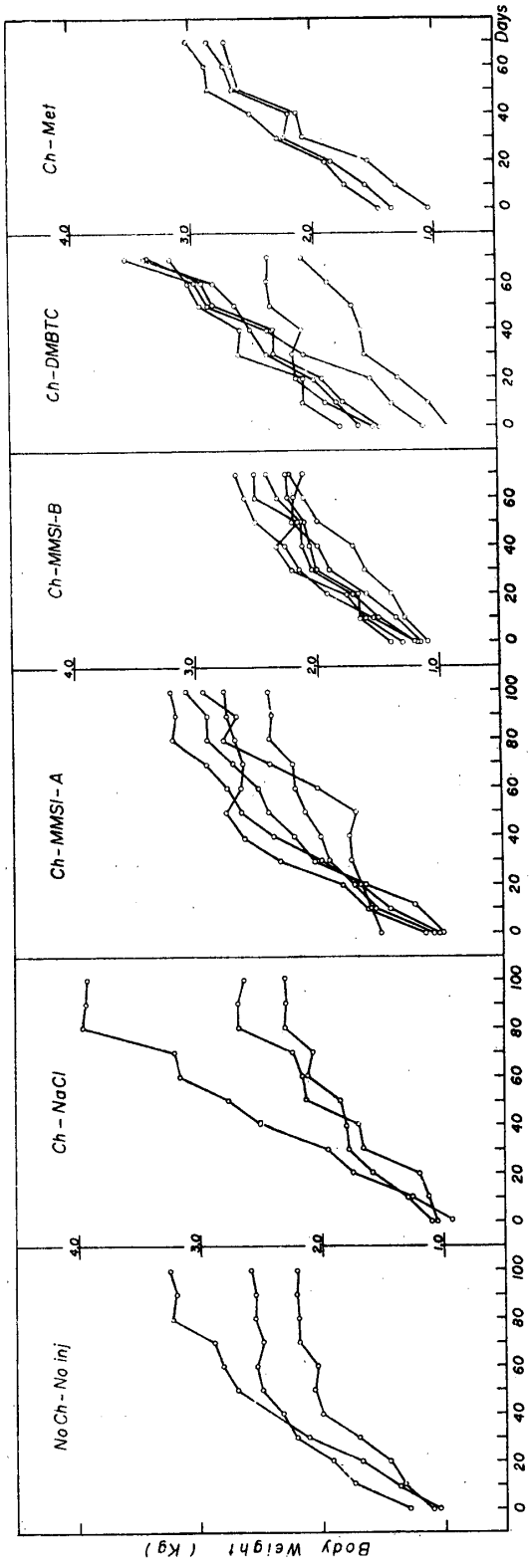

Fig. 1. Growth curves of the rabbits.

The ratio of the liver weight to the whole body weight of the No Ch-No inj group was less than that of the other groups, whereas the ratio of the carcass to the whole body weight of this group was larger. This may be caused by the high accumulation of fat in their adipose tissue of the No Ch-No inj group. These ratios of the liver and the carcass to the whole body weight are

Table 3. Ratios of the carcass and the liver to the whole body weight (%).

Group	Ratio of carcass	Ratio of liver
I No Ch-No inj	83 71 76 77±3.2*	2.73 3.46 2.73 2.97±0.26*
II Ch-NaCl	72 73 60 68±4.5	3 39 4.09 2.97 3.48±0.30
III Ch-MMSI-A	72 71 70 72 72 71±0.4	3.65 3.03 3.35 2.71 3.29 3.34±0.29
IV Ch-MMSI-B	66 73 68 75 72 76 72±1.7	4.10 3.42 3.68 3.15 3.20 2.33 3.31±0.31
V Ch-DMBTC	69 72 67 71 65 63 68±1.2	3.00 3.89 2.93 3.01 2.61 2.69 3.02±0.25
VI Ch-Met	71 69 70 70±0.6	3.18 3.56 3.06 3.26±0.15

* Mean±standard deviation

shown in Table 3.

The hypertrophic alternation of the adrenals was recongnized in the cholesterol-fed groups, but it is not so distinct in comparison of that of the previous experiment (3).

Serum cholesterol: The cholesterol levels in the sera of the total and the free forms were determined at 10 days intarvals. The results are presented in Tables 4-A and 4-B, and illustrated in Fig. 2, plotting of the mean values of each group.

In the Ch-NaCl group, the serum cholesterol of both forms revealed rapid and abrupt increase by the cholesterol feeding, and rose to five times in the total, and four times in the free form to that of the No Ch-No inj group, at the final stage of the experimental period. These two groups of the Ch-MMSI-A

Table 4-A. Total cholesterol in the sera, determined every 10 days.

(mg/*dl*)

Group \ Day	10	20	30	40	50	60	70	80	90	100
I No Ch-No inj	24	30	44	50	40	30	44	50	75	80
	36	30	44	40	30	50	40	48	60	60
	30	35	40	44	40	24	35	48	75	60
	30±3*	32±2	43±1	45±3	36±5	35±3	40±3	49±1	70±5	67±7
II Ch-NaCl	40	60	—	84	188	212	190	200	315	400
	23	40	60	65	121	200	195	250	220	325
	35	80	80	72	150	125	175	280	340	370
	33±5	60±12	70±7	73±6	153±19	179±27	187±6	243±23	258±29	365±23
III Ch-MMSI-A	44	80	96	125	150	170	192	108	124	150
	50	92	102	185	206	250	273	242	238	162
	24	70	92	150	192	240	284	292	285	240
	24	84	70	96	106	122	116	120	138	144
	32	80	50	75	92	102	128	140	138	126
	35±5	81±4	82±10	126±6	149±23	176±30	199±35	180±37	185±32	164±15
IV Ch-MMSI-B	24	75	92	70	100	100	150	—	—	—
	25	60	80	75	80	90	122	—	—	—
	20	43	40	44	70	60	88	—	—	—
	25	80	100	125	135	140	121	—	—	—
	30	50	75	100	110	115	120	—	—	—
	40	63	80	142	132	120	112	—	—	—
	27±3	62±5	77±8	93±8	105±8	104±11	119±2	—	—	—
V Ch-DMBTC	45	55	64	100	135	120	148	—	—	—
	40	60	84	104	152	143	180	—	—	—
	45	70	84	158	145	120	121	—	—	—
	30	55	88	122	100	125	118	—	—	—
	40	77	80	92	130	122	135	—	—	—
	50	84	94	120	125	170	130	—	—	—
	42±3	67±5	82±4	116±9	131±6	133±8	137±9	—	—	—
VI Ch-Met	40	80	125	212	232	225	210	—	—	—
	40	80	136	198	205	235	218	—	—	—
	40	94	130	172	210	220	212	—	—	—
	40±0	85±5	130±3	194±12	216±8	227±4	213±2	—	—	—

* Mean±standard deviation

and the Ch-NaCl showed a parallel increase in their serum cholesterol level, during the first period of the feeding, preceeding the initiation of MMSI injection in the former group. It was noteworthy, that the cholesterol level of the former group increased slowly, and ceased to elevate in the following 30 days, in spite of continuous cholesterol supply. On the 100th day, the difference of the mean value of the total serum cholesterol level between the Ch-MMSI-A group and the Ch-NaCl group was statistically significant. (P: 0.05-0.02). The free cholesterol level, however, did not reveal a significant difference between these two groups.

The Ch-MMSI-B group did not show so rapid elevation of the serum cholesterol level as the Ch-NaCl group. The difference of the mean values between these two groups was significant in the total serum cholesterol, (P:

Table 4-B. Free cholesterol in the sera, determined every 10 days.

(mg/*dl*)

Group \ Day	10	20	30	40	50	60	70	80	90	100
I No Ch-No inj	16	12	25	35	28	15	22	25	35	48
	14	13	30	20	18	25	28	24	30	34
	16	15	20	24	20	14	18	24	34	28
	15±1*	13±1	25±3	26±4	22±3	18±4	23±3	24±0	33±1	37±6
II Ch-NaCl	22	24	—	46	94	104	92	140	184	194
	10	15	34	48	61	112	95	132	110	184
	18	40	36	36	75	90	84	182	170	128
	17±3	26±7	35±1	43±3	77±9	102±5	90±3	151±16	154±23	169±21
III Ch-MMSI-A	12	40	62	70	98	125	60	72	94	114
	15	52	30	42	57	65	72	80	85	92
	15	48	36	80	100	140	80	136	150	140
	10	42	37	40	50	62	56	64	64	76
	16	38	20	44	41	50	72	70	74	64
	14±1	44±3	37±5	55±8	69±13	88±17	68±4	84±11	94±15	97±14
IV Ch-MMSI-B	15	35	68	35	68	72	86	—	—	—
	15	30	40	35	40	60	70	—	—	—
	18	24	20	20	32	55	60	—	—	—
	12	35	40	38	45	80	72	—	—	—
	20	35	48	45	60	70	92	—	—	—
	14	20	40	40	85	65	65	—	—	—
	16±1	30±2	43±6	36±3	55±8	67±4	74±5	—	—	—
V Ch-DMBTC	20	25	24	54	72	74	72	—	—	—
	20	18	42	50	80	71	85	—	—	—
	24	45	55	72	75	60	77	—	—	—
	18	18	35	80	48	66	90	—	—	—
	15	18	49	45	62	59	76	—	—	—
	14	18	48	54	78	80	80	—	—	—
	19±5	24±4	42±5	59±6	69±5	68±4	80±6	—	—	—
VI Ch-Met	25	45	75	84	98	120	110	—	—	—
	30	40	70	104	112	114	124	—	—	—
	30	38	63	98	94	105	140	—	—	—
	28±2	41±2	69±3	95±6	101±5	113±4	125±9	—	—	—

* Mean ± standard deviation

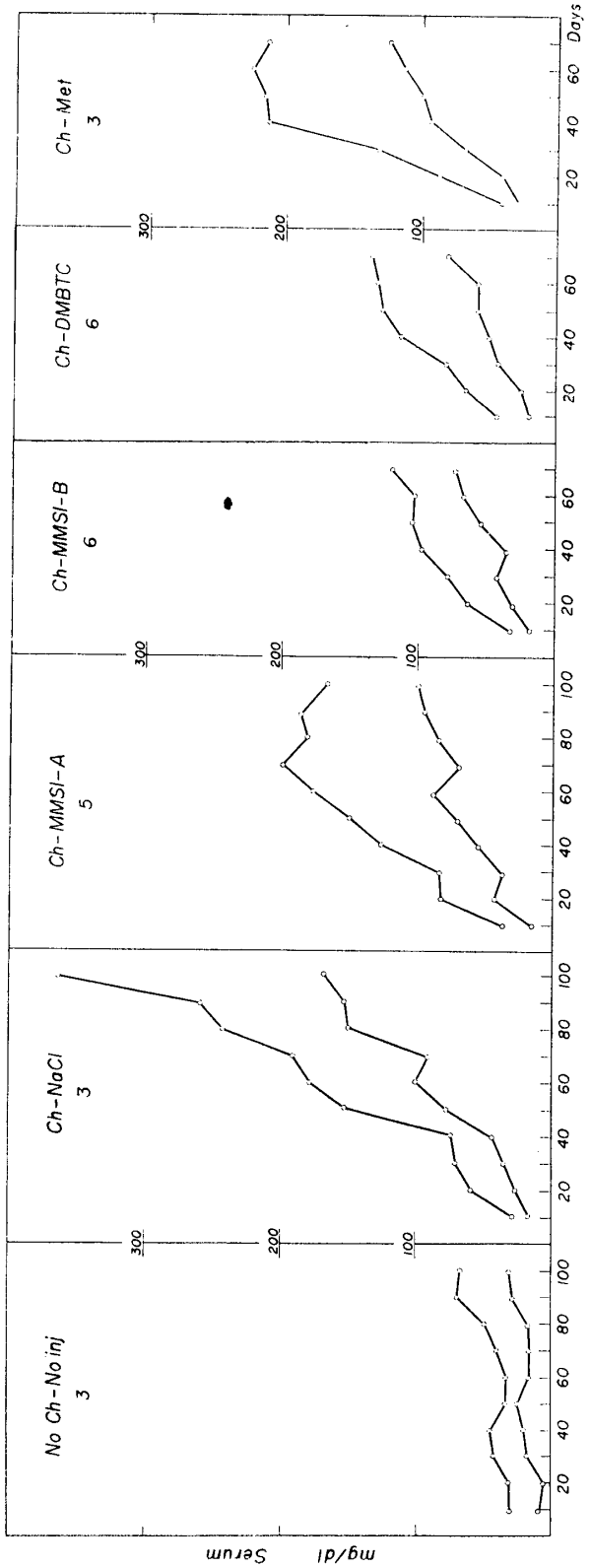

Fig. 2. Changes of serum cholesterol.
Upper curve: Total cholesterol, Lower curve: Free cholesterol.
The mean values of each lot are shown in the Figure.

0.02-0.01), but not significant in the free form.

In the Ch-DMBTC group, the rate of the elevation of serum cholesterol level was slower than that of the Ch-NaCl group, and the level on the 70th day was significantly less than that of the Ch-NaCl group, but slightly higher than that of the Ch-MMSI-B group.

It was of interest that the cholesterol level of this group was remarkably lower than that of the Ch-Met group.

The Ch-Met group showed considerably rapid and abrupt increase of the serum cholesterol, and there was no significant difference between this group and the Ch-NaCl group on the 70th day. The No Ch-No inj group was low and unchanged in both forms of the serum cholesterol level, throughout the experimental period.

Table 5. Lipid content of the liver (%).

Group	Lipid content*
I No Ch-No inj	3.72 4.38 4.00 4.03±0.60**
II Ch-NaCl	3.72 6.11 5.64 5.16±0.73
III Ch-MMSI-A	3.96 4.06 3.23 3.68 3.58 3.70±0.17
IV Ch-MMSI-B	3.08 4.00 5.26 6.12 3.28 4.92 4.44±0.48
V Ch-DMBTC	7.82 5.83 5.12 4.10 6.60 7.74 6.20±0.47
VI Ch-Met	6.74 8.03 8.94 7.90±0.64

* Percentage to fresh matter.
** Mean±standard deviation.

Total lipid in the liver: The content of the total lipid in the liver, extracted with hot ethanol-ether, is shown in Table 5.

It appeared reasonable that the high level of the lipid in the Ch-NaCl group was detected, however, the notable accumulation of the lipid in the liver from the Ch-Met group was an unpredictable fact. These two groups, administrated with MMSI, showed no significant increase in the liver lipid, comparing with the No Ch-No inj group, and less than that of the Ch-NaCl group, but the difference between the Ch-MMSI-B and the Ch-NaCl group was scarcely significant.

In the liver from the Ch-DMBTC group, the content of the lipid was found to be larger than that in the No Ch-No inj group.

Cholesterol content of the aorta: The cholesterol content of the aorta is

Table 6. Cholesterol content of the aorta (%).

Group	Total cholesterol	Free cholesterol
I No Ch-No inj	0.57 0.73 0.63 0.64±0.05**	0.21 0.49 0.27 0.32±0.08**
II Ch-NaCl	3.20 5.70 5.98 4.96±0.89	2.57 3.36 3.40 2.78±0.32
III Ch-MMSI-A	2.18 3.26 3.19 3.12 2.80 2.91±0.26	1.42 1.06 1.52 1.72 1.67 1.48±0.12
IV Ch-MMSI-B	1.86 1.10 1.60 1.51 2.39 2.40 1.81±0.21	0.82 0.65 0.67 0.73 0.88 0.90 0.76±0.05
V Ch-DMBTC	4.45 4.85 3.49 2.57 3.33 3.33 3.67±0.34	1.30 1.55 1.77 1.50 1.35 1.28 1.46±0.08
VI Ch-Met	3.91 2.77 4.32 3.67±0.46	2.47 1.73 1.96 2.05±0.22

* Per fresh matter

** Mean±standard deviation

presented in Table 6.

The cholesterol content of the aorta in cholesterol-fed groups was approximately three times or six times as much as that in the No Ch-No inj group. Among then, the highest content in both forms of cholesterol was found in the Ch-NaCl lot. The cholesterol levels in the MMSI administrated groups were not only considerably lower than that of the Ch-NaCl lot, but also lower than that of the Ch-Met group and the Ch-DMBTC group.

The differences of the mean values, between the Ch-NaCl group and the Ch-Met group or the Ch-DMBTC group, were slightly significant, and so, the cleaning effect of these substances to sweep the cholesterol deposition may be weaker than that of MMSI. These differences in the groups, determined by chemical analysis of cholesterol, could be traced in the histological observation of these arterial tissues.

Serum protein levels: The mean values of serum protein levels of the sera, determined every 10 days, are shown in Fig. 3.

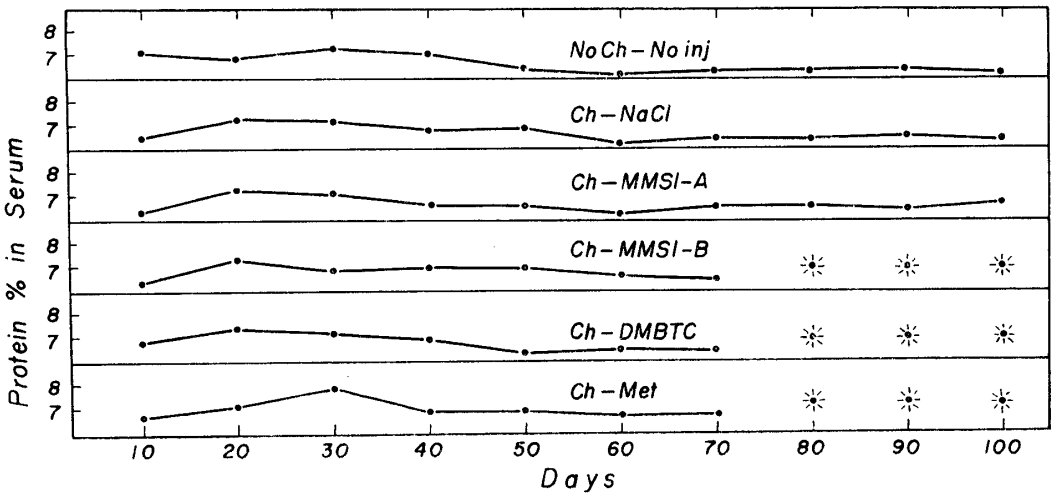

Fig. 3. Serum protein levels.
The mean values are presented in the figure.

There was neither significant nor abnormal change in each lot and *inter se*.

Histological findings: The aortae from all animals were sectioned, stained with Sudan III, and observed histologically under 150× field.

All three casses in the Ch-NaCl group revealed severe pathological alternation of *Tunica intima, i.e.*, atheroma, observed as thick deposition of sudanophilic substance. In the Ch-MMSI-A group and the Ch-MMSI-B group, the aorta was perfectly clear from the sudanophilic deposition in the *Tunica intima*. The erosion, however, was found in some cases of these groups, in spite of the disappearance of atheroma. In the Ch-DMBTC group, sudanophilic depositon of considerable degree was observed in two cases, and the remaining four cases

were free from it, but all cases had erosions of *Tunica intima.* Two cases in the Ch-Met group were atheromous with high depositions of sudanophilic substance, but another case was slightly atheromous.

These results of histological investigations are summarized in Table 7, and illustrated in Plates 1 and 2.

Table 7. Histological changes of the aorta.

Group	Sudanophilic deposition	Erosion of *Tunica intima*
I No Ch-No inj	−	−
	−	±
	−	−
II Ch-NaCl	++	+
	+	±
	++	+
III Ch-MMSI-A	−	+
	−	++
	±	+++
	−	+
	−	++
IV Ch-MMSI-B	−	±
	−	−
	−	+
	−	−
	−	+
	−	+
V Ch-DMBTC	−	+
	−	+
	−	++
	−	+
	++	+
	+	±
VI Ch-Met	+	++
	+	±
	+	−

− : No histological alternation was detectable.

+ : Number of the crosses represents the approximate degree of the sudanophlic deposition or the erosion of the tissues. (*Vid.* the photographes in Plates)

Conclusion and Discussion

As reported in the previous papers (1-3), MMSI has an ability to adjust the excess level of serum cholesterol and sweep the sudanophilic deposition off the aorta, in the animals with hypercholesteremia and atherosclerosis, produced by cholesterol feeding. And the nutritional and pharmacological significance of MMSI, which was administrated preventively as well as therapeutically, for the hypercholesteremia and atherosclerosis, was confirmed in this experiment. On Met, which was the maternal substance of MMSI, this effect was weak, as it had been described in the last paper (3). The analogous substance of MMSI,

DMBTC, with a dimethyl sulfonium pole but free from amino group, may be recognizable as an anti-hypercholesteremic substance, even though it has incomplate ability to sweep out the deposit of the arterial tissue.

It is noteworthy that these two substances of analogous structure show similar activity on hypercholesteremia and atherosclerosis. It may, therefore, suggest that this effect depends upon the specific conformation of dimethyl sulfonium, attached to γ-situation of butyric acid skelton, but the amino group in MMSI is relatively independent for this effect.

However, the mechanism of the anti-hypercholesteremic effect of MMSI remains masked. The authors feel that this anti-cholesteremic action of these substances will produce an attractive problems in the biological transmethylation concept, introduced by Cantoni (7).

Summary

1) Young and male albino rabbits were fed on 0.4 g cholesterol every day. The animals, being fed for 90 days, were intravenously administrated with, *a*) nothing and was not supplied with cholesterol; *b*) isotonic saline; *c*) *s*-methyl methionine sulfonium iodide (MMSI) 20 mg/day, from the 61st experimental day through the experimental period. The others were fed for 60 days and administrated with, *d*) MMSI 20 mg/dose; *e*) γ-dimethyl butyrothetine chloride (DMBTC) 12 mg/dose; *f*) DL-methionine 10 mg/dose; every two days.

2) The serum cholesterol levels of the total and the free form were determined every 10 days. Cholesterol content of the aorta, lipid content of liver and histological examination of the aorta was performed in all animals.

3) The total cholesterol levels of the groups *c* and *d*, which were given MMSI revealed a decrease or a little increase. A reduced elevation of the serum cholesterol was also observed in the group *d* with DMBTC administration; the anti-cholesteremic effect was hardly recongnized in the methionine group (*f*).

4) The arterial tissues (*Aorta descendans thoracica* and *A. abdominalis*), obtained from the groups *c* and *d*, were clean from any sudanophilic substance.

In two of the six animals, atheromous lesions and similar change was found in two of the three cases of the group *f*. In the lot *b*, all animals showed an atheroma in their aorta.

5) These data of cholesterol determination of serum and aorta, and histological observation of the aorta indicate that MMSI has a preventive and therapeutic action for hypercholesteremia and atherosclerosis, caused by cholesterol feeding. Similar action was also found in DMBTC.

Acknowledgement

The authors with to acknowledge their indebtness to Mr. MURAI of The Akita-Yuri Hospital, for his valuable discussion on this study.

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Plate 1.

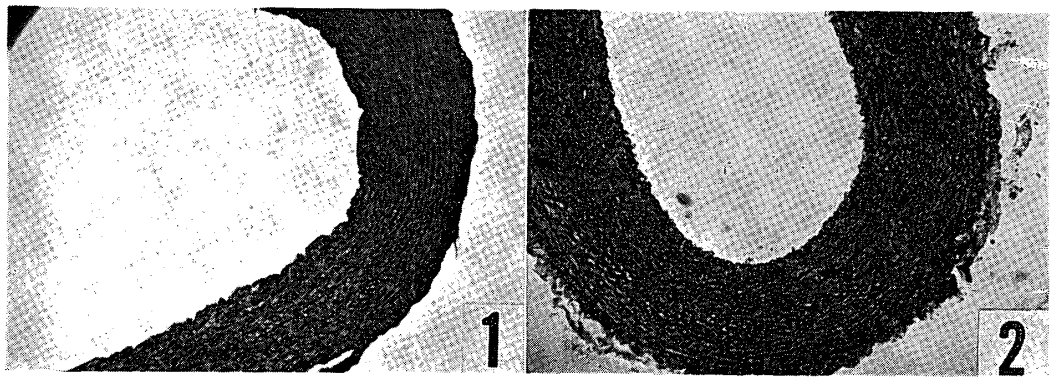
Explanation of the Figures

Histological sections of the aortae (*Aorta desendans thoracica* and *A. abdominalis*) from the rabbits. Stained with Sudan III. 20 μ slices freezed. 150× in the original figures.

Figs. 1 & 2. The No Ch-No inj group. Animal No 1 & 2.
Neither sudanophilic deposition nor erosion on *Tunica intima* is detectable.

Figs. 3 & 4. The Ch-NaCl group. Animal No. 5 & 6.
Representative atherosclerosis with thick deposition of sudanophilic deposition is detected.

Figs. 5 & 6. The Ch-MMSI group. Animal No. 8 & 9.
Sudanophilic deposition on *Tunica intima* is perfectly disappeared, but the erosion of the tissue is distinctly observed in the former case.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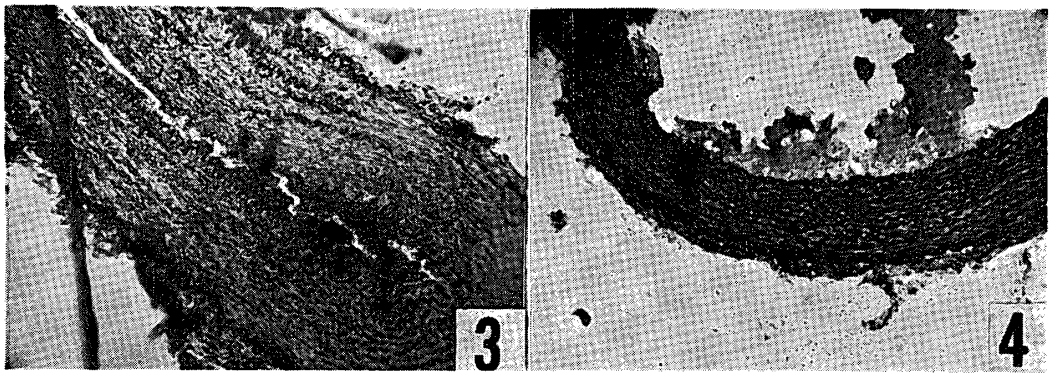
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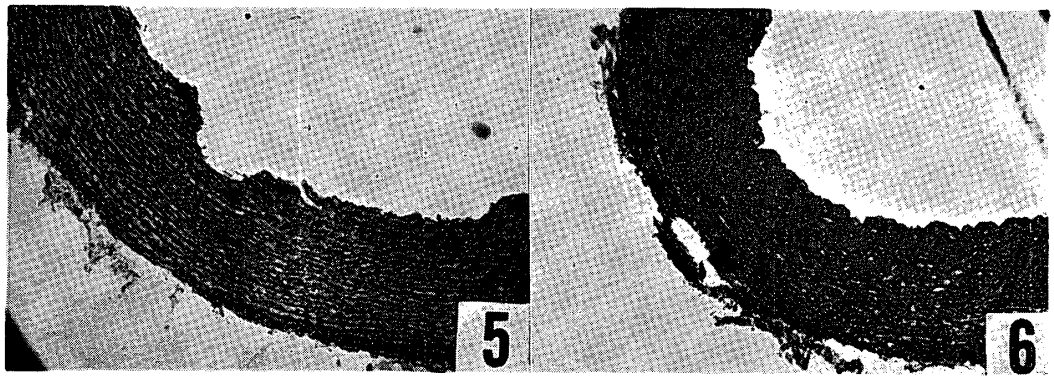
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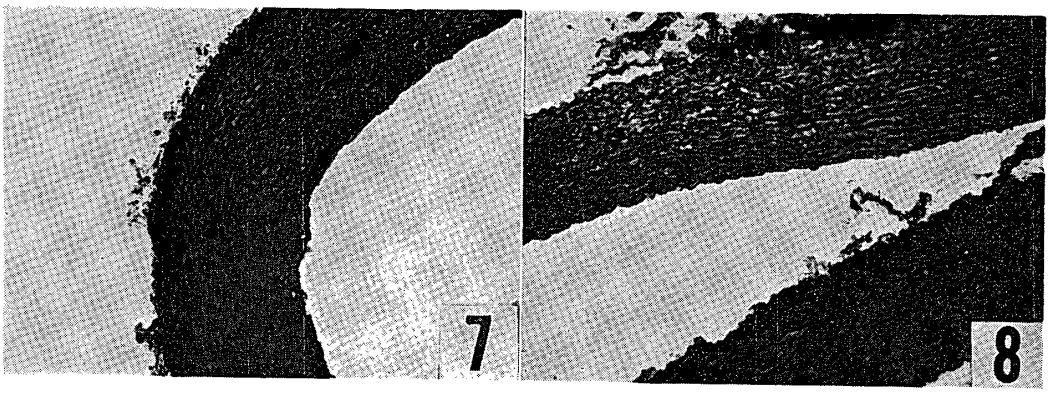
Plate 2.

Explanation of the Figures

Figs. 7 & 8. The Ch-MMSI-B group. Animal No. 14 & 17.
There is no sudanophilic deposition on *Tunica intima*, but slight erosions are detectable in both cases.

Figs. 9 & 10. The Ch-DMBTC group. Animal No. 22 & 23.
Some specks of sudanophilic substances are detectable, slight erosion are also, in both cases.

Figs. 11 & 12. The Ch-Met group. Animal No. 24 & 26.
Thick but partial depositions of sudanophilic substance is detectable in the former case, and massive erosion of *Tunica intima* is observed in the later case.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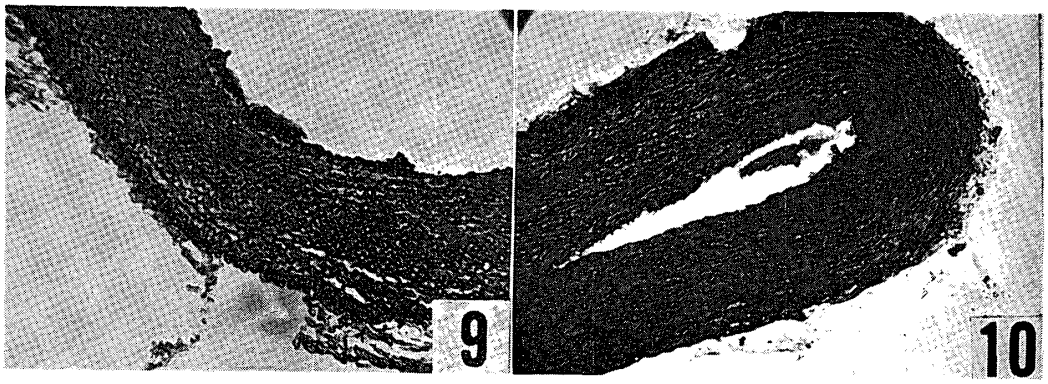
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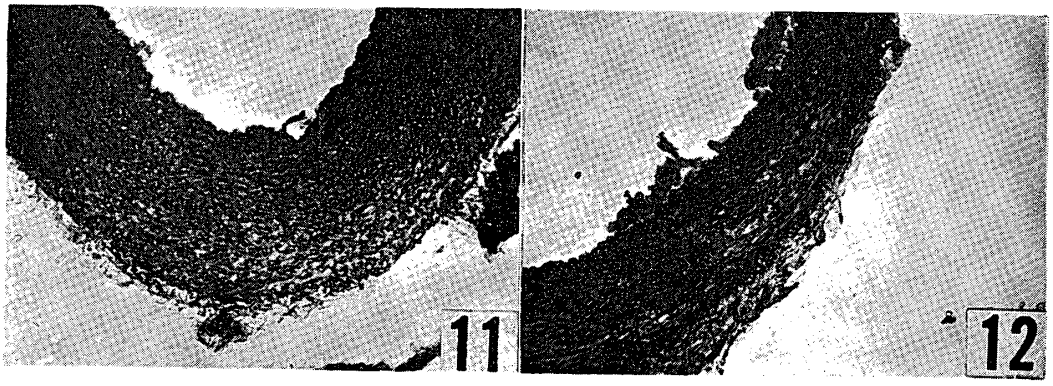
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