

from *B. abortus*, a non-ubiquitous Gram-negative organism, is not cytotoxic *in vitro* unless macrophage donors have first been sensitized by infection with live *B. abortus*.

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### Prevention by Methionine Feeding of Atherosclerosis and Thrombosis in Hyperlipemic Rats.\* (30802)

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It has been observed in species such as rat (1,2), fowl(3,4) and man(5,6) that the serum cholesterol level can be markedly decreased by increasing the dietary level of protein. As a result, it was possible to suppress atherosclerotic lesions in chicken by feeding high protein diets(3) and it may be that the intake of animal protein also accounts for unexplained variances in the death rate of man from atherosclerosis(7). This effect of proteins could be ascribed to their content in sulfur amino acids, since methionine has been shown to exert hypocholesterolemic effects in the rat(1,2) and the monkey(9), and even to inhibit atherosclerosis in the latter(9).

A recent study from this laboratory(10) reported that in hooded rats fed certain hyperlipemic diets a marked reduction in the cholesterolemia, the degree of atherosclerosis and the incidence of disseminated thrombosis could be obtained by increasing the dietary level of casein. The purpose of the present experiments was to investigate whether this preventive effect of casein could be attributed to its methionine content. The effect of this amino acid was compared with that of others and also of choline, which is known to be

as effective as methionine in preventing hepatic lipidosis and necrosis(11). Although no definite relationship appears to have been established between fatty infiltration of the liver and atherosclerosis, rats, like other species, exhibit marked hepatic fatty changes when fed high-fat diets to produce early lesions of atherosclerosis.

*Materials and methods.* A total of 132 male hooded rats (Quebec Breeding Farm, St. Eustache, Qué.) with an initial body weight of 130-140 g were utilized for this study. At the beginning of the experiment each group comprised 12 rats, except Group 1, which contained 24 rats. When the first experiment had been terminated, Groups 1, 2 and 6 were repeated under the same conditions with 12 rats per group and the results listed in the Table constitute the data obtained from both sets of observations. For various reasons, several animals were eliminated in the course of these experiments and the number of animals actually utilized in each group is listed in the Table.

The rats were housed 6 per cage and given, *ad libitum*, tap water alone or, from time to time when deemed necessary, tap water to which was added 187.5 mg per 1,000 ml of oxytetracycline to prevent pulmonary infections.

The basic diet given as such only to Group

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TABLE I. Comparative Effects of Certain Amino Acids and Choline on the Production of Thrombosis and Atherosclerosis in the Rat.

Group	No. of animals	Substances added to the diet	Cholesterol* (mg %)		Thrombosis		Fatty streaks		Survival* (days)	Mortality (%)
			9th wk	20th wk	Incidence (%)	Severity (0-3)	Incidence (%)	Severity (0-3)		
1	29	None	247 ± 15	959 ± 166	48	1.3	88	2.0	159 ± 6	62
2	21	Methionine	239 ± 37	475 ± 76	14	.4	38	.9	180 ± 1	19
3	10	Tryptophan	346 ± 63	1081 ± 123	60	1.5	90	2.6	167 ± 5	80
4	10	Histidine	218 ± 23	1227 ± 363	80	1.7	90	1.6	156 ± 6	80
5	10	Cystine	244 ± 24	1332 ± 224	70	1.5	20	.4	144 ± 7	80
6	21	Choline	275 ± 31	812 ± 141	38	.8	86	2.0	169 ± 5	62
7	11	Cystine-choline	269 ± 19	798 ± 234	27	.5	54	1.0	171 ± 5	36

\* Mean ± S.E.

Basic diet (wt %): butter 43, casein 10, cellulose 24, cholesterol 5, salt mixture 4, sodium cholate 2, sucrose 10, vitamin mixture 2. Choline (1 g) was added at the expense of cellulose, and amino acids (1 g) at the expense of casein, per 100 g diet.

Comparison of Group 2 vs 1. Serum cholesterol (20th wk)  $P < .05$ . Survival  $P < .01$ .

Duration of experiment = 26 wk.

1 was composed (weight %) of butter 43, casein 10, cellulose (Alphacel, Nutritional Biochemicals Co., Cleveland, Ohio) 24, cholesterol 5, salt mixture "Wesson" (Nutritional Biochemicals Co.) 4, sodium cholate 2, sucrose 10 and vitamin mixture (Nutritional Biochemicals Co.) 2. The amino acids given to the animals of Groups 2, 3, 4, 5 and 7 as indicated in the Tables were added to the basic diet at the expense of casein, at the level of 1 g per 100 g of diet. Choline was added, 1 g per 100 g, to the diet of Groups 6 and 7, at the expense of cellulose. All the diets were fed *ad libitum*. After 9 and 20 weeks of dietary feeding 1.5 ml of blood was removed from the jugular vein, under ether anesthesia, after 18 hours' fasting, for serum total cholesterol determination, performed on each rat in duplicate, by the Trinder method(12).

After 26 weeks the survivors were killed with chloroform and most of the organs were examined under a stereoscopic microscope, particularly the aorta after being opened longitudinally. All hearts, aortae, kidneys and livers were examined histologically, the hearts being stained with the periodic-acid-Schiff technic for easy recognition of the thrombi, and the other organs with hematoxylin-phloxine-saffron. Each heart was sectioned at 3 different levels and the severity of the thrombosis graded in terms of a scale of 0 to 3 according to the extent and the number of thrombi. Gradation of the aortic fatty streaks was done solely under stereoscopic microscope examination.

*Results.* In all the groups, most of the animals that died in the course of the study presented at autopsy some tiny or large white patches on their heart surface, frequently surrounded by small foci of hemorrhage. At histology, one large or several small areas of cardiac necrosis were found, surrounded and infiltrated by inflammatory cells. These lesions were apparently due to the presence of thrombi located in the small or large coronary arteries and/or even in the cardiac cavities as reported in detail elsewhere(10,12,13). The fatty streaks, seen in the aorta under stereoscopic microscope examination, consisted of several layers of foam cells on the

intimal surface, covered by endothelial cells with little fibrosis(10,12).

As compared with Group 1 (Table I) fed the basic diet, the animals whose diet was supplemented with methionine (Group 2) presented a significantly reduced cholesterol level at 20 weeks ( $P < 0.05$ ) and a significantly prolonged survival time ( $P < 0.01$ ). In addition, the incidence and severity of thrombosis and of aortic fatty streaks as well as the mortality rate was much lower in this group than in the controls. In contrast to this, addition to the diet of tryptophan (Group 3), histidine (Group 4) or of cystine (Group 5) did not result in a reduction of the serum cholesterol, of the mortality rate, or of the production of thrombosis but, rather, in a slight aggravation of all 3 factors. The incidence of the fatty streaks was approximately the same in Groups 3 and 4 as in Group 1, but was much lower in Group 5, where cystine supplements were given. The lesions, survival time, mortality and cholesterolemia of Group 6 fed choline were quite comparable to those of the controls, except perhaps for thrombosis, the incidence and the severity of which appeared to be milder, although probably not significantly so. Finally, as compared with Group 1, the combined addition to the diet of choline and cystine (Group 7) reduced mortality rate, incidence and the severity of thrombosis and of fatty streaks although it did not significantly affect the serum cholesterol and survival time.

*Discussion.* In the hooded rat, addition of methionine to a hyperlipemic diet afforded marked protection against the production of hypercholesterolemia and also of thrombosis and early lesions of atherosclerosis. These results confirm those reported by others, not only concerning the serum cholesterol in several species(1-8), but also with regard to atherosclerosis in the chicken(3) and the monkey(9); in addition, they extend previous observations to include a thrombotic phenomenon. Although all the amino acids of casein have not been tested in this respect, results of the present experiment indicate that the protective effect of this protein on thrombotic and atherosclerotic lesions in the rat(10) is probably due to its methionine con-

tent. A surprising finding is that neither cystine, another sulfur amino acid, nor choline, which is known to exert effects that are similar to those of methionine on hepatic lipidosis and cirrhosis(11,14), could when added singly to the diet, reproduce the remarkable protection afforded by methionine. However, cystine was able markedly to lower the incidence of atherosclerotic lesions and choline did exert some effect on the production of thrombosis. Therefore, either these substances were not utilized at a proper dosage in the present experiments, they affect different phenomena, or their effect is similar to that reported for hepatic injury(15); whereas, methionine alone is capable of preventing the lesions, choline seems to act through the mediation of cystine. Under the conditions of the experiment reported here, the production of thrombosis and atherosclerosis could be partly inhibited by addition of choline plus cystine; but this combined treatment did not appear to be as effective as methionine.

*Summary.* In the rat, addition of methionine to a high-fat low-protein diet markedly prevented the production of thrombotic and atherosclerotic lesions and the hypercholesterolemia normally induced by such dietary feeding. This marked protective effect of methionine could not be reproduced by other amino acids, such as tryptophan, histidine and cystine, or by choline. Combined addition to the diet of cystine and choline afforded some protection, but was not as effective as methionine.

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### Teratogenic Effect of Chlormadinone Acetate in Mice and Rabbits. (30803)

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Among steroids, glucocorticoids(1,2) and estrogens(3,4) are known to induce fetal abnormality, such as cleft palate and open eye, when administered to pregnant animals in large amounts. For progestogens such teratogenicity has not been reported, though some of them are known to cause masculinization of the female fetuses when administered during pregnancy(5-8). However, it is noteworthy that progesterone showed an antimitotic effect on the cultured cells or mammalian eggs(9-11). Hence, a study was undertaken to ascertain whether or not progestogens, remedies commonly used for the therapy of threatened abortions or as oral contraceptives, administered at various stages of pregnancy produce any fetal malformation other than female pseudohermaphroditism.

**Materials and methods.** We used for our experiments 3 groups of colony bred stock animals: Japanese ddS mice from Aburahi Farm, Shionogi & Co., Ltd., Aburahi, Shiga Prefecture; CF#1 mice maintained at this laboratory; and Japanese albino rabbits purchased from Funabashi Farm, Funabashi, Chiba Prefecture. Animals were kept on a diet of pellets: ddS mice on those prepared by Aburahi Farm (crude protein, 23.7%; crude fat, 6.7%; carbohydrate, 49.6%; total

ash, 3.3%); CF#1 mice on N.M.F. pellets supplied by Oriental Co., Tokyo (crude protein, 26.5%; crude fat, 6.1%; crude fiber, 4.1%; N.F.E., 49.8%; total ash, 6.5%); and albino rabbits on those supplied by Funabashi Farm (crude protein, 24.9%; crude fat, 4.1%; crude fiber, 17.0%; N.F.E., 43.3%; total ash, 5.6%). In addition, fresh tap water was given *ad libitum*.

Suspension of chlormadinone acetate (CA), of norethisterone (NE), and of norethynodrel (ND) in 0.5% sodium carboxymethyl cellulose (CMC) aqueous solution was prepared at various concentrations.† In mice nulliparous females at 12 to 16 weeks of age were used and the day when the vaginal plug was found was designated as day 0 of gestation. The suspension was given at 10 ml/kg of body weight by an esophageal tube once a day during day 8-15, 14-17 or 8-17 of gestation. In rabbits females at 8 to 10 months of age were used and the day when copulation was observed was described as day 0. The suspension was given at 1 ml/kg by a gastric tube daily from day 8 to day 20 of gestation. Sodium carboxymethyl cellulose solution without the tested compound was given to the control animals in the same way as in the respective experiment. Mice were sacri-

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† NE and ND contain mestranol as an additive at the rate of 1 and 2% respectively. The CMC aqueous solution contains Tween 80 at 0.5%.