1956

Investigations of Methyl Donors and Linseed Oil Meal in Alleviating Selenium Poisoning

Eriks Leitis

INVESTIGATIONS OF METHYL DONORS AND LINSEED OIL MEAL IN ALLEVIATING SELENIUM POISONING

by

Eriks Leitis

A thesis submitted
in partial fulfillment of the requirements for the
degree of Master of Science at South Dakota
State College of Agriculture
and Mechanic Arts

May 1956

INVESTIGATIONS OF METHYL DONORS AND LINSEED OIL MEAL IN ALLEVIATING SELENIUM POISONING

This thesis is approved as a creditable, independent investigation by a candidate for the degree, Master of Science, and acceptable as meeting the thesis requirements for this degree; but without implying that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Thesis Advisor

Head of the Major Department

ACKNOWLEDGMENT

The author wishes to express his appreciation to Dr. O. E. Olson for helpful suggestions and assistance in organizing this work, to Mr. Kenneth Schneider for the selenium analysis and to Mrs. Lavona Snodgress for typing the thesis. The author further wishes to acknowledge that this work was made possible by a research grant (A-813) from the National Institute of Arthritis and Metabolic diseases, National Institutes of Health, Public Health Service.

TABLE OF CONTENTS

	Page
INTRODUCTION	1
STUDIES WITH METHIONINE AND RELATED COMPOUNDS	
EXPERIMENTAL	5
RESULTS	7
DISCUSSION	21
SUMMARY	23
STUDIES WITH LINSEED OIL MEAL	
EXPERIMENTAL AND RESULTS	24
DISCUSSION	34
SUMMARY	36
LITERATURE CITED	37

LIST OF TABLES

	Page
Table I. Basal diet used in studies with methionine and related compounds	5
Table II. The effect of DL-methionine on the toxicity of a naturally seleniferous diet (wheat)	18
Table III. Composition of basal diet used in linseed oil meal studies	24
Table IV. Absolute ethanol in the removal of activity from Fraction II	28
Table V. Absolute methanol in the removal of activity from Fraction II	30
Table VI. Ether-absolute methanol in the removal of activity from Fraction III	33

LIST OF FIGURES

Page

Figure 1. Effect of various levels of DL-methionine on average daily gain in weight, on liver size and on survival of rats on non-seleniferous and seleniferous diets 8

Figure 2. Effect of various levels of choline chloride on average daily gain in weight, on liver size and on survival of rats on non-seleniferous and seleniferous diets 10

Figure 3. Effect of various levels of betaine on average daily gain in weight, on liver size and on survival of rats on non-seleniferous and seleniferous diets 12

Figure 4. Effect of various levels of DL-homocystine on average daily gain in weight, on liver size and on survival of rats on non-seleniferous and seleniferous diets 14

Figure 5. Effect of various levels of L-methionine on average daily gain in weight, on liver size and on survival of rats on non-seleniferous and seleniferous diets 16

Figure 6. Effect of various levels of different supplements on the selenium content of livers of rats on seleniferous diets 19

Figure 7. Preliminary extraction of linseed oil meal according to method described by Halverson, Hendricks and Olson (17) . 25

Figure 8. Schematic summary of fractionation of Fraction II with ethanol 27

Figure 9. Schematic summary of methanol extraction and subsequent fractionation of Fraction II 32

INTRODUCTION

The problem of selenium poisoning has been extensively reviewed by Moxon (1), Moxon and Rhian (2), and Trelease and Beath (3). Although a considerable amount of work has been done on this problem, the mechanism of the toxic action of selenium is not understood. This work was undertaken as part of an effort to determine the nature of the protective effect of certain substances against selenium poisoning, in that this might aid in understanding its toxic action.

Moxon (1) reported that proteins minimized the severity of selenium poisoning and others have reported similar findings (4, 5, 6, 7, 8.) Some work discussed by Trelease and Beath (3), has indicated that selenium and sulfur metabolism by living organisms may follow similar routes. In view of this and the protective effect of proteins, methionine as a possible selenium antagonist has received considerable attention, and much work with albino rats on this problem has been reported as discussed below.

Smith (6) showed that the effect of feeding naturally occuring selenium to albino rats depends on dietary factors and that the extent of damage is correlated with the percent of protein in the diets. He also concluded that it is the protein-selenium ratio rather than the level of selenium intake that influences selenium toxicity most.

Lewis, Schultz and Gortner (7) found that the addition of methionine to diets containing 6 percent protein in the form of casein increased the resistance of rats to the toxic effects of 25, 35, and 50 ppm of selenium in the form of sodium selenite. The addition of cystine, however, had no effect. On the other hand both methionine and cystine

were equally effective in increasing growth when added to the control group containing 6 percent casein. These authors suggested that methionine might possibly function as a source of methyl groups in the detoxification of selenium. Nevertheless a comparison between a high protein diet (30 percent casein) and low protein diet (6 percent casein) supplemented with methionine to give the same percent of methionine as the high protein diet negated methionine as the only factor in protein supplements responsible for the diminished toxicity of selenium.

Smith and Stohlman (8) reported that lysine and methionine at levels of 1.7 percent and 0.8 percent respectively were ineffective in preventing poisoning caused by naturally occuring wheat selenium at a level of 10 p. p. m. Further they found that methionine and cystine when fed simultaneously at levels of 0.5 and 1.0 percent respectively were ineffective in preventing selenium poisoning caused by sodium selenite at a level of 15 p.p.m. of selenium in a diet containing 4 percent casein.

Sellers, You, and Lucas (9) showed that methionine at a level of 0.5 percent gave protection against selenium poisoning on a diet which was adequate for normal growth and which contained 20 p.p.m. selenium as sodium selenite but only in the presence of α-tocopherol. They also reported that choline chloride (0.15 percent) afforded slight protection while cystine (0.4 percent) was ineffective.

Klug and Harshfield (10) found that 2 percent of methionine was not effective in preventing symptoms of selenium poisoning in 250 gram rats on a naturally seleniferous diet (23 p.p.m. selenium). Later, Klug et al. (11) reported that 2 percent of methionine, alone or with 0.05 percent added α-tocopherol, did not afford protection against 23 p.p.m.

of naturally occurring selenium. In view of the failure of the great excess of methionine to prevent selenium poisoning, the authors discarded the idea of a metabolic antagonism between selenium and the sulfur of methionine or the in vivo inactivation of methionine by selenium.

Unpublished work, which was undertaken at the South Dakota Agricultural Experiment Station after Baron and Allison (12) reported increased tissue regeneration caused by a mixture of glycocyamine and methionine, indicated that glycocyamine alone was not effective against selenium in the form of selenite while methionine alone was effective. A mixture of methionine and glycocyamine was more effective than methionine alone. The role of glycocyamine in this latter finding was interpreted as that of a protective agent against the toxicity of methionine itself at the relatively high levels used.

Fels and Cheldelin (13) found that methionine was specific in the detoxification of selenate by yeast and that only the naturally occurring L form was active. DL-homocystine plus a methyl donor such as choline or betaine did not reverse the inhibition caused by selenate.

The conflicting findings concerning the effectiveness of methionine against selenium poisoning were obtained with a variety of diets and under a variety of other experimental conditions. It appeared, therefore, that certain unknown factors or conditions might be necessary for methionine to be active as a protectant. It was decided that a semi-purified diet containing 10 p.p.m. of selenium as sodium selenite and male albino rats weighing about 70 grams should be used for further study of the problem. With such standardized conditions and with a diet of constant and well-defined composition the unknown factors might be determined. Part of the

work discussed here deals with the effectiveness of methionine and related compounds against selenium poisoning under these conditions.

In his studies of several protein supplements, Moxon (14) found that linseed oil meal gave the most consistent protection against selenium poisoning. Halverson, Peterson and Klug (15) reported that flax embryo seemed to be more effective than the flax hull against naturally occurring selenium (10 p.p.m.). Later, Schuchardt, Halverson and Clagett (16) made a similar study and found that both hull and embryo were about equally protective when an inhibitory effect of the hull on the growth of rats was considered.

In 1955, Halverson, Hendrick and Olson (17) reported that the protective principle could be extracted from linseed oil meal with hot 50 percent ethanol-water mixture. Ashing destroyed the activity of the extract. The protective principle was soluble in water and it could not be precipitated by lead, making it unlikely that it was protein in nature. It was active against both inorganic and naturally occurring selenium. Halverson and Hendrick (18) also established that there was no relationship between this factor and the anti-vitamin B_6 principle of linseed oil meal (18). Further studies on the fractionation of linseed oil meal are reported here.

STUDIES WITH METHIONINE AND RELATED COMPOUNDS

EXPERIMENTAL

All of the experiments with methionine and related compounds were carried out using male albino rats of the Sprague-Dawley strain. The animals were put on the experiment when they weighed about 65-75 grams. They were kept on experiment in individual wire bottom cages for about four weeks and received food and water ad libitum. Their weight was re-

Table I

Basal diet used in studies with methionine and related compounds.

Major Constituents		Vitamin mix	
Dracket protein [1]	20%	Thiamine . HCl	0.60 gm.
Lard	3%	Riboflavin	0.60 gm.
Salt Mix 2	3%	Pyridoxine . HCl	0.60 gm.
Solka floc 3	3%	Calcium pantothenate	4.00 gm.
Corn starch	71%	Nicotinic acid	2.00 gm.
		Inositol	100 .00 gm.
Vitamin mix 0.14 g./100 g. diet. [4]		Pteroylglutamic acid	0.20 gm.
		Biotin	0.01 gm.
		2-methyl-1,4-napthoquinone	3.00 gm.
		Para aminobenzoic acid	30.00 gm.
		Vitamin B_{12}	0.002gm.
			144.012gm.

[1]Purified soybean protein
[2]U.S.P. XIV, Nutritional Biochemicals Corporation
[3]A cellulose product of Brown Company
[4]Vitamin A, D and E supplement orally once a week: 600 IU Vitamin A, 85 IU Vitamin D, and 0.8 mg. α-tocopherol.

corded once a week and at the end of the experimental period they were weighed and sacrificed. Their livers were then removed, weighed and their

selenium content for a group as a whole determined. The weights of the livers, expressed as the percent of body weight were used to express liver damage numerically. Average daily gain was calculated for all the animals on an experiment.

A semi-purified diet low in methionine was used in these studies. The composition of this diet is shown in Table I. It should be noted that choline has been omitted from the vitamin supplement.

In the diets containing added methionine or other similar supplements, the weight of supplement replaced an equal weight of starch, other ingredients remaining the same. When selenium was to be fed, it was incorporated into the diets as sodium selenite by dissolving the salt in 70 percent ethanol and sprinkling the solution on the other ingredients. In all experiments the level of selenium was 10 p.p.m. All diets were well mixed and stored at 3°C (± 2°).

RESULTS

DL-Methionine: Although a response to DL-methionine at the 1 and 2 percent level had been shown using a corn type seleniferous diet (unpublished work, South Dakota Agricultural Experiment Station), it was decided that a similar study was necessary with the semi-purified diet. This diet contained no choline and had a calculated methionine content of only 0.20 percent but contained adequate amounts of other essential amino acids and vitamins. Three levels of added DL-methionine were used here: 0.30, 0.80, and 1.80 percent. Both non-seleniferous and seleniferous diets were thus supplemented with the amino acid. The results of this experiment are given in Figure 1.

With rats on the non-seleniferous diet, some stimulation of growth was obtained at the 0.30 percent added DL-methionine level. This could be expected in view of the low methionine content of the basal diet. Less stimulation was obtained at the 0.80 percent level, and at the 1.80 percent level growth was actually retarded below that of the rats on the diet with no added methionine. At no level was the liver:body weight ratio increased over that obtained on the basal diet, and all rats in all groups survived.

On the seleniferous diet, all levels of DL-methionine gave better growth than the basal without added DL-methionine, although the 1.80 percent level was not quite as good as the 0.80 percent level. All levels of DL-methionine also gave better liver:body weight ratios than the basal diet, but at 1.80 percent, the ratio was not quite as good as at 0.80 percent. As to survival, only the 1.80 percent level seemed to prevent deaths.

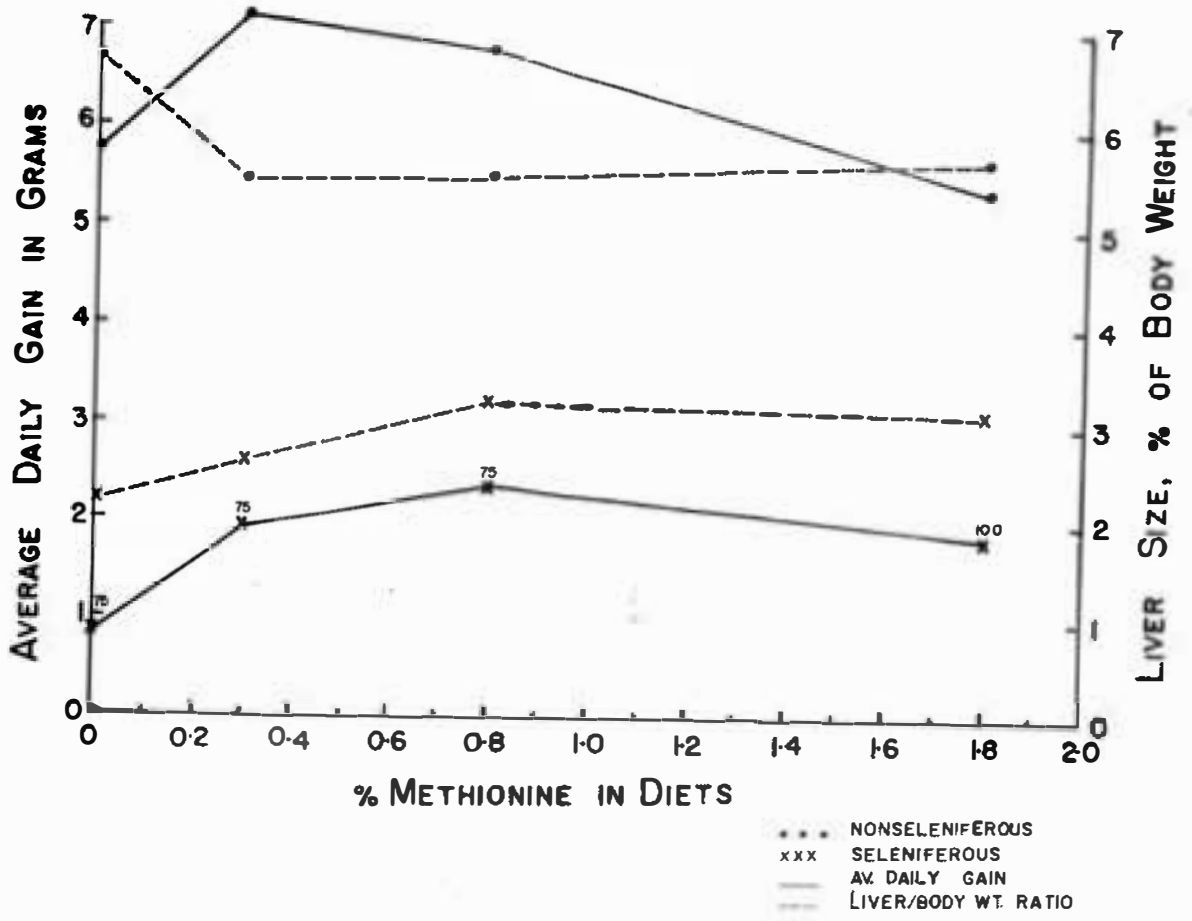

Figure 1. Effects at various levels of DL-methionine on average daily gain in weight, on liver size and on survival of rats on non-seleniferous and seleniferous diets.

There were 8 rats per group with the average initial weight of 62.6 grams. The experiment was terminated at the end of 26 days. On the non-seleniferous diet all animals in all levels survived and on the seleniferous diet the survival is indicated by the small numerals above each point on the average daily gain line.

A point on the graph represents an average value for all rats in a group.

Data for average daily gain indicated some protection at the 1.80 percent level. At this level rats on the seleniferous diet gained better than those on the same diet without added DL- methionine, while for those on the non-seleniferous diet the reverse was true. Considering survival, again it was only at the 1.80 percent level where any effect was apparent. The liver data, however, indicated that the 0.80 percent and possibly the 0.30 percent levels also gave some protection against selenium. Nevertheless, the results were not decisive and did not clearly demonstrate a protective effect.

Choline: Although DL-methionine did not give clearly defined protection, the suggestion of protection, and the earlier results already mentioned, made trials with other methyl donors seem advisable. Lewis, Schults and Gortner (7) have postulated that methyl groups might detoxify selenium, and the finding that dimethyl selenide is exhaled by rats injected with inorganic selenium (20) makes this seem quite possible. Choline chloride, another methyl group donor, was used at levels of 0.10, 0.20, 0.40, and 0.80 percent. It was added to both the seleniferous and non-seleniferous diets and the results are given in Figure 2.

The non-seleniferous diet produced increased growth of rats at the 0.10, 0.20, and 0.40 percent levels. The 0.10 percent level was the best while 0.80 percent gave decreased growth. The liver:body weight ratio showed the largest increase at the 0.10 percent level with all other levels being only slightly more effective than the basal. All rats in all groups survived.

Protection from selenium by choline chloride was more pronounced than by DL-methionine, especially as measured in terms of the liver:body

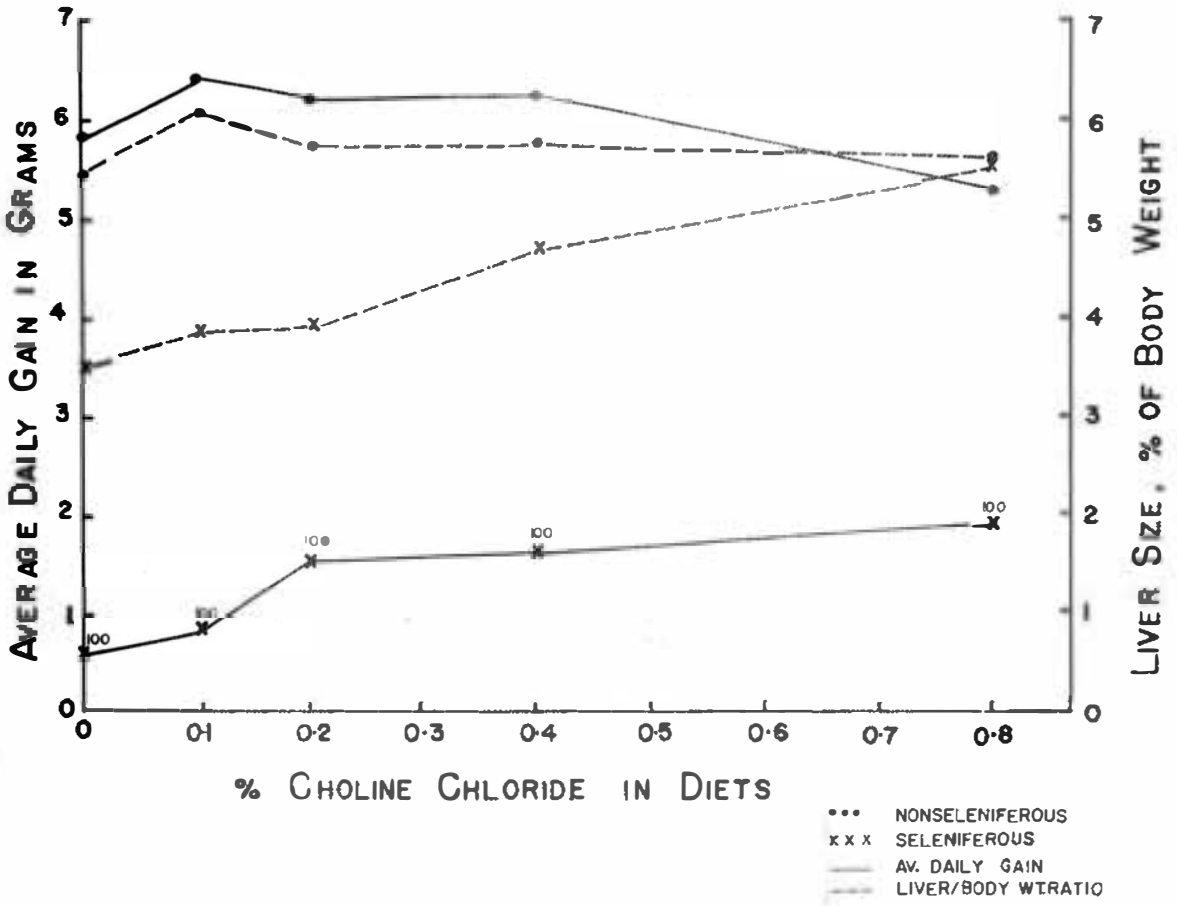

Figure 2. Effects at various levels of choline chloride on average daily gain in weight, on liver size and on survival of rats on non-seleniferous and seleniferous diets.

There were 7 rats per group with the average initial weight at 75.6 grams. The experiment was terminated at the end of 28 days. All animals in all groups of the non-seleniferous and seleniferous diets survived.

A point on the graph represents an average value for all rats in a group.

weight ratio. Protection as expressed by the average daily gain was again apparent only at the highest level.

Betaine: In view of the results with choline chloride it was decided that betaine should be studied next. It was added to both the seleniferous and non-seleniferous diets at 0.084, 0.17, 0.34 and 0.67 percent levels. These levels were used in order to make the number of moles of betaine per unit weight of the diet in each level equal to the number of moles of choline chloride at 0.10, 0.20, 0.40, and 0.80 percent levels respectively. The results of the betaine experiment are shown in Figure 3.

Rats on the non-seleniferous diet showed increased growth at the 0.084, 0.17, and 0.34 percent levels, while at the 0.67 percent level the growth was about the same as for the rats on the diet without added betaine. The stimulation of growth at the 0.17 percent level was better than that obtained at the 0.084 percent level and about the same as that of the 0.34 percent level. The liver:body weight ratios on the 0.17 and 0.34 percent levels were about the same as that on the basal diet, while the 0.084 and 0.67 percent levels decreased the liver:body weight below that of the basal diet. These variations are probably not of significance. All rats in all groups of the non-seleniferous diets survived.

On the seleniferous diet all the levels of betaine produced better growth than the basal, with the 0.084, and 0.17 percent levels being about equally effective. Further increase of the betaine supplement resulted in an additional growth stimulation. All levels gave a better liver:body weight ratio than the basal diet. As concerns the data on survival, all the supplemented levels gave better protection than the basal, but only at the 0.67 percent level was 100 percent survival found. There was apparent-

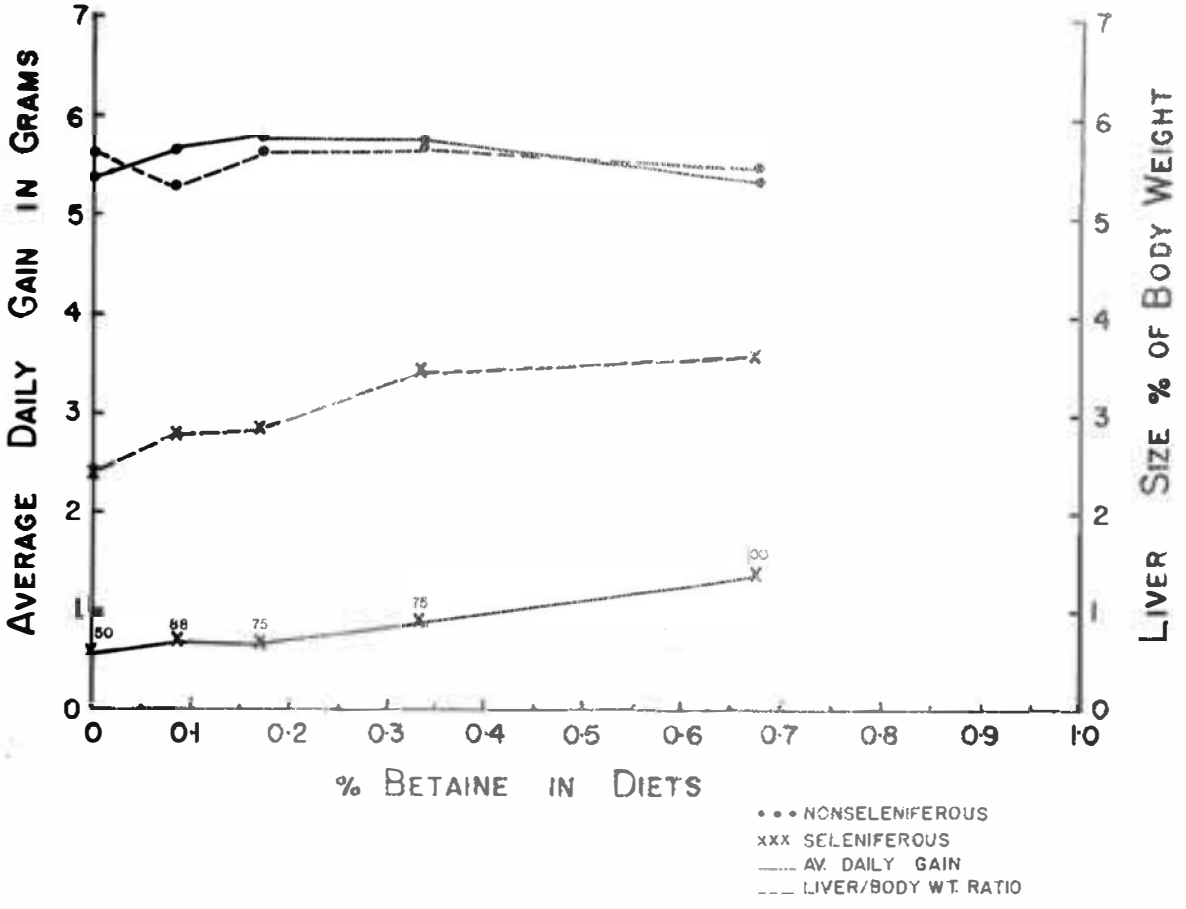

Figure 3. Effects at various levels of betaine on average daily gain in weight, on liver size and on survival of rats on non-seleniferous and seleniferous diets.

There were 8 rats per group with the average initial weight at 67.8 grams. The experiment was terminated at the end of 28 days. On the non-seleniferous diet all animals at all levels survived and on the seleniferous diet the survival is indicated by the small numerals above each point on the average daily gain line.

A point on the graph represents an average value for all rats in a group.

ly some protection from selenium by betaine in this experiment, although it was very slight. There was some indication that higher than 0.67 percent of betaine in this type of diet might be more effective.

DL-Homocystine: To ascertain whether methyl group was responsible for the apparent protection observed with methionine, an experiment with DL-homocystine was undertaken. It was added to the seleniferous and non-seleniferous diets at 0.27, 0.72, and 1.62 percent levels, which correspond on the basis of homocystine content to the 0.30, 0.80, and 1.80 percent DL-methionine added in the earlier experiment. The results of this experiment are given in Figure 4.

Rats on the non-seleniferous diet displayed a steady decrease in growth with increasing amount of homocystine added. Liver:body weight ratios showed a considerable increase at all levels of added DL-homocystine, with the greatest increase at the 0.72 percent level. All rats in all groups survived.

On the seleniferous diet the homocystine supplements caused a decrease of growth at the 0.27 percent level. There was no change in the growth rate at the 0.72 percent level, but a slight increase at the 1.62 percent level. The liver:body weight ratio decreased at the 0.27 and 0.72 percent levels and was increased somewhat at the 1.62 percent level. There was no protection as measured both by the average daily gain and the liver:body weight ratios at the 0.27 percent and 0.72 percent levels. At the 1.62 percent level the average daily gain indicated a slight protection. Increase in the liver:body weight ratio, because of a similar trend in the corresponding control groups, cannot be taken as a sign of protection from selenium. In spite of the largely negative results, it cannot be definite-

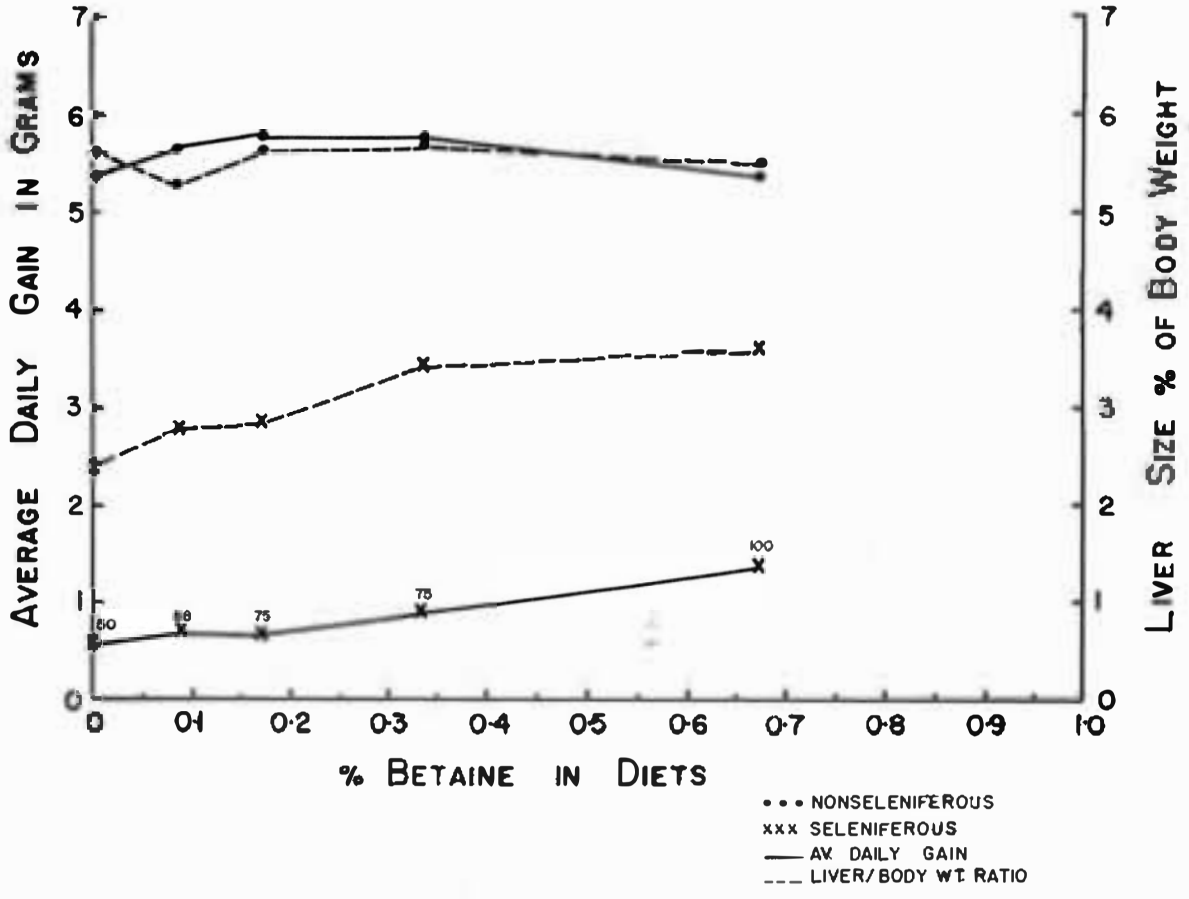

Figure 3. Effects at various levels of betaine on average daily gain in weight, on liver size and on survival of rats on non-seleniferous and seleniferous diets.

There were 8 rats per group with the average initial weight at 67.8 grams. The experiment was terminated at the end of 28 days. On the non-seleniferous diet all animals at all levels survived and on the seleniferous diet the survival is indicated by the small numerals above each point on the average daily gain line.

A point on the graph represents an average value for all rats in a group.

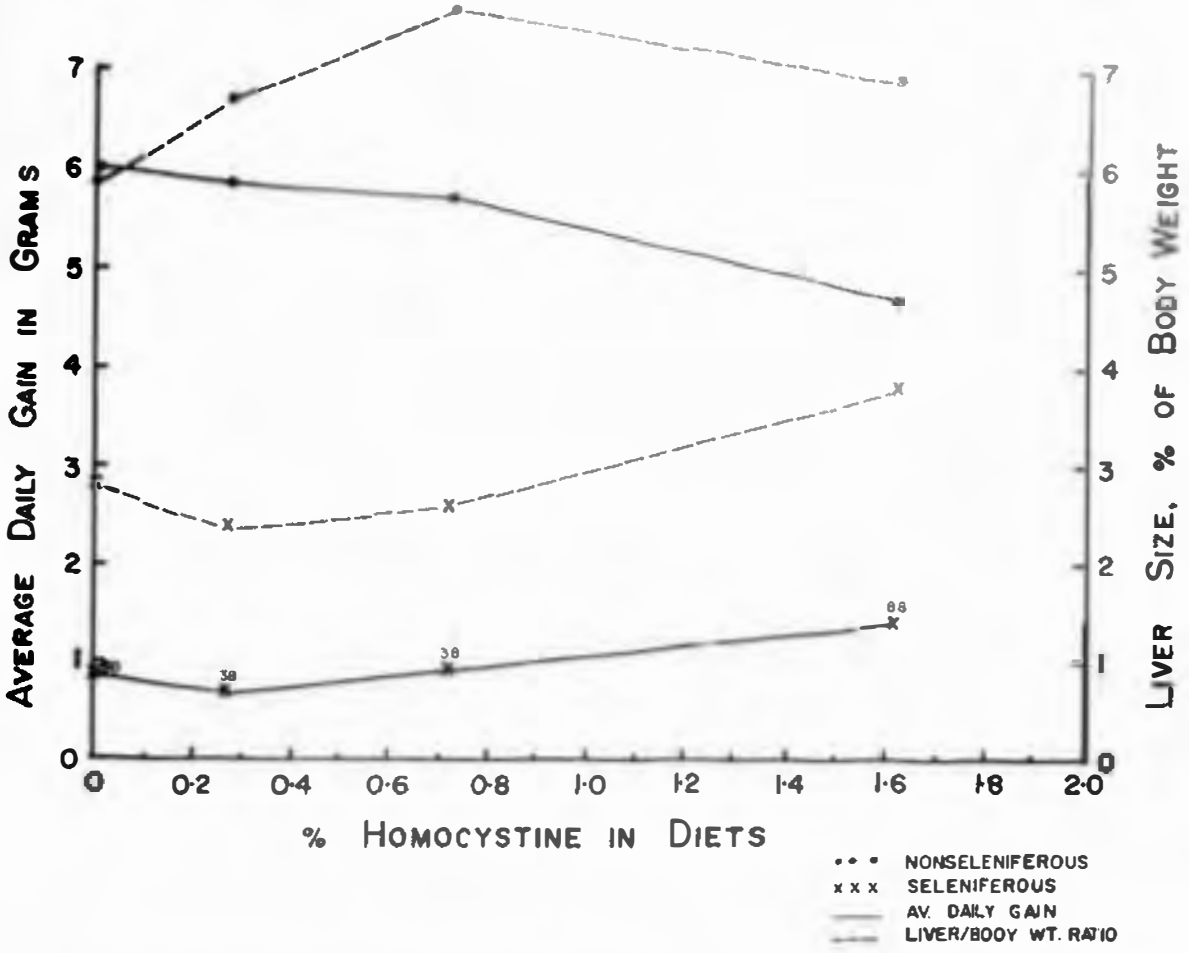

Figure 1. Effects of various levels of DL-homocystine on average daily gain in weight, or liver size and on survival of rats on non-seleniferous and seleniferous diets.

There were 8 rats per group with the average initial weight at 6.2 grams. The experiment was terminated at the end of 29 days. On the non-seleniferous diet all animals at all levels survived and on the seleniferous diet the survival is indicated by the small numerals above each point on the average daily gain line.

A point on the graph represents an average value for all rats in a group.

ly stated that homocystine has no protective effect whatsoever.

L-Methionine: In order to compare the physiological and chemical factors in the protective effect of methionine a study with L-methionine was undertaken. It was added to both the seleniferous and non-seleniferous diets at 0.15, 0.40, and 0.90 percent levels in order to provide the same number of moles of L-methionine as in 0.30, 0.80, and 1.80 percent levels of DL-methionine respectively. The results are given in Figure 5.

In the non-seleniferous diet the added L-methionine produced increased growth of rats at all levels. However, the increase at the 0.90 percent level was less than at either the 0.15 or the 0.40 percent levels which were about equally effective. The liver:body weight ratios showed a slight increase at the 0.15 and the 0.40 percent levels, but decreased somewhat at the 0.90 percent level to below that for the unsupplemented diet. All rats in all groups survived.

The seleniferous diet produced increased growth at all levels of L-methionine, the rate of increase being about the same throughout. The liver:body weight ratio decreased slightly at the 0.15 percent level but increased at the 0.40 and the 0.90 percent levels above that for the non-supplemented level. The survival in the seleniferous diet was 71 percent in all levels.

In view of the similar trend in both the non-seleniferous and the seleniferous diets at the 0.15 and the 0.40 percent levels no protection can be said to be demonstrated at these levels. At the 0.90 percent level, however, some protection as expressed by the average daily gain and liver: body weight ratio was evident.

DL-Methionine and Naturally Seleniferous Wheat: As mentioned earlier here,

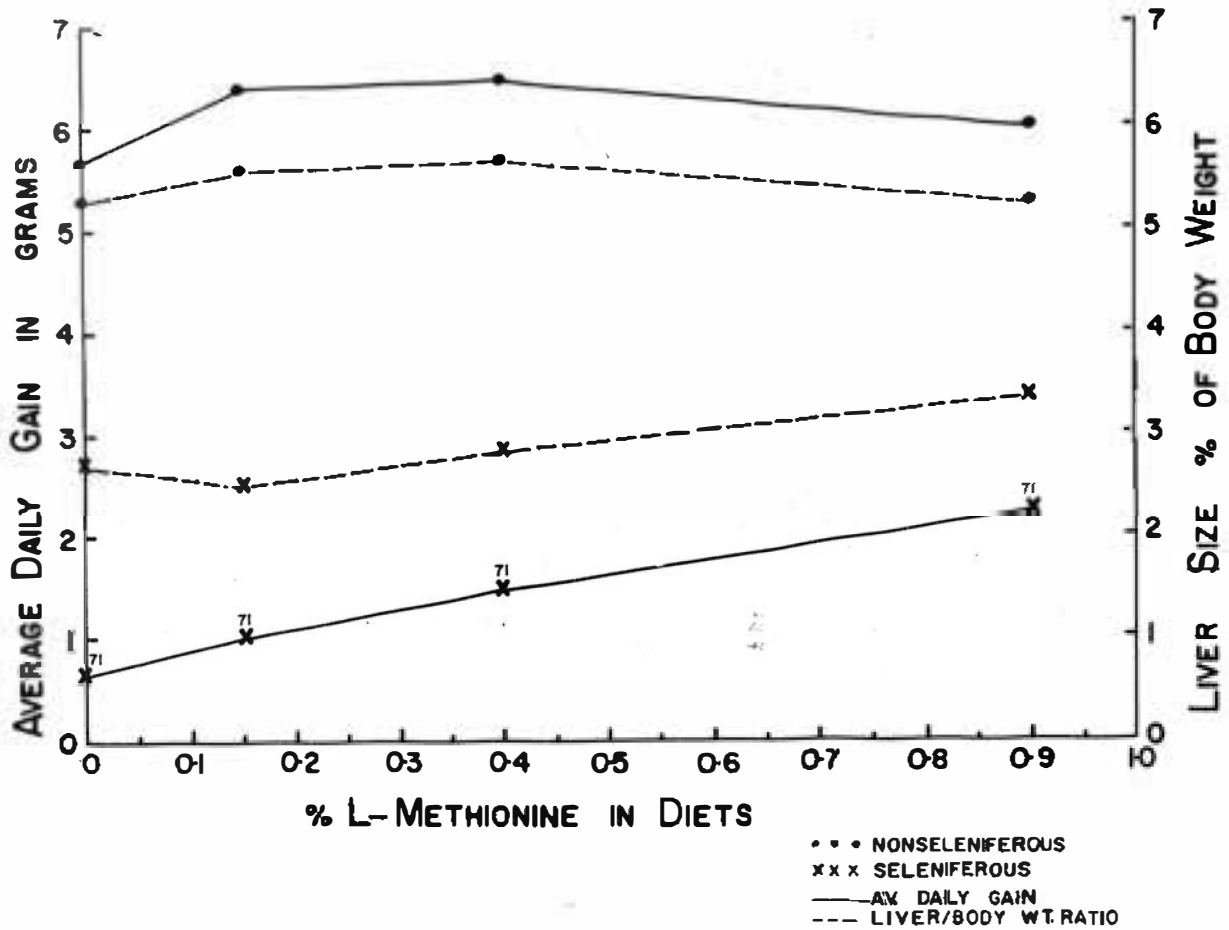

Figure 5. Effects of various levels of L-methionine on average daily gain in weight, on liver size and on survival of rats on non-seleniferous and seleniferous diets.

There were 7 rats per group with the average initial weight of 64.3 grams. The experiment was terminated at the end of 28 days. On the non-seleniferous diet all animals at all levels survived and on the seleniferous diet the survival is indicated by the small numerals above each point on the average daily gain line.

A point on the graph represents an average value for all rats in a group.

DL-methionine had been found to protect against selenium poisoning to a fairly high degree (unpublished data). In these early experiments a corn-casein type diet and sodium selenite were used. On the semi-purified diet used here, the methionine effect was slight. It was decided that the effect of DL-methionine on a wheat type diet as used by Klug et al. (11) should be further investigated. An experiment using seleniferous wheat was therefore undertaken.

The results of the experiment and the composition of the diets used are given in Table II. In the non-seleniferous diet 2 percent of added DL-methionine decreased both the average daily gain and the liver:body weight ratio, although the latter decrease was not of significance. All rats in all groups lived.

In the seleniferous diet, 2.0 percent of added methionine gave an increase in the average daily gain, about 50 percent greater liver:body weight ratio and a much higher survival. Some protection from naturally occurring selenium by DL-methionine at the 2.0 percent level was indicated by all of the criteria used. Although this protective effect was unmistakable, it was again rather small.

Effects of Various Compounds on Selenium in Livers: The livers of all rats except those in the choline chloride and the seleniferous wheat experiments were analyzed for selenium at the conclusion of each experiment and the data are presented in Figure 6. The method of Klein (23) was used for the selenium determination, and the livers were analyzed without drying.

The data in Figure 6 show that even small amounts of a methyl donor produced a large decrease in the liver selenium content but that more than

Table II

The effect of DL-methionine on the toxicity of a naturally seleniferous diet (wheat).

Percent of DL-methionine added to diet[1]	Results on non-seleniferous diets[2]			Results on seleniferous diets[2]		
	Average daily gain	Liver weight	Survival	Average daily gain	Liver weight	Survival
	gm.	% of body weight	%	gm.	% of body weight	%
0	6.14	5.58	100	0.15	2.76	12
2	3.81	5.48	100	0.95	3.35	86

[1] Basal diet as described by Klug et al. (11). Seleniferous wheat was used in an amount to give 10 p.p.m. of selenium in the seleniferous diet. Vitamins A and D were given orally once a week.

[2] All groups of rats composed of eight animals except for the seleniferous diet containg 2.0 percent of added DL-methionine where seven rats were used. Average initial weight of all groups was 66.5 grams. Animals were on experiment for 21 days.

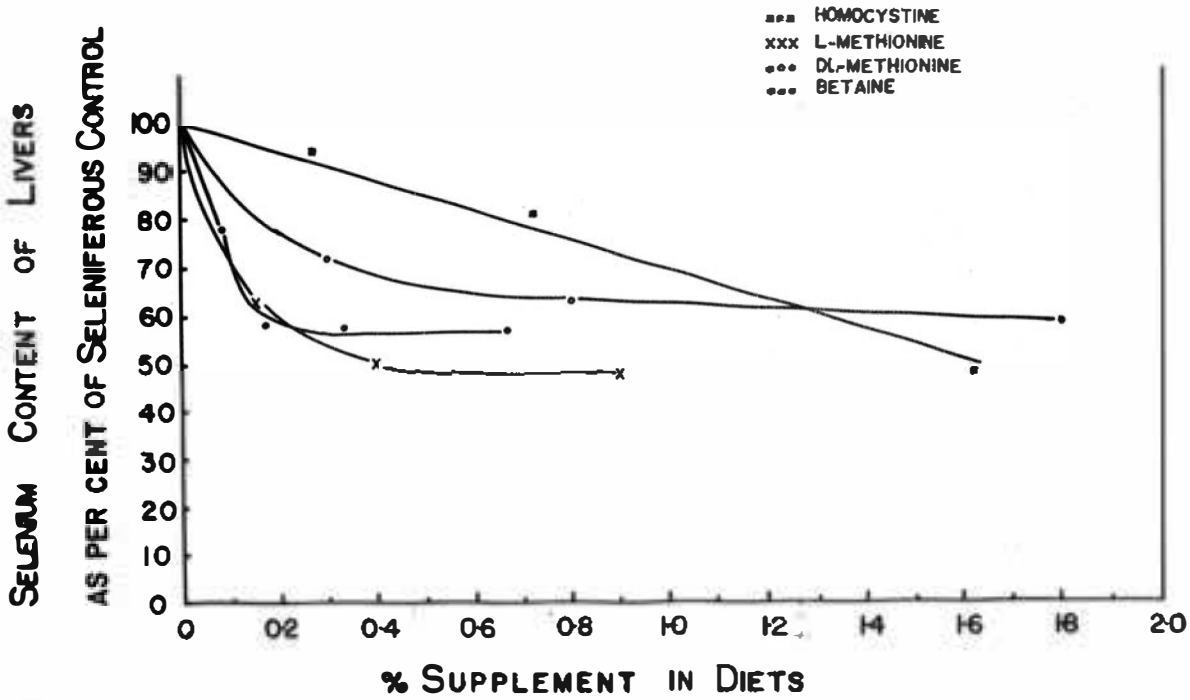

Figure 6. Effect of various levels of different supplements on the average selenium content of livers of rats on seleniferous diets.

about 0.4 percent of any supplement resulted in only a slight further decrease. In contrast to this, the data obtained with DL-homocystine gave a straight line relationship between selenium content of the livers and the amount of DL-homocystine added.

DISCUSSION

It is obvious from the results presented here that none of the methyl donors used gave good protection against selenium poisoning on the semi-synthetic diet. However, there is a certain consistency to the data that does indicate a protective effect of small magnitude.

Considering the growth data for rats on non-seleniferous diets, all of the methyl donors gave increased growth rates at the lower levels with a subsequent decrease at the high level to almost or even below that of the unsupplemented diet. For the rats on seleniferous diets, the growth rates were again increased at the lower levels of supplementation. Except in the case of DL-methionine, they continued to increase even at the highest level. With DL-methionine, however, a protective effect is also evident since the highest level of this compound decreased growth on the non-seleniferous diet to below that of the basal level, while on the seleniferous diet the growth rate was still over twice that of the seleniferous basal.

As to the data for liver:body weight ratios, the different levels of the various methyl donors had no consistent effect on the non-seleniferous diets. On the seleniferous diets, however, the liver:body weight ratios increased with good consistency with increasing methyl donor level.

The data with DL-methionine and L-methionine are of interest because they indicate that both forms of this amino acid are almost identical in their action in either non-seleniferous or seleniferous diets.

The data with the various methyl donors may substantiate the opinion of Lewis, Schultz, and Gortner (7) that methyl groups are active in detoxifying selenium. The results obtained with DL-homocystine may seem to refute this, but they may still be explained on the basis of methyl group

transfer if one accepts the general scheme of metabolic interrelationships in the synthesis of methionine and related compounds as visualized by Stekol (21, page 510). In support of this, it has been reported (22) that dietary homocysteine increases the amount of C^{14} incorporated into choline from formate and serine.

In view of the similar make up of betaine and choline, one might expect protection to about the same extent, but such was not the case here. In this connection, it must be emphasized that the rats receiving choline chloride were about 7 grams heavier at the beginning of the experiment than those receiving betaine. Because of this variation between these two, as well as between other groups, a strictly quantitative comparison between the various supplements cannot be made.

If the selenium content of the livers of rats on seleniferous diets can be taken as a measure of the relative protection against selenium by the various supplements, then the methyl donors are obviously more effective at lower levels than the homocystine. Since the high levels of homocysteine and the methyl donors were about equally effective, there appears to be no deficiency in methyl groups available (from serine and formate) for transfer through homocysteine. The relatively low effectiveness of homocysteine may then be the result of its inefficient conversion to homocysteine.

In conclusion it might be said that although the data do indicate a protective role for methionine and related compounds, this is perhaps of little practical significance. In the first place, the amount of protection is small. Secondly, the levels of the various compounds required to give this slight protection is considerably above what could be considered practical.

SUMMARY

The effects of DL-methionine, L-methionine, choline chloride, betaine and homocystine in alleviating selenium poisoning in rats on semi-purified diets containing sodium selenite were investigated. One experiment using DL-methionine and a diet containing naturally seleniferous wheat was included in this study.

All of the substances mentioned appeared to give a relatively small but consistent degree of protection, but only at levels that depressed growth in rats on diets containing no selenium. Since all of the compounds used may act as methyl group donors or in their transport, the possibility that the protective effect resulted from detoxification of selenium through dimethyl selenide is discussed.

Data on the effects of some of the compounds on the selenium content of the livers are presented and discussed.

STUDIES WITH LINSEED OIL MEAL

EXPERIMENTAL AND RESULTS

The conditions for the rat work were the same as those described earlier here except that the basal diet shown in Table III was used. Linseed oil meal and its fractions were added at the expense of the corn and selenium was again added as sodium selenite (10 p.p.m. selenium) as previously described.

Table III

Composition of basal diet used in linseed oil meal studies

Diet Component [1]	Percent
Corn	88.9
Casein (purified)[2]	12.0
Brewer's yeast[2]	2.0
Salt mixture (U.S.P. XVI)[2]	2.0
Wesson oil	3.0
Animal protein factor[2]	0.1
	100.0

[1] Vitamins A and D orally once a week: 600 IU vitamin A and 85 IU vitamin D.

2 Nutritional Biochemicals Corporation

Halverson, Hendrick and Olson (17) have discussed the treatment of linseed oil meal to obtain a fraction active against selenium poisoning. The method they used was essentially that shown in Figure 7. The same authors found that the activity could not be removed from a water solution of Fraction II by lead acetate and that ashing destroyed the activity of the fraction. The work discussed here concerns attempts to further concentrate and purify the active principle from Fraction II. The procedures

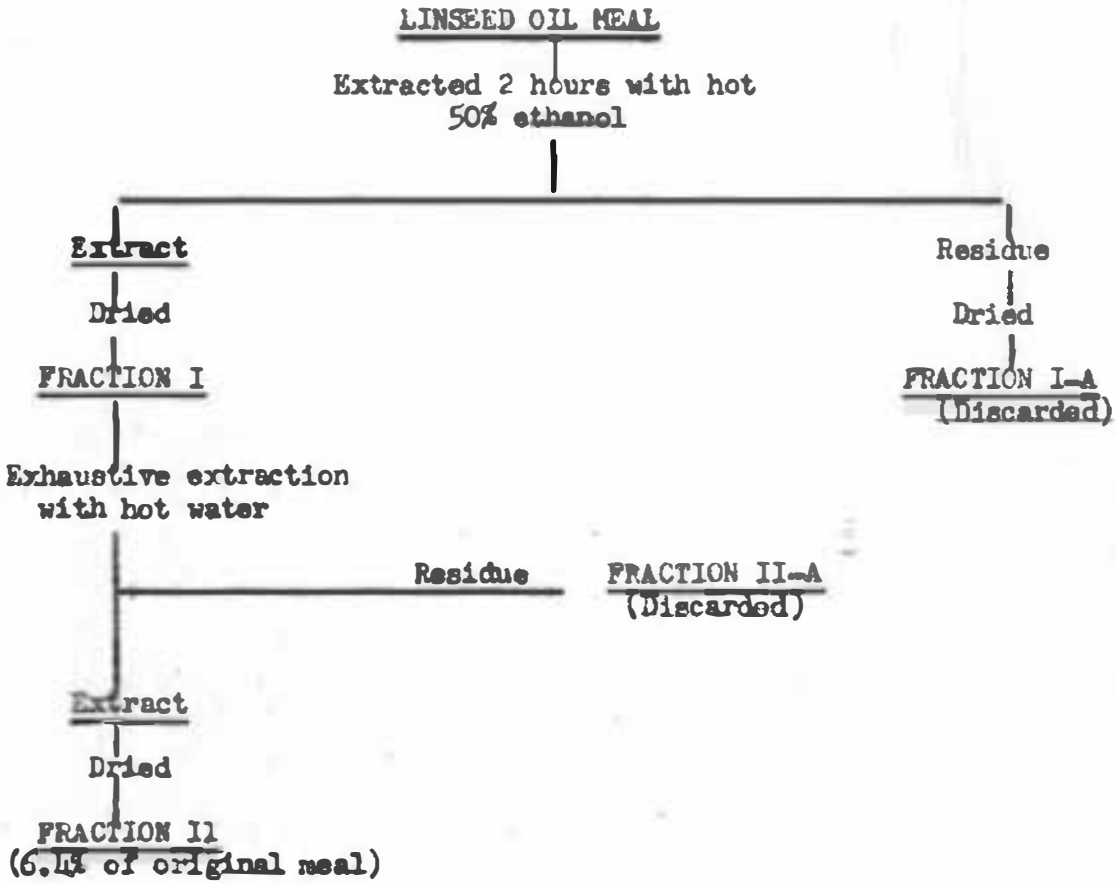

Figure 7. Preliminary extraction of linseed oil meal according to method described by Halverson, Hendricks and Olson (17).

used are discussed below.

Extraction with absolute ethanol. In an attempt toward the isolation of the protective factor from Fraction II a treatment with absolute ethanol was used. Two liters of commercial absolute ethanol in a flask were heated to boiling and 100 grams of Fraction II was added slowly to it. The mixture was boiled for 10 minutes with stirring, filtered immediately with suction, evaporated to about one-fifth of its original volume and then stored at -10°C overnight. The residue was saved for re-extraction. On standing overnight at -10°C, a flocculent material separated from the alcohol solution. The mixture was filtered with suction while still cold and the precipitate was washed with cold absolute ethanol. The precipitate (Fraction II-1) was dried in vacuo. The filtrate and washings were concentrated and during the concentration a crystalline material separated. The crystals (Fraction II-2) which precipitated out as the volume was reduced were filtered off and dried in vacuo. The filtrate was then concentrated in vacuo to a black tar (Fraction II-3).

The residue was re-extracted twice using 200 grams the first time and 400 grams the second time per two liters of absolute ethanol. The procedure from then on was the same as that described above. In all, 15 portions of 100 grams each of Fraction II were so treated. The residue after three extractions as well as all fractions obtained from the 1500 grams of Fraction II are given in figure 8, a summary of the procedure used.

The results of the feeding trial are given in Table IV. The data show that the alcohol treatment removed the activity since Fraction II-4 was ineffective against selenium. The moist tar (Fraction II-3) was the most effective fraction. However, some activity appeared in Fractions II-1

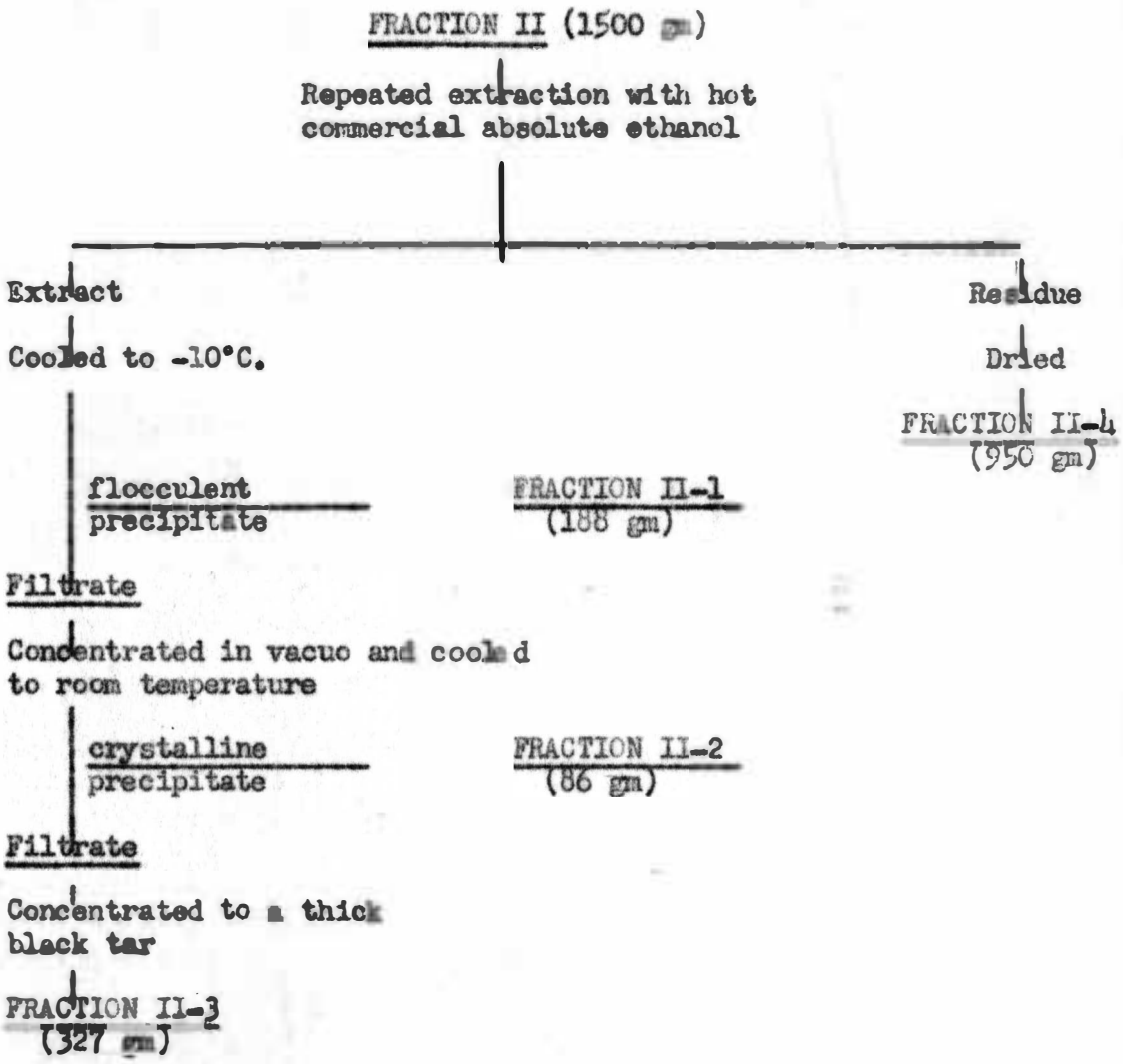

Figure 8. Schematic summary of fractionation of Fraction II with ethanol.

and II-2, indicating that partial separation had resulted during the steps by which they were obtained. These results indicated that the active fraction was somewhat soluble in absolute ethanol, and it was felt that work with methanol might prove even more fruitful.

Table IV

Absolute ethanol in the removal of activity from Fraction II

5 rats per group for 27 days

Fraction	Average daily gain	Liver weight	Survival
	gm.	% of Body wt.	%
None (Non-seleniferous basal)	6.44	5.19	100
None (Seleniferous basal)	1.68	2.93	60
II-1 (4% of diet)	3.77	4.35	60
II-2 (4% of diet)	2.68	3.73	80
II-3 (4% of diet)	4.80	6.32	100
II-4 (4% of diet)	1.69	2.39	60

In extracting Fraction II with absolute methanol (commercial) one part of the fraction was heated on the steam bath with 5 volumes of the solvent for 45 minutes. It was filtered hot with suction. The residue was extracted again with 3 volumes and then with 2 volumes of absolute methanol as before. The residue, Fraction III-A (15.7 percent of original Fraction II), was dried for feeding. The combined filtrates were added to 3 1/2 volumes of acetone (further acetone addition gave no more precipitate) and after standing over night the precipitate, Fraction III-B, was filtered by suction and washed with acetone-methanol (3 1/2:1). The precipitate was dried in

vacuo after air drying (38.3 percent of original Fraction II). The filtrate (Fraction III) was dried on the steam bath and then in vacuo at 80°C. to give a brown, hard substance (41.6 percent of original Fraction II).

The various fractions were fed in two trials (except for Fraction III-B). In the first trial (Table V) none of the three fractions seemed to have appreciable activity, except that Fraction III gave excellent liver protection. It appeared possible that in addition to its protective effect this fraction might also possess growth or appetite depressing characteristics. Therefore, a second experiment using graded levels (2, 4 and 6 percent of the diets) of Fractions II, III and III A was run (Table V). Fraction II gave increasing growth and liver protection with increasing levels. The same was true for Fraction III-A, but its protective effect was not great at any level. Fraction III gave the best growth at the 4 percent level, being better in this respect than any level of the other two fractions. Liver damage protection was best at the 4 percent level, but was also better at the 6 percent level than that given by the other fractions. The findings in these two trials indicated that absolute methanol was a rather good solvent for the protective factor, that the factor could not be effectively precipitated from methanol by acetone and that a general growth or appetite depressing factor was also showing its effect.

An effort was next made to extract the active principle from linseed oil meal itself with hot absolute methanol. This was not found to be a practical procedure. The reason for the failure to efficiently extract the active principle is unknown.

In an attempt to effect a further separation of the protective factor from Fraction III, this fraction was placed in cold methanol and the

Table V

Absolute methanol in the removal of activity from Fraction II.

Fraction added [1]	Amount added	Average daily gain	Liver weight	Survival
	%	gm.	% of body wt.	%
(5 rats per group for 21 days) Trial I				
None (Non-seleniferous basal)	—	7.07	5.24	100
None (Seleniferous basal)	—	1.24	3.23	80
Fraction II	10	5.72	5.97	100
Fraction III	10	2.29	6.73	100
Fraction III-A	10	2.01	3.71	100
Fraction III-B	4	1.30	3.26	100
Trial II (5 rats per group for 20 days)				
None (non-seleniferous control)	—	6.85	5.28	100
None (seleniferous control)	—	2.37	3.65	100
Fraction II	2	3.40	4.38	100
Fraction II	4	4.13	5.10	100
Fraction II	6	4.25	5.32	100
Fraction III	2	3.98	4.78	100
Fraction III	4	5.25	7.00	100
Fraction III	6	3.29	5.70	100
Fraction III - A	2	1.68	3.00	100
Fraction III - A	4	2.03	3.78	100
Fraction III - A	6	3.39	4.88	100

1 All diets contained 10 p.p.m. selenium except the non-seleniferous controls.

soluble part of it poured into ten volumes of diethyl ether (further ether addition did not produce more precipitate). The insoluble part probably belonged with Fraction III-C. After standing overnight the precipitate settled out and the ether-methanol mixture was decanted. The flocculent precipitate (Fraction IV) was centrifuged for half an hour, decanted, washed with methanol-ether solution (1:10) and again centrifuged for half an hour. The combined washings were added to the originally decanted methanol-ether mixture. This mixture was filtered by gravity, evaporated under partial vacuum and the residue finally concentrated in vacuo. This residue (a tar) constituted Fraction IV-A.

Fraction IV after centrifuging and washing was redissolved in cold methanol and again precipitated with ether as described above. Finally it was dried in vacuo at a temperature of 70°C. The amounts of the various fractions obtained from Fraction II by absolute methanol and consequent treatment are given in Figure 9, which summarizes the procedure used for obtaining the protective factor in the highest concentration thus far. In the course of preparing more of Fraction III this fraction, while still in the methanol-acetone mixture, was left standing for a period of about five days. During this time a precipitate appeared in the form of white crystals having a sweetish taste. These crystals constituted Fraction III-C (6.7% of the original Fraction II). The three fractions IV, IV-A, and III-C were used in feeding trial, the results of which are given in Table VI. Fraction III-C gave the same average daily gain as the basal seleniferous diet and a slightly smaller liver:body weight ratio. Thus it appears that this fraction did not contain the protective factor.

Fraction IV-A gave a slightly larger liver:body weight ratio and a

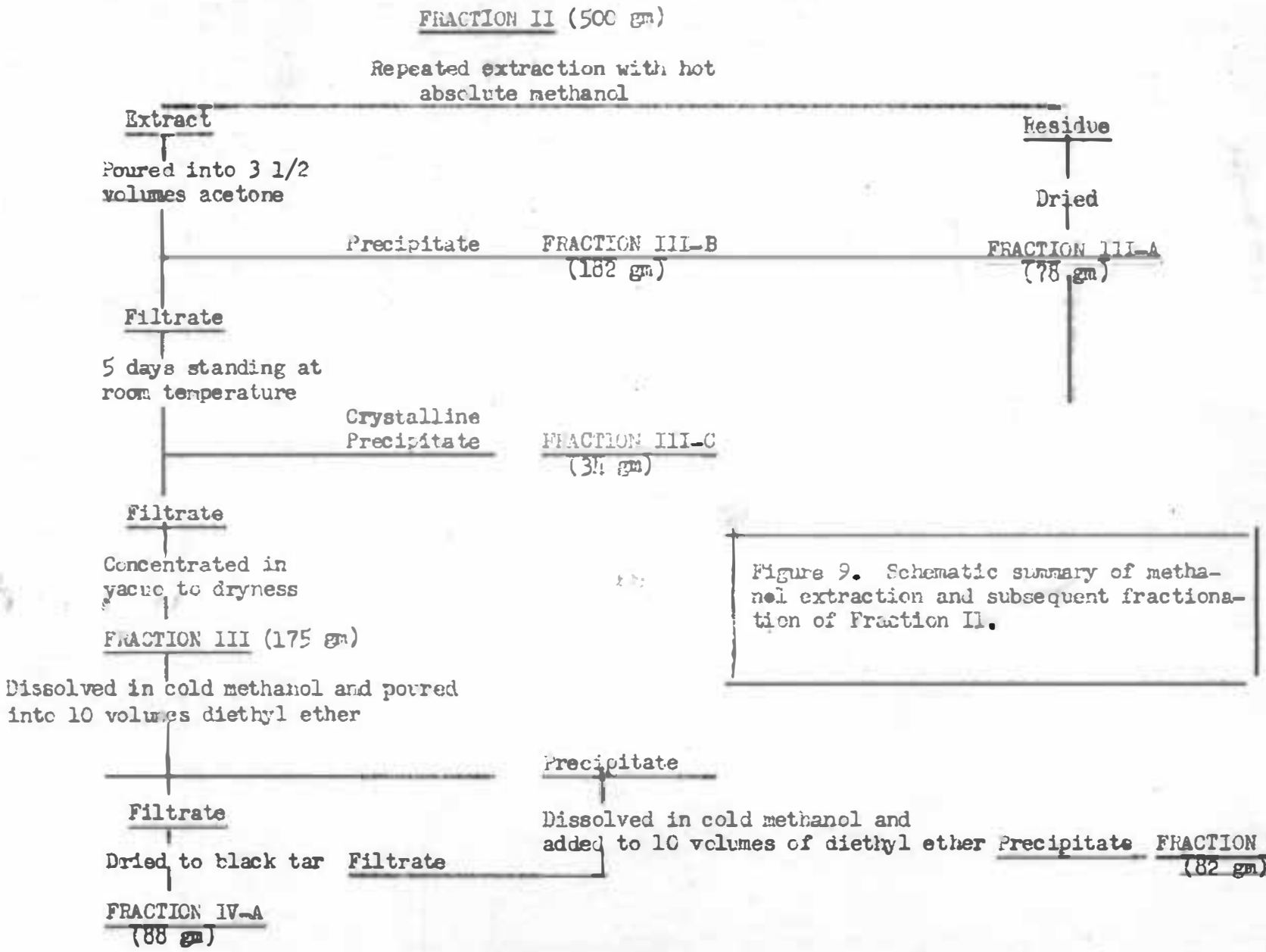

Figure 9. Schematic summary of methanol extraction and subsequent fractionation of Fraction II.

TABLE VI

Ether-absolute methanol in the removal of activity from Fraction III

Fraction added	Amount added	Average daily gain	Liver weight	Survival
	%	gm.	% of body weight	%
	(4 rats per group for 23 days)			
None (Seleniferous basal)	—	1.89	3.74	100
Fraction IV	2	4.18	5.48	100
Fraction IV-A	2	3.10	4.06	75
Fraction III-C	2	1.89	3.45	75

somewhat better average daily gain. However, the data on mortality show that 25 percent of the animals in this group died before the end of the experiment and 67 percent of the surviving were almost dead, when the experiment was terminated. Hence the protective effect of this fraction is questionable or at least it is minimized by the toxicity of the fraction.

Fraction IV gave values for the average daily gain and liver:body weight ratio that were considerably above the seleniferous control. All rats in this group survived. Therefore, it appears that the protective factor was most concentrated in this fraction.

DISCUSSION

The work presented here, although it does not identify the substance or substances present in linseed oil meal that protect against selenium, has yielded some information that may be helpful in further isolation studies.

Rat feeding trials showed that the active principle can be removed from Fraction II with hot absolute ethanol or methanol. Treatment of a methanol solution of the active principle with acetone gave an inactive principitate while ethyl ether gave a precipitate (Fraction IV) that was protective. All results obtained thus far indicate that the active principle is insoluble in non-polar solvents.

Fraction IV is the most active (per gram of dry weight) of any obtained to date. This fraction constitutes about 1 percent of the original linseed oil meal. However, it is only about 10 times as active as the original meal, which means that considerable loss of activity occurs during the procedure used here. The occurrence of these losses is further substantiated by the results of the rat feeding trials, which indicated that few of the procedures used gave clear-cut separations. However, chemical examination of the fractions obtained may yield an assay useful in future studies, provided some chemical entity can be correlated with the activity of these fractions.

It has been found that Fraction IV partially dissolves in hot ethanol. Upon concentration and cooling of the solution, there was found a precipitate which appeared to be crystalline. It is possible that this particular step might yield an active material pure enough for the purpose of identification. The preparation of a large quantity of Fraction IV and

the subsequent solution and crystallization procedure seems to offer an excellent approach toward the final solution of this problem of isolation.

SUMMARY

Previous work on the fractionation of linseed oil meal for the purpose of isolating the factor or factors responsible for protection against selenium poisoning in rats was continued. A 50 percent ethanol-water, water soluble fraction from the meal was used as a starting material for these studies. Albino rats were used to test various fractions obtained for activity.

Hot absolute methanol was found to be more satisfactory in removing the active principle from Fraction II than was hot absolute ethanol. Treatment of the methanol soluble material with acetone removed inactive materials from solution. Subsequent treatment of a methanol solution with diethyl ether gave a fraction 1 percent by weight of the original meal and about 10 times as active. Further purification of this fraction with absolute ethanol seems possible.

LITERATURE CITED

1. Moxon, A. L. Alkali disease or selenium poisoning. S. Dak. Agri. Expt. Sta. Tech. Bull. 311 (1937).

2. Moxon, A. L. and Rhian, M. Selenium poisoning. Physiol. Rev. 23:305-329 (1943).

3. Trelease, S. F. and Beath, O. A. Selenium. Published by the authors, New York (1949).

4. Gortner, R. A., Jr. Chronic selenium poisoning of rats as influenced by dietary protein. Jour. Nutrition 19:105-112 (1940).

5. Rosenfeld, I. and Beath, O. A. The influence of protein diets on selenium poisoning. I. Am. F. Vet. Res. 7:52-56 (1946).

6. Smith, M. I. The influence of diet on the chronic toxicity of selenium. Pub. Health Rpts. 54:1441-1453 (1939).

7. Lewis, H. B., Schultz, J. and Gortner, R. A., Jr. Dietary protein and the toxicity of sodium selenite in the white rat. Jour. Pharm. Exptl. Thera. 68:292-299 (1940).

8. Smith, M. I. and Stohlman, F. E. Further observations on the influence of dietary protein on the toxicity of selenium. Jour. Pharm. Exptl. Thera. 70:270-278 (1940).

9. Sellers, A. E., You, W. R. and Lucas, C. C. Lipotropic agents in liver damage produced by selenium or carbon tetrachloride. Proc. Soc. Exptl. Biol. Med. 75:118-121 (1950).

10. Klug, H. L. and Harshfield, R. H. Methionine and selenium toxicity in rats. Proc. S. Dak. Acad. Sci. 28:99-102 (1949).

11. Klug, H. L., Harshfield, R. D., Pengra, R. M., and Moxon, A. L. Methionine and selenium toxicity. Jour. Nutrition 48:409-420 (1952).

12. Baron, H. and Allison, J. B. Effect of methionine and glycocyamine on growth of rats. Fed. Proc. 13:450 (1954).

13. Fels, C. I. and Cheldelin, V. H. Methionine in selenium poisoning. Jour. Bio. Chem. 176:819-826 (1948).

14. Moxon, A. L. Ph. D. Thesis, University of Wisconsin (1941).

15. Halverson, A. W., Peterson, D. V., and Klug, H. L. Fractionation of the selenium protective factor in flaxseed. Proc. S. Dak. Acad. Sci. 30:97-102 (1951).

16. Schuchardt, P. A., Halverson, A. W., and Clagett, C. O. Occurrence of

the selenium protective principle of flax in hull and embryo fractions. Proc. S. Dak. Acad. Sci. (in press).

17. Halverson, A. W., Hendrick, C. M., and Olson, O. E. Observations on the protective effect of linseed oil meal and some extracts against chronic selenium poisoning in rats. Jour. Nutrition 56:51-60 (1955).

18. Halverson, A. W. and Hendrick, C. M. Negative relation of the selenium protective factor and the anti-vitamin B_6 principle of linseed oil meal. Proc. of S. Dak. Acad. Sci. 33:95-97 (1954).

19. Kratzer, F. H. and Williams, D. E. The effect of pyridoxine upon growth of chicks fed linseed oil meal. Poultry Sci. 27:671 (1948).

20. McConnell, K. P. and Portman, O. W. Excretion of dimethyl selenide by the rat. J. Biol. Chem. 195:277(1952).

21. McElroy, W. D. and Glass, H. B. A Symposium on Amino Acid Metabolism. The Johns Hopkins Press (1955)

22. Stekol, J. A., Weiss, S., Smith, P. and Weiss, K. The synthesis of choline and creatine in rats under various dietary conditions. J. Biol. Chem. 201:299 (1953).

23. Klein, A. K. Report on selenium. J.A.O.A.C. 24:363 (1941).