Changes in fatty acid composition in nutritional fatty degeneration of the liver

3.* Effect of ethionine†

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The role of ethionine in intermediary metabolism and its effect on the production of fatty livers has received extensive attention (Farber, 1959). Several studies (Simpson, Farber & Tarver, 1950; Farber, 1955; Farber, Simpson, & Tarver, 1950; Farber, Koch-Weser & Popper, 1951; Farber & Segaloff, 1955; Sidransky & Farber, 1958) go far towards explaining the mechanism of action of ethionine administered parenterally in the rat. The primary effect of ethionine appears to be on protein metabolism: there is an inhibition of methionine incorporation into protein (in female rats only) and a reduction in transmethylation from methionine to choline (in both male and female rats). These effects occur before any increase in liver lipids becomes apparent. The accumulation of lipids takes place only in the female rat liver; the male seems to be protected by androgens. The accumulation of liver lipid in the female rat cannot be prevented by dietary choline, but the fatty liver condition can be prevented by simultaneous injection of methionine or glucose along with the ethionine.

Little is known about the nature of the lipid found in the ethionine-induced fatty liver except that, unlike that in normal rat liver, the extracted fat is liquid rather than solid. The investigation now described had a twofold purpose: (1) to extend the study of the effects of ethionine to the chicken, particularly since we have shown this species to react differently from other animals to factors that give rise to a fatty liver condition (Feigenbaum & Fisher, 1963*a*); and (2) to study the fatty acid composition of liver fat in animals given ethionine in the hope of elucidating thereby the origin of the fat and possible reasons for its accumulation.

EXPERIMENTAL

Procedure. Day-old female chickens from a Columbian × New Hampshire cross were used in all experiments. The composition of the basal diet is shown in Table 1; its methionine content was just adequate for optimal growth on this diet. The source and quantity of fat and the addition of DL-methionine (or methionine hydroxy analogue)

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and of DL-ethionine, in the different experiments, are shown in the tables. All dietary additions were made at the expense of glucose.

Birds, in selected groups of five to represent the mean weight of the original group, were killed with chloroform for liver fat analyses at the end of a 2-week feeding period. The livers were prepared and the fat was analysed as previously described (Feigenbaum & Fisher, 1963*a*).

Table 1. *Composition of basal diet*

Ingredient	Amount (%)
Defatted soya-bean meal (50% protein)	40·00
Mineral mixture*	4·94
Cellulose†	3·00
Vitamins‡	0·25
Antioxidant§	0·01
DL-methionine or methionine hydroxy analogue‖	Varied
Fat	Varied
Glucose monohydrate	To 100

* For composition see Fisher, Griminger, Leveille & Shapiro (1960).
† Solka Floc, Brown and Co., Berlin, New Hampshire, USA.
‡ For composition see Fisher & Johnson (1956).
§ Santoquin (6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline), Monsanto Chemical Co., St Louis, Mo., USA.
‖ MHA, Monsanto Chemical Co., St Louis, Mo.

Expt 1. This experiment was designed to determine a dietary ethionine level that would produce a fatty liver degeneration without killing the birds within the 2-week feeding period. All diets used in Expt 1 contained 2% maize oil.

Expt 2. This experiment was designed to explore further the dienoic acid-concentrating effect which appeared to be an integral part of the accumulation of liver fat due to ethionine. The effect of the following dietary lipids was investigated: (1) coconut oil, a highly saturated fat; (2) olive oil, which is predominantly mono-unsaturated; (3) maize oil, which contains approximately equal amounts of mono- and di-enoic acid; and (4) safflower-oil fatty acids containing predominantly dienoic acid (72·9%). These lipids (and glycerol for a control group) were given at a level of 3·5% of the diet. The lipid supplements were added to a diet containing 0·2% DL-ethionine and 0·3% supplementary DL-methionine (or methionine hydroxy analogue). On this diet, mortality due to ethionine toxicity was kept low, and accumulation of liver fat was considerable.

Expt 3. We had previously found that high levels of dietary fat were beneficial in preventing the accumulation of liver fat in chickens refed after a period of starvation (Feigenbaum & Fisher, 1963*b*). Since in Expt 2, maize and coconut oil had caused the least accumulation of fat in the livers of ethionine-fed chickens, in Expt 3 higher levels of these oils were used.

Expt 4. In this final experiment we tested the effects of dietary linoleic acid and of triolein on the ethionine-induced fatty liver condition. In Expt 4, a lower level of ethionine (0·05%), that would permit a fairly good rate of growth, was used as well as the 0·2% level which had been used in Expts 2 and 3.

RESULTS

Table 2 gives values for the liver fat of day-old chickens. The small liver size relative to body-weight, the high fat content of liver, and the particular distribution of the fatty acids, specifically the saturated, mono- and di-enoic acid fractions should be noted.

Table 2. *Expt* 1. *Effect of increasing levels of* DL-*ethionine, with and without added methionine, on liver fat of chickens and its fatty acid composition*

Measurement	Day-old chickens	Chickens given DL-ethionine at dietary level (%) of:									
		0	0·025	0·05	0·10	0·20	0	0·025	0·05	0·10	0·20
		Without added methionine					With added methionine*				
Consumption:											
ethionine (mg/14 days)	—	0	51	86	91	90	0	58	100	143	134
diet (g/14 days)	—	247	205	172	91	45	249	233	200	143	67
Body-weight (g)	45	164	143	113	72	54	160	164	151	124	64
Liver weight as percentage of body-weight	1·8	4·6	4·7	6·1	5·8	4·5	4·3	4·4	4·5	4·6	5·3
Liver fat:											
as percentage of wet liver weight	16·4	4·6	4·6	7·6	7·7	8·1	5·6	5·3	6·0	6·0	7·5
as mg/liver	136	343	309	522	320	198	385	382	410	336	272
iodine value	81	61	68	68	62	76	67	79	71	72	73
Fatty acids:											
as percentage of fat											
saturated	34·7	70·7	72·7	65·3	70·7	57·7	66·5	60·2	67·8	67·3	56·0
monoenes	55·3	11·0	4·7	13·8	6·0	15·0	13·4	17·1	9·3	5·6	19·8
dienes†	**3·7**	**8·1**	**10·0**	**11·1**	**14·8**	**19·6**	**9·4**	**10·3**	**11·1**	**16·7**	**17·0**
trienes	0	1·2	0·8	1·2	1·3	1·2	1·1	1·0	0·8	1·2	1·5
tetraenes	2·7	4·8	6·4	5·0	4·4	3·6	5·6	6·5	6·3	5·6	3·8
pentaenes	0·8	1·5	2·2	1·7	1·4	1·1	1·6	2·1	2·2	1·9	0·9
hexaenes	2·8	2·7	3·1	1·9	1·4	1·7	2·4	2·8	2·4	1·6	1·1
as mg/g liver											
saturated	57·0	32·3	33·4	49·8	54·6	46·9	37·4	32·0	40·7	40·0	42·1
monoenes	90·9	5·0	2·2	10·5	4·6	12·2	7·5	9·1	5·6	3·3	14·9
dienes	6·1	3·7	4·6	8·5	11·4	15·9	5·3	5·5	6·7	9·9	12·8
trienes	0	0·5	0·4	0·9	1·0	1·0	0·6	0·5	0·5	0·7	1·1
tetraenes	4·5	2·2	3·0	3·8	3·4	2·9	3·2	3·5	3·8	3·4	2·8
pentaenes	1·3	0·7	1·0	1·3	1·1	0·9	0·9	1·1	1·3	1·2	0·6
hexaenes	4·6	1·2	1·4	1·5	1·1	1·4	1·3	1·5	1·5	1·0	0·8

The basal diet included 2% maize oil.

* Added as 0·15% of diet.

† Diene values are in bold-faced type to draw attention to the increasing concentration with increased ethionine.

Expt 1. The results are shown in Table 2. Comparison of the values for the 2-week-old chickens receiving no ethionine and for the day-old ones showed the normal changes in liver fat during the first 2 weeks of life. The liver increased in weight about two and a half times as fast as the total carcass. During the same period, the relative amount of liver fat decreased by two-thirds. There were also large changes in amounts of the various fatty acid fractions. On a relative basis (percentage of fat), saturated, di-, tetra-, and penta-enoic acid fractions all doubled during the 2 weeks. The monoenoic acid fraction was the only one to show a marked decrease (when expressed on a relative basis). On an absolute basis the change was even more striking (from 91 to 6 mg/g liver).

Supplementation with methionine clearly exerted some protection against ethionine toxicity. A comparison of the groups that received diets with the same level of ethionine showed that those on diets with added methionine grew better and accumulated less fat in the liver; only the groups receiving 0 and 0·025 % ethionine deviated from this generalization. With these groups, the addition of methionine resulted in slightly increased accumulation of liver fat. This observation, however, is in agreement with that of Shils, de Giovanni & Stewart (1955), who found that high levels of supplementary methionine reduced accumulation of liver fat whereas small methionine additions enhanced it.

In this connexion it should be noted that the degree of growth depression and the extent of accumulation of liver fat depended on the relative amount of ethionine and methionine consumed and not on the consumption of ethionine alone. This relationship becomes clear from a comparison of the values for food consumption and ethionine consumption. The groups receiving the three highest levels of ethionine (0·05, 0·1 and 0·2 %) in the diets without methionine supplementation consumed about equal amounts of ethionine. Similarly, for the groups given the two highest ethionine levels (0·1 and 0·2 %) in the methionine-supplemented diets, the ethionine consumption was about equal, whereas in both groups total food intake decreased by about 50 % with each higher level of ethionine. Since the methionine level in all diets was held constant as a percentage of the diet, the methionine consumption and, therefore, the ratio, methionine:ethionine consumed, decreased as increasing levels of ethionine depressed food and methionine intake.

The most dramatic change in the composition of the liver fatty acids due to increasing level of ethionine was the increase in the dienoic acid fractions both on a relative (as a percentage of fat) and on an absolute (mg/g liver) basis. The preferential accumulation of dienoic acid in the development of the ethionine-induced fatty liver condition did not occur in the fatty liver which resulted from realimentation of starved chickens, in which monoenoic acid accumulated (Feigenbaum & Fisher, 1963*b*). The accumulation of dienoic acid, which the bird cannot synthesize (Murty, Williams & Reiser, 1960), suggests that depot fat is the origin of the liver fat in the ethionine-fed bird.

Expt 2. The results are shown in Table 3. The effect of ethionine in this experiment is summarized in the second and third columns, variations due to type of dietary fat being disregarded. The decrease in food consumption and rate of weight increase due to ethionine was considerable. Liver size was significantly greater and liver fat (as percentage of wet liver weight) was almost twice that of the controls when ethionine was given. Once again there was an increase in concentration of dienoic acid with the ethionine-supplemented diets.

Among the dietary supplements, maize and coconut oil resulted in the least accumulation of liver fat, followed by glycerol, the safflower-oil fatty acids, and olive oil. The dienoic acid-concentrating effect of the dietary lipids is best illustrated by the differences among the dienoic acid concentration (mg/g liver) of the groups given or not given ethionine within each lipid treatment. The order of increasing dienoic acid-concentrating effect for the dietary fats was similar to that for accumulation of liver fat except for a reversal in the positions of olive oil and the safflower-oil fatty acids. The very large build-up of dienoic acid with the latter may well have been due to the giving of free fatty acids rather than glycerides. This point was further investigated in Expt 4.

Table 3. *Expt 2. Effect of various dietary fats (3·5 % of the diet), with and without ethionine, on liver fat of chickens and its fatty acid composition*

	Mean value, all diets		Glycerol		Coconut oil		Olive oil		Maize oil		Safflower-oil fatty acids	
Ethionine added to diet (%)	0	0·2	0	0·2	0	0·2	0	0·2	0	0·2	0	0·2
Consumption:												
ethionine (mg/14 days)	—	151	0	174	0	132	0	164	0	166	0	118
diet (g/14 days)	219	75	230	87	241	66	231	82	196	83	195	59
Body-weight (g)	160	72	162	71	175	75	175	75	140	77	150	60
Liver weight as percentage of body-weight	4·8	6·7	5·6	7·0	4·3	5·6	5·0	7·1	4·1	6·3	5·2	7·3
Liver fat:												
as percentage of wet liver weight	5·8	10·5	6·0	9·8	5·0	8·9	6·2	13·6	5·2	8·7	6·7	11·4
as mg/liver	461	501	544	486	381	370	549	730	301	422	530	499
iodine value	70	78	73	78	69	76	73	82	65	59	69	93
Fatty acids:												
as percentage of fat												
saturated	47·8	37·2	40·8	34·0	51·8	38·5	46·4	28·9	52·2	54·0	47·7	29·8
monoenes	39·6	47·4	48·4	53·4	34·4	47·5	40·7	57·3	34·9	32·9	39·6	46·0
dienes	6·1	11·2	5·0	8·4	6·6	9·5	5·7	8·7	7·0	9·7	6·1	19·5
trienes	1·5	1·1	1·4	1·1	1·6	1·0	1·4	1·2	1·4	1·4	1·6	1·0
tetraenes	2·8	1·7	2·2	1·6	2·7	1·9	3·1	1·9	2·9	0·6	3·0	2·3
pentaenes	0·8	0·5	0·8	0·5	1·0	0·6	0·9	0·5	0·7	0·3	0·7	0·5
hexaenes	1·5	0·9	1·4	0·9	1·9	1·0	1·9	0·6	1·1	1·1	1·2	0·9
as mg/g liver												
saturated	27·7	37·8	24·5	33·4	26·0	34·1	28·8	40·6	27·1	46·9	32·1	34·1
monoenes	23·3	50·7	29·1	52·4	17·2	42·1	25·3	78·0	18·1	28·6	26·7	52·6
dienes	3·5	11·9	3·0	8·3	3·3	8·4	3·6	11·9	3·6	8·4	4·1	22·3
trienes	0·8	1·2	0·8	1·1	0·8	0·9	0·8	1·6	0·7	1·2	1·1	1·2
tetraenes	1·6	1·9	1·3	1·6	1·4	1·7	1·9	2·8	1·5	0·6	2·1	2·6
pentaenes	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·7	0·3	0·3	0·5	0·5
hexaenes	0·9	0·9	0·9	0·9	1·0	0·9	1·2	0·9	0·5	0·9	0·8	1·0

The fatty acid pattern of the liver with all treatments varied only slightly, even though the fatty acid patterns of the diets varied greatly. This observation is well illustrated by the relative amounts of the polyunsaturated acids of the groups receiving ethionine.

Expt 3. Since the effect of ethionine in diets containing 10 % fat on food consumption, weight gain, relative liver size, and relative amount of liver fat was similar to that observed previously (Expts 1 and 2) with lower levels of dietary maize or coconut oil, the details are not reported here; the noteworthy differences due to the high-fat diets are summarized in Table 4. As the level of dietary fat was increased the accumulation of dienoic acid in the livers of birds given ethionine became less than in the control group both with maize and coconut oil. Apparently, therefore, the dienoic acid-concentrating effect is not specifically characteristic of the ethionine-induced fatty liver condition since the content of this fatty acid fraction can be reduced without a concomitant reduction in total liver fat. In fact, Table 4 shows that the higher level of dietary fat in combination with ethionine resulted in very marked increases in amount of liver fat, whereas the amount of liver fat in the controls decreased as dietary fat increased.

Table 4. *Expt* 3. *Effect of the level of dietary fat on liver fat and dienoic acid in ethionine-fed chickens and controls*

Dietary fat (%)	DL-ethionine in diet (%)	Liver fat (as percentage of wet liver weight)	Liver dienoic acid (as percentage of fat)
		Maize oil	
2	0	5·6	9·4
2	0·2	7·5	17·0
3·5	0	5·2	7·0
3·5	0·2	8·7	9·7
		Coconut oil	
3·5	0	5·0	6·6
3·5	0·2	8·9	9·5
10	0	4·7	8·8
10	0·2	12·1	8·0

Values at 2 and 3·5 % of fat levels are taken from Tables 2 and 3, respectively.

Expt 4. The results are shown in Table 5. Despite an intake of about 100 mg ethionine/bird in 14 days, the birds given the 0·05 % ethionine diets maintained relatively high levels of food consumption and weight gain. By comparison, the birds receiving the higher level of ethionine consumed only about one-third more ethionine, with a striking reduction in food consumption and weight gain.

Increasing the ethionine level within each dietary lipid treatment generally increased liver size, liver fat (as percentage of wet liver weight) and unsaturation of the liver fat as assessed by iodine value. Although the dienoic acid levels (fat basis) in groups on all diets except that containing 4 % triolein were slightly lower for the birds that received the low-ethionine diets than for those on the ethionine-free diets, the diet with high ethionine content (0·2 %) did result in marked accumulation of dienoic acid. On an absolute basis (mg/g liver) the dienoic acid fraction did increase gradually with both levels of dietary ethionine.

The combination of 4 % dietary linoleic acid and ethionine resulted in less accumulation of liver fat than did the 2 % linoleic acid diet. With triolein, the results were reversed, the high level of dietary fat producing the greater accumulation of liver fat. Triolein gave rise to a fattier liver than linoleic acid and the combination of dietary lipids was similar to triolein in this regard.

Table 5. *Expt 4. Effect of various dietary levels of* DL-*ethionine, linoleic acid and triolein on liver fat of chickens and its fatty acid composition*

Dietary fat: type ...	Linoleic acid						Linoleic acid + triolein, 2 of each			Triolein					
amount (%) ...	2			4						2			4		
Ethionine added to diet (%)	0	0·05	0·20	0	0·05	0·20	0	0·05	0·20	0	0·05	0·20	0	0·05	0·20
Consumption:															
ethionine (mg/14 days)	0	88	132	0	99	128	0	108	142	0	98	114	0	104	126
diet (g/14 days)	222	175	66	211	198	64	223	216	71	222	195	57	212	208	63
Body-weight (g)	172	142	67	163	156	66	171	169	64	165	144	60	158	160	66
Liver weight as percentage of body-weight	5·0	5·9	7·7	5·4	5·9	7·2	4·7	5·7	8·7	5·9	5·5	7·2	5·3	6·0	8·1
Liver fat:															
as percentage of wet liver weight	3·9	8·1	10·2	4·3	5·9	9·5	4·5	6·1	12·5	5·4	6·6	11·8	6·3	8·3	12·3
as mg/liver	339	671	509	376	544	451	365	588	699	530	521	511	523	791	656
iodine value	70	72	83	84	79	92	77	82	89	73	73	74	70	71	76
Fatty acids:															
as percentage of fat															
saturated	56·3	44·7	40·6	46·2	43·2	34·8	46·9	36·6	29·2	40·3	37·5	37·4	39·9	36·2	34·0
monoenes	26·0	40·6	36·1	30·3	37·3	36·8	33·7	47·0	50·1	49·0	52·8	50·7	51·2	55·8	54·2
dienes*	**9·5**	**9·3**	**18·4**	**15·1**	**13·0**	**23·9**	**12·1**	**10·4**	**16·8**	**5·0**	**4·6**	**8·0**	**4·4**	**4·4**	**8·1**
trienes	1·2	1·4	1·2	1·5	1·5	1·3	1·6	1·4	1·2	1·5	0·5	0·8	1·3	1·1	0·8
tetraenes	4·3	2·2	2·0	4·3	3·3	1·7	3·3	2·9	1·5	2·2	1·9	1·6	1·7	1·3	1·5
pentaenes	1·0	0·5	0·5	0·8	0·6	0·4	0·7	0·5	0·4	0·6	0·6	0·5	0·5	0·4	0·5
hexaenes	1·8	1·2	1·1	1·7	1·1	1·1	1·6	1·2	0·8	1·5	1·2	1·1	1·0	0·9	0·9
as mg/g liver															
saturated	22·1	36·0	41·3	19·9	25·7	32·9	21·2	22·4	36·6	21·8	24·6	44·2	24·9	30·0	41·9
monoenes	10·2	32·7	36·7	13·0	22·2	34·8	15·2	28·7	62·8	26·5	34·6	60·0	32·0	46·2	66·7
dienes	3·7	7·5	18·7	6·5	7·7	22·6	5·5	6·4	21·1	2·7	3·0	9·5	2·8	3·6	10·0
trienes	0·5	1·1	1·2	0·6	0·9	1·2	0·7	0·9	1·5	0·8	0·3	0·9	0·8	0·9	1·0
tetraenes	1·7	1·8	2·0	1·8	2·0	1·6	1·5	1·8	1·9	1·2	1·2	1·9	1·1	1·1	1·8
pentaenes	0·4	0·4	0·4	0·3	0·4	0·4	0·3	0·3	0·5	0·3	0·4	0·6	0·3	0·3	0·6
hexaenes	0·7	1·0	1·1	0·7	0·7	1·0	0·7	0·7	1·0	0·8	0·8	1·3	0·6	0·7	1·1

* Diene values are in bold-faced type to draw attention to the increasing concentration with increased ethionine.

In this experiment, in which free linoleic acid was added to the diet, there was much greater accumulation of liver dienoic acid than, for example, with 10% maize oil (Table 4) although the latter provided at least as much glyceride-bound dienoic acid. Thus, the earlier results with the safflower-oil fatty acids (Table 3) can probably be explained on the basis of the glyceride binding of fatty acids. The triolein supplements resulted in higher levels of mono-unsaturated fatty acids, and the linoleic acid–triolein combination in raised levels of both mono- and di-enes in liver fat.

DISCUSSION

Our studies showed that the growing female chicken, like the female rat, develops a fatty liver when ethionine is added to the diet. The work described here also supports the finding of a protective effect from dietary methionine, although we cannot draw conclusions of a quantitative nature in this regard because, since the methionine level of the diet was held constant, the intake decreased with increased levels of dietary ethionine.

As expected, detailed analysis of the liver fatty acids provided some clues to the origin of the ethionine-induced fatty liver. Our earlier studies (Feigenbaum & Fisher, 1963 *a*, *b*) suggested that dietary fat, and to a lesser degree dietary free fatty acids, by-pass the liver and are deposited in the fat depots. The study corroborated this suggestion even for the ethionine-treated animals, since the fatty acid pattern of the liver bore little relationship to the composition pattern of the dietary fats. On the other hand, the great reduction in food intake due to ethionine creates a demand for energy which must be met from the body fat stores. Harris & Robinson (1961) have suggested that this effect may also be a factor in the development of the fatty liver. The ethionine-induced fatty liver may therefore result from an inability of the birds to oxidize properly the fat infiltrating from the fat depots, possibly because of decreased enzyme activity (impaired protein synthesis), as the work of Artom (1959) suggested. The accumulation of liver fat might also arise from *de novo* liver synthesis in an attempt to maintain the physical characteristics of liver fat due to the influx of large quantities of dienoic acid from fat depots. In this connexion the small variation in iodine values on widely differing treatments should be noted, particularly in those instances in which dienoic acid levels increased sharply.

SUMMARY

1. Day-old female chickens were given diets varying in amounts of ethionine, methionine and different fats.

2. The chicken, like the rat, developed a fatty liver when treated with ethionine.

3. The fatty acid composition of the liver fat in the ethionine-treated bird was characterized by a relatively high level of dienoic acid that bore little relationship to the composition of the dietary fat.

4. Our study suggested that upon ethionine treatment fat moves from body stores to liver in response to increased caloric need due to reduced food intake. In turn, either reduced fat oxidation or increased liver synthesis to balance the accumulation of dienoic acid may explain the ethionine-induced fatty liver condition.

REFERENCES

Artom, C. (1959). *J. biol. Chem.* **234**, 2259.
Farber, E. (1955). *Fed. Proc.* **14**, 402.
Farber, E. (1959). *AMA Arch. Path. Lab. Med.* **67**, 1.
Farber, E., Koch-Weser, D. & Popper, H. (1951). *Endocrinology*, **48**, 205.
Farber, E. & Segaloff, A. (1955). *J. biol. Chem.* **216**, 471.
Farber, E., Simpson, M. V. & Tarver, H. (1950). *J. biol. Chem.* **182**, 91.
Feigenbaum, A. S. & Fisher, H. (1963*a*). *Brit. J. Nutr.* **17**, 31.
Feigenbaum, A. S. & Fisher, H. (1963*b*). *Brit. J. Nutr.* **17**, 39.
Fisher, H., Griminger, P., Leveille, G. A. & Shapiro, R. (1960). *J. Nutr.* **71**, 213.
Fisher, H. & Johnson, D. Jr. (1956). *J. Nutr.* **60**, 261.
Harris, P. M. & Robinson, D. S. (1961). *Biochem. J.* **80**, 352.
Murty, N. L., Williams, M. C. & Reiser, R. (1960). *J. Nutr.* **72**, 451.
Shils, M. E., de Giovanni, R. & Stewart, W. B. (1955). *J. Nutr.* **56**, 95.
Sidransky, H. & Farber, E. (1958). *Proc. Soc. exp. Biol., N.Y.*, **98**, 293.
Simpson, M. V., Farber, E. & Tarver, H. (1950). *J. biol. Chem.* **182**, 81.

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