Effect of Antioxidants on Dietary Necrotic Liver Degeneration.[1] (24232)

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Necrotic liver degeneration is readily produced in rats by *Torula* yeast diets which are simultaneously deficient in cystine*, Vit. E, and Factor 3(1,2). Since the disease is prevented by physiological amounts of tocopherol (3,4), it belongs to those Vit. E deficiency syndromes which show extensive anatomical damage and which are fatal. Other such diseases are encephalomalacia, exudative diathesis, muscular dystrophy, and acute heart degeneration(5).† It is not fully known what causes Vit. E deficiency to document itself in such a wide variety of pathological lesions. However, it is certain that the supply of Factor 3-selenium‡ has a decisive effect on the pathology produced by Vit. E-deficient diets.

In vitro, various forms of Vit. E are effective as antioxidants, although greatly divergent in biological potency(6,7). This has been the basis for much conjecture as to their mode of action *in vivo,* without leading to an accepted, clearly understood concept for the biological mechanism of action of Vit. E(8). The present paper deals with effects of various groups of antioxidants on dietary necrotic liver degeneration. A total of 13 such compounds was tested. Liver necrosis was found very effectively prevented by a few of these substances, but not by others.

Methods. Assays reported here were carried out on rats of the Fischer 344 strain(9).§ This albino strain has been brother-sister mated through more than 60 generations and is very uniform in responding to the basal, liver necrosis-producing diet. Under our standard conditions, Fischer animals require an average of 21 days for development of liver necrosis, as compared to 45 days for the Sprague-Dawley strain referred to previously. For protection against liver necrosis, Fischer rats need only approximately half as much dietary Vit. E as Sprague-Dawley rats. Necrotic liver degeneration was produced by a Vit. E-free, 30% *Torula* yeast diet, as described previously(1). The predepletion method was used(2). Supplementation of the diet with the various antioxidants started after the animals had been kept for 12 days on the basal ration. In all experiments, unsupplemented groups of rats were maintained as controls. Food and tap water were given *ad libitum.* Supplements were either added directly to the whole diet (D), or suspended in Vit. E-free lard before incorporation of the latter in the ration (L). Diets were made up in 1 or 2 kg lots and fed in porcelain cups holding approximately 80 g; these were checked daily. Unused diets were stored at +4°C. All animals were autopsied, and a few animals which died from causes other than liver necrosis were omitted. The results were evaluated by comparison of numbers of survivors, and also of survival times. The latter were used for calculation of average velocity (V_{st} = 100/survival time) of death from liver necrosis, as described previously (3,10). For estimation of the protection caused by a supplement, the V_{st} of the supplemented group was compared to that of the controls. "Per cent protection" was calculated by expressing the reduction of V_{st}, resulting from the supplement, in per cent of

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* Since submission of this paper it has been shown that Factor 3 is an organic selenium compound (Schwarz, K., Foltz, C. M., *J. Am. Chem. Soc.,* 1957, v79, 3292). The protective effect of L-cystine is caused by a trace contamination with Factor 3-active selenium. To be published.

† Multiple necrotic degeneration (heart, liver, kidney and muscle necrosis) in the mouse also belongs into this category. (DeWitt, W. B., Schwarz, K., *Experientia,* 1958, v14, 28).

‡ Various selenium compounds show greatly different biopotencies (Schwarz, K., Foltz, C. M., *J. Biol. Chem.,* 1958, v233, 14). The term "Factor 3" is applied to designate the biologically active, selenium containing substance, or substances, in material of natural origin.

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the V_{st} of the unsupplemented controls (% protection = 100 − (100 X V_{st} exp.)/V_{st} cont.). This method permits one to express, in mathematically correct terms, protective effects obtained with groups of animals. It is more accurate than the simple comparison of numbers of survivors, since it gives consideration to length of survival of individual

TABLE I. Effect of Antioxidants on Dietary Necrotic Liver Degeneration.

Supplement: Dose (g/100 g of diet)	Supplement: Mode of suppl.*	No. of exp.	No. of animals	No. of survivors†	Duration (days)	V_{st}, avg velocity‡	% protection§
			Ascorbic acid**				
.0	C	2	20	1		5.2 ± .21–.6	
.5	D	1	10	2	30	3.4 ± .5	33
2.0	D	1	10	1	30	3.7 ± .5	28
			Methylene blue chloride††				
.0	C	1	10	1		4.3 ± .6	
.1	L	1	10	0		4.9 ± .1	
.2	L	1	9	3		2.8 ± .5	35
.5	L	1	10	2	35	2.7 ± .4	36
1.0‖	L	1	—	—	35		
			Di-tert-amylhydroquinone‡‡				
.0	C	1	8	0		5.2 ± .3	
.25	D	1	10	9	30	.3 ± .3	93
			Santoquin§§				
.0	C	3	29	2		4.5 ± .4–.6	
.10¶	L	2	20	0		3.8 ± .1	(16)
.25	L	2	19	12	35	1.2 ± .3–.4	73
			Santoflex B‖‖				
.0	C	3	25	2		4.5 ± .5–.6	
.005	L	1	10	0		4.3 ± .3	
.05	L	1	10	1		3.2 ± .4	28
.10	L	2	20	1		3.6 ± .1–.5	19
.25	L	2	20	2	40	3.5 ± .3–.6	22
			DPPD (N,N′-diphenyl-p-phenylenediamine)¶¶				
.0	C	4	35	0		5.2 ± .2–.7	
.00062¶	L	1	10	0		4.8 ± .2	
.00125	L	2	20	0		3.4 ± .1–.2	34
.0025	L	1	9	0	42	2.8 ± .1	47
.0050	L	2	20	13	42	.6 ± 0–.2	89
.0200	L	1	10	10	60	0	100
.0	C	3	25	2		5.0 ± .5–.7	
.00125	D	1	8	1		3.4 ± .1	32
.0025	D	2	19	1	42	2.5 ± .3	50
.0050	D	1	10	10	42	0	100
			D,L-α-tocopheryl acetate				
.0	C	2	16	1		5.1 ± .5–.6	
.00125	L	1	9	8	42	.6 ± .6	88
.0025	L	1	9	9	42	0	100

* C = control, D = added directly to diet, L = premixed in lard before addition to diet.

† If not specially indicated, experiments were terminated between 25th and 35th day.

‡ Mean ± stand. error. Where several groups are combined, lowest and highest stand. error are listed.

§ $100 - \frac{V_{st}\ \text{exp.} \times 100}{V_{st}\ \text{control}}$.

‖ Toxic.

¶ Lower levels tested and found inactive.

** USP, Merck and Co.

†† National Aniline Division, Allied Chemical and Dye Corp.

‡‡ 2,5-di-tert-amylhydroquinone, Eastman Kodak Co.

§§ 6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline, Monsanto Chemical Co.

‖‖ 6-Phenyl-1,2-dihydro-2,2,4-trimethylquinoline, Monsanto Chemical Co.

¶¶ Food grade, B. F. Goodrich Chemical Co.

animal, and permits calculation of averages and statistical treatment of the data where survival times themselves cannot be averaged, due to animals which are maintained alive. With increasing duration of survival, velocity of death approaches zero (V_{st} of survivors $\longrightarrow$ 0).

Results. Results are presented in Table I. Data obtained in different experiments were pooled for each antioxidant, and control groups pertaining to tests of each substance were also combined. Growth was not significantly affected. Ascorbic acid and methylene blue afforded a small measure of protection in that they somewhat delayed development of the disease. However, rather high levels of the supplement were required; the effects were relatively small and seemed to reach a ceiling, so that with elevated doses no further increases in protection were obtained. The effect of 2% ascorbic acid in the diet was practically identical to that of .5%. Methylene blue protected 3 out of 9 animals when supplied at a .2% level. With .5% of the dye, 2 out of 10 animals survived; higher levels were clearly toxic.

The following substances were without significant influence. All were tested at dose levels up to .5% in the diet:

Antabuse (tetraethylthiuram disulfide)‖
Hydroquinone¶
NDGA (nordihydroguaiaretic acid)**
n-Propylgallate (propylester of gallic acid)††
DBPC (2,6-di-tert-butyl-4-methyl-phenol)‡‡
Butylated hydroxyanisole§§
Propylparasept (propylester of p-hydroxy-benzoic acid)‖‖

The last 5 of these compounds are commercial, widely used, multiple-purpose antioxidants of phenolic character.

‖ Purified, Ayerst Laboratories, Inc.
¶ Mallinckrodt Chemical Works.
** Nordigard Corp.
†† Purified, Heyden Chemical Corp.
‡‡ Food grade, Koppers Co., Inc.
§§ Mixed 2- and 3-tert-butyl-4-methoxyphenol, Tenox BHA, Eastman Kodak Co.
‖‖ Purified, Heyden Chemical Corp.

Hydroquinone (.5%) was without effect but di-tert-amylhydroquinone prevented liver necrosis almost completely at a level of .25%. Effects were also seen from 2 dihydro-quinoline derivatives: Santoquin at a .1% level delayed onset of the disease, and .25% protected 12 out of 19 animals for more than 35 days. Santoflex B, only slightly different from Santoquin in its constitution, was much less active and showed a ceiling in its effectiveness.

Most conspicuous results were obtained with N,N′-diphenyl - p - phenylenediamine (DPPD). Activity of this antioxidant was of the same order of magnitude as that of D,L-α-tocopheryl acetate. Doses of 1.25 and 2.5 mg% had a pronounced delaying action on development of the disease. Out of 20 animals receiving 5 mg %, 13 survived for an experimental period of 42 days. When .02% DPPD was furnished in the diet all 10 animals survived; they were killed after 60 days. There was essentially no difference between diets prepared by mixing of the DPPD into lard before addition to the diet, and 2 groups which received 2.5 and 5 mg % of DPPD supplemented by addition directly to the complete basal ration. Control experiments with D,L-α-tocopheryl acetate listed in Table I were run simultaneously with some of the DPPD experiments.

Discussion. The present studies evidently deal with those aspects of necrotic liver degeneration which are related to Vit. E. This is also apparent from a subsequent paper, describing the effects of intraportal injection of antioxidants on respiratory decline(11). The effects of antioxidants such as methylene blue, Antabuse, ascorbic acid, and nordihydroguaiaretic acid (NDGA) on various experimental Vit. E deficiency diseases in chickens and rats were initially investigated by Dam and collaborators, as reviewed elsewhere(12,13). Liver necrosis was reported to be delayed by several of these substances. Moore, *et al.*, extended Dam's studies by testing a series of known oxidation-reduction indicators, such as dyes related to methylene blue, in Vit. E-deficient rats(14,15). Singsen, Jungherr, and collaborators subsequently detected that some commercial antioxidants prevented encephalo-

malacia in chickens. DPPD was practically as active as α-tocopherol(16). Contrasted with these findings is the observation that exudative diathesis, another form of Vit. E deficiency in the chicken, is much less responsive to DPPD supplements(17).

From the results presented here it follows that dietary necrotic liver degeneration belongs to those Vit. E deficiency diseases which are preventable by certain antioxidants, but various antioxidants are widely different in their effects. There is no apparent correlation between antioxidant activity and potency seen against liver necrosis. Roughly, the tested substances can be divided into 3 different groups: (I) Ascorbic acid, methylene blue, and Antabuse. These may act as antioxidants, but are better known for other properties. Antabuse is inactive at .2%, while the other 2 are required in rather large doses and afford only a limited, but measurable amount of protection. The results with methylene blue confirm those of Dam and Granados(18). In our experiments the effect does not surpass the 30 to 40% level of protection, even when increased levels of these agents are supplied. (II) A group of 5 antioxidants which are most widely and effectively used for stabilization of dietary fats and other materials, and which are without influence on necrotic liver degeneration at the tested levels. These antioxidants all have phenolic properties. (III) Antioxidants which are active, namely, di-tert-amylhydroquinone, Santoquin, and especially DPPD. These are chemically not related to each other. It is at present not possible to define the specific properties which enable them to protect, like Vit. E, against dietary necrotic liver degeneration. Singsen, Jungherr, and collaborators have tested a variety of antioxidants against exudative diathesis in chickens(16), and have reported a scale of relative activities similar to that described here. The only substance obviously different is DBPC, which is active in the chicken, but inactive in the rat.

It is inferred that the protective compounds function within the tissues and that the results are not simply due to stabilization of the diet against rancidity. This conclusion is warranted in spite of the fact that *Torula* yeast has been reported to contain several per cent of unsaturated fatty acids(19), for the following reasons: (1) Autoxidation of the diet is not involved in production of necrotic liver degeneration. The basal diet is stable against rancidity; this is due to the presence of antioxidants in the yeast(20); (2) The deficiency can be produced readily by fat-free rations(21)¶¶; (3) If a general antioxidant action against rancidity of the diet were involved, the 5 inactive antioxidants listed in group II should be effective. Evidence will be presented that the active antioxidants, when injected intraportally, are like Vit. E, effective *within* the liver tissue in reverting respiratory decline in the Warburg apparatus (11).

Summary. Thirteen antioxidants were tested at varying dose levels against dietary necrotic liver degeneration produced in rats by a Vit. E-deficient, 30% *Torula* yeast ration. The substances can roughly be grouped in 3 different classes: (I) Ascorbic acid (.5%) and methylene blue (.5%) showed activity, but did not afford more than 30 to 40% protection, even at higher dose levels. Antabuse (.2%) was ineffective. (II) Inactive antioxidants: NDGA (nordihydroguaiaretic acid), n-propylgallate, DBPC (2,6-di-tert-butyl-4-methylphenol), BHA (mixed 2- and 3-tert-butyl-4-methoxyphenol), hydroquinone, and others were without effect at dose levels up to .5% in the diet. (III) Antioxidants protecting against liver necrosis: Di-tert-amylhydroquinone and Santoquin (6-ethoxy-1,2-dihydro - 2,2,4-trimethylquinoline) inhibited the deficiency strongly at .25% levels in the diet, while Santoflex B (6-phenyl-1,2-dihydro-2,2,4-trimethylquinoline, .05 to .25%) was slightly active. DPPD (N,N'-diphenyl-p-phenylenediamine, food grade) came close to Vit. E in activity. 5 mg % DPPD were approximately as effective as 1.25 mg % D,L-α-tocopheryl acetate.

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¶¶ Incidence and velocity of development of liver necrosis are unchanged when fat is eliminated from our basal *Torula* yeast diet.

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1. Schwarz, K., PROC. SOC. EXP. BIOL. & MED., 1951, v77, 818.
2. ———, *ibid.*, 1951, v78, 852.
3. ———, *Z. physiol. Chem.*, 1944, v281, 109.
4. ———, *Ann. N. Y. Acad. Sci.*, 1954, v57, 878.
5. Mason, K. E., in Sebrell, W. H., Harris, R. S., *The Vitamins*, Academic Press, N. Y., 1954, Vol. III, p514.
6. Hove, E. L., Hove, Z., *J. Biol. Chem.*, 1944, v156, 623.
7. Gottlieb, H., Quackenbush, F. W., Steenbock, N., *J. Nutrition*, 1943, v25, 433.
8. Mattill, H. A., in Sebrell, W. H., Harris, R. S., *The Vitamins*, Academic Press, N. Y., 1954, Vol. III, 490.
9. Jay, G. E., Jr., in *Rat Quality, a Consideration of Heredity, Diet and Disease*, Nat. Vitamin Foundation, N. Y., 1953, 98.
10. Schwarz, K., PROC. SOC. EXP. BIOL. & MED., 1952, v80, 319.
11. Mertz, W., and Schwarz, K., *ibid.*, 1958, v98, 808.
12. Dam, H., *Experientia*, Supplementum I (Symposium on Present Problems in Nutrition Research), 1953, 195.
13. Anonymous, *Nutrition Rev.*, 1952, v10, 149.
14. Heard, D. H., Moore, T., Sharman, I. M., *Biochem. J.*, 1952, v52, I.
15. Moore, T., Sharman, I. M., Ward, R. J., *ibid.*, 1954, v58, Part 1, VII.
16. Bunnell, R. H., Matterson, L. D., Singsen, E. P., Potter, L. M., Kozeff, A., Jungherr, E. L., *Poultry Sci.*, 1955, v34, 1068.
17. Scott, M. L., Hill, E. J., Norris, L. C., Dobson, D. C., Nelson, T. S., *J. Nutrition*, 1955, v56, 387.
18. Dam, H., Granados, H., *Acta Pharmacol. Toxicol.*, 1952, v8, 47.
19. Dam, H., Nielsen, G. K., Prange, I., Sondergaard, E., *Experientia*, 1957, v13, 493.
20. Muster, S., *U. S. Patents*, 1942, v282, 2, 792.
21. Schwarz, K., *Z. physiol. Chem.*, 1944, v281, 101.

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Effect of Cortisone and Growth Hormone upon Ductular Cell Proliferation in Liver Ethionine Intoxication.* (24233)

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Recently interest was focused upon accumulation of interstitial cells between liver cell plates, as apparent in biliary obstruction and dietary disorders(1), and in intoxication with carcinogenic drugs(2). These interstitial cells have been considered to be epithelium of bile ductules(2). Injection of bile ducts demonstrated that interstitial cells are to a large extent bile ductular cells even though morphologically they resemble mesenchymal elements (3). The interstitial cell reaction has been considered a response of the liver to injury, and this raised the question whether the reaction can be suppressed by anti-inflammatory agents, such as cortisone. Since this reaction can be elicited with reproduceable regularity in ethionine intoxicated rats(4), the influence of simultaneous cortisone administration was studied in view of known effect of cortisone upon inflammatory reactions in the liver(5,6). For comparison the effects of administration of growth hormone upon ethionine intoxication was also studied because of its influence on liver cells(7) and its antiphlogistic effect possibly antagonistic to cortisone(8).

Methods. Female Sprague-Dawley rats weighing approximately 175 g were placed in individual cages for 25 to 35 days and kept on synthetic diet containing vitamin free case-

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