Prevention of Respiratory Decline in Necrotic Liver Degeneration by Antioxidants *in vitro*. (25319)

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The latent phase of dietary necrotic liver degeneration in the rat is characterized by respiratory decline, a metabolic lesion readily observed long before onset of gross pathological changes. Normal-appearing slices of livers of such animals are not capable of maintaining their initial oxygen consumption in the Warburg apparatus(1). The disturbance is *prevented* by feeding of factors which protect against liver necrosis, *i.e.*, Vit. E and Factor 3(1). It is also *reversed* by injection of Vit. E emulsions into the portal vein immediately prior to extirpation of the liver(2). In previous investigations with 13 different antioxidants it was shown that several of the compounds prevented dietary liver necrosis more or less effectively when fed in the diet, while others were completely inactive(3). The same antioxidants were then tested for capacity to reverse the tendency to respiratory decline after injection into the liver *via* portal vein(4); by this method almost the same order of relative potencies was obtained. DPPD (N, N'-diphenyl-p-phenylenediamine) was most effective. By the portal route it was approximately twice as potent as D,L-α-tocopherol in reversing the tendency to respiratory failure. The current paper deals with prevention of respiratory decline by direct *in vitro* incubation of liver slices with the antioxidants. D,L-α-tocopherol or its esters leave respiratory decline completely unaffected when added to the Warburg medium. Two tocopherol metabolites, however, isolated by Simon *et al.*(5) from urines of rabbits and humans, possessed high *in vitro* activity(6). We discovered that synthetic antioxidants are also effective when applied to liver slices directly. A quantitative comparison *in vitro* of the 13 antioxidants screened previously in the diet and by the intraportal technic is given below.

* Studies performed in 1955 during tenure of a Brewer's Yeast Council Research Fellowship.

Methods. Most experimental details have been described(4). Weanling rats of inbred Fischer 344 strain were maintained on 30% *Torula* yeast diet(7). Under our conditions, this ration produces necrotic liver degeneration with practically 100% incidence, after average survival time of 21 days. Animals for Warburg experiments were taken between 14th and 17th day, at average weight of 45 g. Liver necrosis was seen occasionally at that time; only normal-appearing liver was used for testing. Rats were sacrificed by decapitation, and approximately 400 mg of slices were prepared by hand from the left lateral liver lobe, divided into 4 equal portions of 90 mg ($\pm$ 15 mg) and used in 4 different flasks. On the average, 6 minutes were required for killing and preparation of the slices. Three ml of oxygenated Krebs-Ringer phosphate buffer/flask was used, with .01 M glucose as substrate. Of 4 flasks containing slices from one liver, 3 received supplements at different doses; the other served as control. Incubation was carried out under O_2 at 37.5° for 140 minutes. *Evaluation.* The formula for calculation of respiratory decline, *i.e.*, failure of liver slices to maintain initial O_2 consumption, has been given previously(4). The effect of supplements is expressed as per cent *prevention* of respiratory decline. It is calculated by comparing the decline of supplemented slices (dB) to the decline of their respective controls (dA), using the formula $P = 100 - (dB \times 100)/dA$. *Antioxidants.* The compounds used are listed in the Tables. Water insoluble substances were dissolved in ethanol to make a 10% solution. A portion of the latter was emulsified in incubation medium with a Potter-Elvehjem homogenizer. Of this emulsion, the required amount was added to the flask. The amount of ethanol present in 3 ml of medium was thus roughly 10 times that of the antioxidant. With one exception (hydroquinone) it never exceeded

.002 ml; for most experiments it was much less.

Results. Control experiments. It is well established that liver slices from normal, unfasted rats maintain their O_2 uptake in the Warburg apparatus at or close to initial values for 4 to 6 hours(1). In livers of rats on complete diets respiratory decline is practically zero, while in rats on 30% *Torula* yeast diet it amounts (on the average) to *ca* 75% of initial rate of O_2 consumption after 90-120 minutes. To ascertain that results were not affected by non-enzymatic oxidation of the supplements, the 2 compounds most likely to react directly with O_2, ascorbic acid and hydroquinone, were incubated in buffer in the absence of slices. O_2 uptake was measurable, but negligible as compared to that of liver slices. With 400 μg of hydroquinone, the value was 6 μl/hour. These findings do not, of course, exclude the possibility that the compounds could stimulate O_2 consumption in the presence of slices. It is noted in the Tables that average initial respiration (0-30 min. interval) is usually somewhat higher with the supplement (column B) than without (column A), but the differences are small and most likely caused by an effect of the supplement on the incipient respiratory decline during the first 30-minute interval.

The effect of DPPD on slices of intact liver was studied with animals protected against necrotic liver degeneration by 50 mg% D, L-α-tocopheryl acetate and .12% D, L-methionine in their diet. Decline of O_2 consumption after 2 hours incubation was not more than 6%, and *in vitro* addition of an optimal dose of DPPD, was without any demonstrable influence on respiration (Fig. 1A). In simultaneous experiments with deficient liver slices (Fig. 1B), the breakdown of respiration was prevented by the supplement. Addition of .02 ml of ethanol alone to deficient slices had no significant influence except for slight stimulation of initial O_2 consumption. When lower amounts were used, the pattern of respiration was not different from that of controls (Table I).

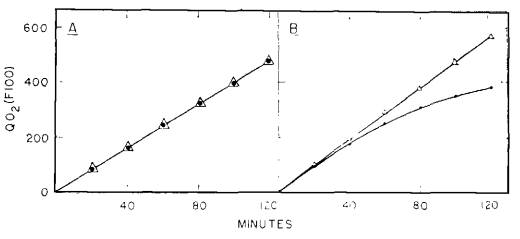

FIG. 1. Effect of *in vitro* addition of 16 μg of DPPD on respiration of liver slices. A. Rats on Vit. E supplemented basal diet (N = 3). ●, Controls; △, with DPPD. B. Rats on 30% *Torula* yeast diet (N = 5). ●, Controls; △, with DPPD.

Effect of antioxidants on respiratory decline. As in the 2 preceding publications(3,4) the compounds used are grouped in 3 categories to facilitate organization of the data. Group 1 consists of ascorbic acid, methylene blue and Antabuse, which can act as antioxidants but are better known for other properties. Substances of phenolic character (NDGA, n-Propylgallate, DBPC, BHA, Propylparasept, hydroquinone) which were ineffective in the preceding tests form Group II. Group III contains compounds (di-tert-amyl-hydroquinone, Santoquin, Santoflex B and DPPD) which protect effectively against dietary necrotic liver degeneration(3) and also reverse respiratory decline when injected intraportally(4). Of the compounds of Group I, ascorbic acid was relatively ineffective *in vitro* (Table I). Antabuse afforded more protection, but its activity was much below that of methylene blue. The dye produced 50% prevention of metabolic lesion when roughly 1 μg was added/flask. This potency *in vitro* is in distinct contrast to the behavior of the compound *in vivo, i.e.,* in dietary and intraportal tests. When injected into the portal vein, methylene blue was rapidly decolorized after reaching the liver and it did not reverse the tendency towards respiratory failure.† When added *in vitro,* on the other hand, the blue color was maintained and respiratory decline was prevented.

Of compounds of Group II, hydroquinone and Propylparasept were ineffective, while a

† The current study evolved from a chance observation: In one instance, during intraportal injection experiments, methylene blue remained unchanged in a liver lobe, possibly due to blocking of circulation after operation. Slices from this lobe maintained normal respiration, whereas other, decolorized lobes of liver demonstrated respiratory failure.

TABLE I. *In Vitro* Effect of Antioxidants on Respiratory Decline of Liver Slices. Group I (ascorbic acid, methylene blue, Antabuse) and Group II (phenolic antioxidants).

Dose in μg/flask	No. of exp.	Q_{O_2} (F100)* A Control 0-30 min.	A Control 100-120 min.	B With supplement 0-30 min.	B With supplement 100-120 min.	% respiratory decline† dA	% respiratory decline† dB	Prevention of respiratory decline‡ in %
				Controls (ethanol)				
1584§	6	277	61	296	57	79	81	0 (−3.5 ± 2.6)‖
50	4	256	62	259	65	76	75	0 (1 ± 1.9)
				Ascorbic acid				
100	4	279	77	277	109	73	61	15 ± 4.1
400	5	286	68	318	165	75	48	37 ±14.6
				Methylene blue chloride				
.4	5	274	82	278	124	70	56	24 ±10.2
1.65	5	280	84	301	215	70	28	62 ± 8.1
6.25	5	328	57	352	254	83	28	65 ± 7.4
25	5	317	65	353	300	79	14	83 ± 2.2
				Antabuse (tetraethylthiuram disulfide)				
6.25	5	320	75	350	121	76	65	17 ±10.1
25	5	"	"	364	178	"	51	34 ± 9.6
100	5	"	"	346	251	"	27	64 ±11.0
				NDGA (nordihydroguaiaretic acid)				
6.25	5	301	109	340	119	64	64	0 (−2 ± 6.2)
25	5	"	"	335	191	"	40	49 ±19.0
100	5	307	50	322	196	84	37	58 ±12.9
				n-Propylgallate (propylester of gallic acid)				
6.25	5	342	89	370	125	75	67	12 ± 5.1
25	5	"	"	389	179	"	55	27 ±11.2
100	5	"	"	378	191	"	50	35 ± 9.0
				DBPC (2,6-di-tert-butyl-4-methylphenol)				
1.56	5	301	88	357	150	74	59	20 ± 6.8
6.25	5	278	74	328	140	76	57	25 ±10.7
25	5	313	101	353	225	68	37	49 ± 6.3
100	4	307	104	325	271	66	14	80 ±12
				BHA (2- and 3-tert-butyl-4-methoxyphenol)				
6.25	5	293	66	324	83	78	75	0 (4 ± 4.8)
25	5	"	"	344	177	"	48	39 ± 6.8
100	5	292	53	325	283	82	12	85 ± 2.4
				Propylparasept (propylester of p-hydroxybenzoic acid)				
100	5	301	70	325	51	80	85	0 (−8 ± 9.3)
				Hydroquinone				
400	5	303	76	319	101	74	69	0 (5 ± 5.4)

* O_2 consumption in μl/hr and 100 mg of liver slices; values calculated for first 30 min. interval and for 100-120 min. interval, respectively.

† Q_{O_2} (0-30) − Q_{O_2} (100-120), expressed in % of Q_{O_2} (0-30).

‡ Prevention = dA minus dB, expressed in % of dA.

§ Equal to .002 ml.

‖ Mean ± stand. error of mean.

measurable degree of prevention was obtained with high levels of NDGA, Propylgallate, DBPC and BHA (Table I). The approximate dose required for 50% protection ranged from 25 to more than 100 μg. DBPC was the most active of the compounds; this is in good agreement with results of intraportal study, where DBPC showed a moderate effect, while the other 5 compounds were without influence at dose levels tested. In the dietary test against liver necrosis, all 6 of these antioxidants had been inactive.

The antioxidants of Group III, which prevented liver necrosis more or less effectively and also reversed respiratory decline upon intraportal injection, proved to be the most

TABLE II. *In Vitro* Effect on Respiratory Decline. Group III (active compounds and vitamin E).

Dose in μg/flask	No. of exp.	Q_{O_2} (F100)* A Control 0-30 min.	A Control 100-120 min.	B With supplement 0-30 min.	B With supplement 100-120 min.	% respiratory decline† dA	% respiratory decline† dB	Prevention of respiratory decline‡ in %
				Amylhydroquinone (2,5-di-tert-amylhydroquinone)				
1.56	5	280	50	312	91	83	71	14 ± 6.9‖
6.25	5	"	"	334	274	"	17	79 ±10.8
25	5	"	"	307	273	"	9	88 ± 7.9
				Santoquin (6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline)				
1.56	5	315	74	352	155	76	56	28 ± 9.7
6.25	5	307	76	332	278	"	15	79 ± 7.2
25	5	"	"	350	287	"	17	76 ± 4.6
				Santoflex B (6-phenyl-1,2-dihydro-2,2,4-trimethylquinoline)				
.0625	5	344	99	355	110	74	69	7 ± 2.7
.25	5	"	"	353	154	"	57	23 ± 5.3
1	5	"	"	358	293	"	19	75 ± 4.0
				DPPD (N,N'-diphenyl-p-phenylenediamine)				
.0156	5	269	59	271	72	78	74	5 ± 3.4
.0625	5	329	79	319	149	76	52	32 ± 5.1
.25	5	"	"	330	255	"	23	70 ± 5.0
1	5	"	"	344	319	"	7	91 ± 1.4
16	5	274	82	288	274	70	2	98 ± 2.2
				D,L-α-tocopherol				
50	5	333	94	364	91	72	77	0 (−6 ± 6.5)
200	5	"	"	350	106	"	70	0 (4 ± 5.2)
				D-α-Tocopheryl Polyethylene Glycol-1000 Succinate				
1000	5	275	58	273	68	79	74	7 ± 5.3
				Tocopherol metabolite§ 2-(3-hydroxy-3-methyl-5-carboxypentyl)-3,5,6-trimethylbenzoquinone				
1.56	5	251	87	290	145	66	51	27 ± 8.6
6.25	5	228	60	258	161	73	39	50 ±20.6
25	4	243	69	294	256	71	13	83 ± 4.4

* O_2 consumption in μl/hr and 100 mg of liver slices; values calculated for first 30 min. interval and for 100-120 min. interval, respectively.

† Q_{O_2} (0-30) − Q_{O_2} (100-120), expressed in % of Q_{O_2} (0-30).

‡ Prevention = dA minus dB, expressed in % of dA.

§ Published previously(6).

‖ Mean ± stand. error of mean.

active compounds in preventing the metabolic lesion *in vitro* as well (Table II). While unsubstituted hydroquinone did not prevent respiratory decline *in vitro,* the substituted, sterically hindered amylhydroquinone proved quite potent. Two quinoline derivatives, Santoquin and Santoflex, also produced a response analogous to that found in *in vivo* assay systems: Santoquin, the 6-ethoxy substituted compound, was slightly less effective than Santoflex, the 6-phenyl form. Of the first, *ca* 3.5 μg was required for 50% prevention, whereas *ca* 0.6 μg of the latter gave the same effect. Activity of Santoquin seems to reach a ceiling at *ca* 6 μg; 4 times this dose did not elicit a higher degree of response.

As in previous studies, DPPD was the most active member of the series. As little as .06 μg, representing *ca* .00025 μmole of DPPD, gave a significant degree of protection, and 1 μg prevented decline almost completely. If this amount were directly oxidized, it would account for less than .01 μl of O_2 consumed. In the presence of .06 μg of DPPD, respiration of liver slices in the last 20 minute interval alone was approximately 23 μl higher than without supplement, which shows clearly that the phenomenon cannot be interpreted as a substrate effect.

In accordance with previously published results(6), 50 and 200 μg of D,L-α-tocopherol, added *in vitro,* did not prevent respiratory de-

cline. This cannot be explained on the assumption of poor solubility; water soluble tocopheryl-polyethylene glycol succinate, highly effective *in vivo,* was equally without effect when 1000 μg were given *in vitro.* The tocopherol metabolite, 2-(3-hydroxy-3-methyl-5-carboxypentyl) - 3,5,6-trimethylbenzoquinone, on the other hand, prevented respiratory failure quite effectively, even though given in the quinone form(6).

Discussion. Comparing these results with those published previously, it is evident that the 13 antioxidants studied produce a similar pattern of response in all 3 test systems. They offer protection against dietary necrotic liver degeneration when fed in the diet(3), reversal of respiratory decline of liver slices upon intraportal injection(4), and prevention of respiratory decline by *in vitro* addition. While earlier studies showed that the site of action was within the organism, the present evidence demonstrates that the effect occurs directly in liver tissue. Since *in vitro* technic is more sensitive and permits study of wider dose ranges, it shows several substances capable of protection against the metabolic lesion which formerly were found inactive. Differences in effective dose levels of various substances are large, but with 2 main exceptions the relative positions of the compounds on the scale of potencies remain unchanged. The exceptions are Vit. E and methylene blue.

Vit. E and its esters, while superior to DPPD in the diet, and almost equal to DPPD in the intraportal injection, are completely inactive *in vitro.* This observation led to the concept that tocopherol is converted in the organism into an "active form"(8), with the subsequent discovery that the 2 Vit. E-metabolites, 2-(3-hydroxy-3-methyl-5-carboxy)-pentyl-3,5,6-trimethyl-benzoquinone and its γ-lactone, are active in the *in vitro* system(6).

Methylene blue, on the other hand, is *in vitro* comparatively much more potent than *in vivo;* it moves up into third place behind DPPD and Santoflex B. This shift is related to the fact that in the aerobic Warburg medium the dye is maintained in the oxidized state, whereas *in vivo* it is readily reduced to the leuco-form.

With reference to respiratory decline and death from liver necrosis, it is clear that the effective compounds of our antioxidant series truly replace Vit. E, or rather its active form, in its function within liver tissue. (In studies with DPPD and resorption sterility of the rat, Draper *et al.* have come to similar results (9)). As discussed previously, a certain degree of structural specificity governs metabolic efficiency of the compounds. For example, hydroquinone is inactive but is converted into a potent compound by substitution of amyl groups in the 2 and 5 positions. This indicates the importance of sterical hindrance for the effect.

Since quantities effective in preventing respiratory decline are too small to yield an effect as substrates, most, if not all of the compounds must act on the catalytic level. The exact site and mode of action of the substances in the liver cell cannot be deduced from the data at hand. It is obvious that physicochemical properties essential for this effect are also important for antioxidant-activity, but there is no parallelism of antioxidant potencies of compounds with their capacity to influence the respiratory lesion. It is probable that the antioxidant function proper, *i.e.,* protection against consequences of peroxide formation, is not involved in prevention of respiratory decline, since a number of the compounds with excellent, antioxidant capacity are ineffective in all 3 test systems even at large dose levels. The most important argument against the simple antioxidant theory of action is derived from the behavior of tocopherol itself. The vitamin is known to have strong antioxidant properties, and yet is completely ineffective on our *in vitro* system (while effective in the diet and after intraportal injection). Vit. E metabolites, on the other hand, are potent agents in prevention of respiratory decline, even though they are used in the quinone form which is worthless as antioxidant. One has to consider the possibility that the active compounds could participate catalytically in some specific aspect of electron transfer which happens to require structural and functional molecular properties similar to those of antioxidants.

Summary. The effect of 13 antioxidants on liver slices during the latent phase of necrotic

liver degeneration, was studied *in vitro*. The characteristic respiratory decline, *i.e.*, breakdown of O_2 consumption in the Warburg apparatus, was prevented by addition of most compounds at catalytic dose levels. With increasing amounts of compounds, dose response curves were obtained. The scale of relative potencies *in vitro* followed closely that previously established *in vivo* for protection against liver necrosis by feeding of the compounds, as well as for reversal of respiratory decline by intraportal injection, with the exception of Vit. E and methylene blue. Tocopherol and its esters were completely inactive *in vitro*, while the tocopherol metabolite showed high potency in preventing the metabolic lesion. Methylene blue, inactive in the *in vivo* systems, was highly potent in the Warburg assay. The most potent substance, DPPD, produced a significant effect with .06 μg/100 mg of liver tissue in 3 cc medium. Santoquin, Santoflex B and amylhydroquinone were effective at doses from less than 1 to 6 μg. Less protection was found with phenolic compounds, such as NDGA, Propylgallate, DBPC and BHA; hydroquinone and Propylparasept were without activity. It is concluded that the substances act directly in the liver cell, perhaps not merely as antioxidants but as catalysts in intermediary metabolism.

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Comparison of Normal and Buffered Saline Solution for Fluid Replacement Following Tourniquet Shock.* (25320)

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Most experimental work dealing with electrolyte solution replacement after tourniquet trauma has been done with isotonic NaCl solution. The parenteral or oral use of adequate amounts of such solution protects against an otherwise lethal tourniquet trauma(1,2). Speculation on whether the therapeutic effect of saline derives from Na or Cl ion or both, is not new. Allen(3) reported that in treatment of tourniquet shock, normal saline gave better survival results than solutions more alkaline or more acid. He suggested that to combat acidosis, which accompanies shock, by alkaline solutions might actually be deleterious. Our method(1) of intravenous infusion in therapy of tourniquet trauma lends itself to a study of this problem. Our experiments were undertaken to compare survival rates, after an otherwise lethal tourniquet trauma, when animals were treated with isotonic NaCl or an alkaline buffered saline solution as used by Moyer(4). Induced pH and electrolyte changes were also measured.

Methods. Male albino rats of Holtzman Farms, weighing 200-250 g were used. Each animal was individually housed in air-condi-

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