tate, bicarbonate, and lactate are far less effective than chloride, and may be toxic in large doses. 3) There is no sanctity in the customary 93:47 or higher meq ratio between chloride and other ions in oral electrolyte solutions used in these experiments. 4) Gluconate appears to serve as a partial substitute for chloride, and it lends palatability; experimental and clinical studies are indicated on gluconate metabolism in man.

This article is not to be construed as reflecting the position of the U. S. Army or any element thereof. The author alone is responsible for data presented and conclusions drawn.

1. Rosenthal, S. M., *Pub. Health Rep.*, 1943, v58, 513.

2. Millican, R. C., Tabor, H., Rosenthal, S. M., *Am. J. Physiol.*, 1952, v170, 179.

3. Fox, C. L., Jr., Mersheimer, W. L., Lasker, S., Winfield, J. M., *Am. J. Surg.*, 1953, v85, 359.

4. Fox, C. L., Jr., *J.A.M.A.*, 1944, v124, 207.

5. Fox, C. L., Lasker, S., Winfield, J. M., Mersheimer, W. L., Silverstein, M. E., *Am. J. Surg.*, 1955, v89, 730.

6. Markley, K., Bocanegra, M., Bazan, A., Temple, R., Chiappori, M., Morales, G., Carrion, A., *J.A.M.A.*, 1956, v161, 1465.

7. Moyer, C. A., *Ann. Surg.*, 1953, v137, 628.

8. Rosenthal, S. M., *Pub. Health Rep.*, 1943, v58, 1429.

9. Tabor, H., Kabat, H., Rosenthal, S. M., *ibid.*, 1944, v59, 637.

10. Millican, R. C., *Am. J. Physiol.*, 1955, v183, 187.

11. Reynolds, M., *ibid.*, 1949, v158, 418.

12. Hamilton, J. I., Hoar, W. S., Haist, R. E., *Canad. J. Res.*, 1946, v24, 31.

13. Rosenthal, S. M., Millican, R. C., *Pharm. Rev.*, 1954, v6, 489.

14. Allen, F. M., *A.M.A. Arch. Surg.*, 1957, v75, 210.

15. Rosenthal, S. M., Lillie, R. D., Verder, E., *Fed. Proc.*, 1952, v11, 375.

16. Meyer, J., Lendrum, B., Katz, L. N., *Am. J. Physiol.*, 1945, v145, 151.

17. Mylon, E., Winternitz, M. C., De Suto-Nagy, G. J., *ibid.*, 1943, v139, 313.

18. Millican, R. C., Stohlman, E. F., Mowry, R. W., *Am. J. Physiol.*, 1952, v170, 173.

19. *Pub. Health Rep.*, 1950, v65, 1317.

20. Rosenthal, S. M., *Pub. Health Rep.*, 1942, v57, 1923.

Received May 8, 1958. P.S.E.B.M., 1958, v98.

Reversal of Respiratory Decline in Necrotic Liver Degeneration by Intraportal Antioxidants.[1] (24192)

WALTER MERTZ* AND KLAUS SCHWARZ
(With technical assistance of Edward E. Roginski)
National Institute of Arthritis and Metabolic Diseases, N.I.H., Bethesda, Md.

A specific metabolic lesion, respiratory decline, has been demonstrated in liver slices from rats maintained on a diet producing necrotic liver degeneration(1,2). Normal-appearing slices of such livers are unable to maintain respiration in the Warburg apparatus after initially normal O_2 consumption for the first half hour. The defect is characteristic for the latent period of the disease; it precedes the acute pathological lesion by several weeks. Respiratory decline is prevented by feeding of those factors which protect against liver necrosis, *i.e.*, cystine†. Vit. E. and Factor 3. The lesion is reversed within minutes after injection of Vit. E into the portal vein (3)‡, but not after that of Factor 3§. Pre-

[1] This manuscript was originally submitted on April 30, 1956.

* These studies were performed during tenure of Brewer's Yeast Council Research Fellowship.

† 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.

‡ *In vitro* addition of α-tocopherol to deficient liver slices, either as an emulsion or in water-soluble forms, has no significant effect on metabolic lesion.

§ Relation of Factor 3-active selenium compounds to respiratory decline will be the subject of a separate report.

viously, evidence was presented for the protective dietary effects of a series of antioxidants, notably of N, N'-diphenyl-p-phenylenediamine (DPPD), against development of necrotic liver degeneration(4). We have investigated the effects of *intraportal injection* of the same series of antioxidants on respiratory decline. In this paper it is shown that certain of these substances cause a reversal of the metabolic lesion identical to that produced by injection of α-tocopherol.

Methods. Details concerning methods and materials have been published(4,5). Rats of the Fischer 344 strain, an inbred albino strain, were placed at weaning on the necrogenic, 30% *Torula* yeast diet. The Warburg experiments were usually performed after 15 to 17 days. The average weight of the animals at that time was 42 g. The livers mostly appeared grossly normal; if there was any necrosis, only normal-looking areas were used. *Two step Warburg experiments.* The technic of partial liver extirpation and intraportal injection followed that of Rodnan, *et al.*(3), except that a light ether anesthesia was used instead of Nembutal. In this procedure each animal serves as its own control. The left lateral lobe was removed from the liver, and slices were immediately prepared and incubated. After removal of the left liver lobe, the emulsion containing the antioxidant was injected into the portal vein. Care was taken to avoid hemorrhage. The wound was closed after instillation of approximately 2 cc of saline into the abdominal cavity in order to counteract shock. The animals recovered rapidly from the operation and behaved normally. Thirty minutes later they were sacrificed by decapitation, the rest of the liver was extirpated, and slices were made from the *right* lateral lobe. This lobe was least disturbed by the preceding ligation of the left lobe. Slicing was done on an ice-chilled tray; the slices were blotted on wet filter paper. In each instance, 2 samples were assayed from the pre- and the post-injection lobe. Each Warburg flask contained approximately 90 mg of tissues ($\pm$ 15 mg), 3 cc of oxygenated Krebs-Ringer phosphate buffer with .01 M glucose, and .2 cc of 30% KOH in the center well. The flask was gassed with oxygen for 30 seconds and, after an initial equilibration period of approximately 4 minutes, incubated at 37.5° for 140 minutes. Readings were taken at 10-minute intervals during the first hour of incubation, and at 20-minute intervals thereafter. *Evaluation.* The results were evaluated by comparing the respiratory decline of the post-injection samples with the corresponding pre-injection controls. Respiratory decline was calculated from the formula: $d = 100 - \frac{Qo_2 \text{ final} \times 100}{Qo_2 \text{ initial}}$. In normal livers, this value for respiratory decline was practically zero. In rats on the necrogenic diet, it ranged from 43% to 85%. From the values for the respiratory decline before and after intraportal injection, reversion (R) of the decline was calculated and expressed in per cent of pre-injection decline: $R = 100 - \frac{dB \times 100}{dA}$, where dA = respiratory decline of initial pre-injection control, dB = respiratory decline of post-injection sample. *Antioxidants.* The compounds used in this investigation have been listed(4). The sequence of presentation is that used in the other report. *Preparation of emulsions.* Ascorbic acid and methylene blue were dissolved in saline. The emulsions of the more water-insoluble antioxidants were prepared from solutions of the substances in minimal amounts of ethanol. These were added to a warm 5% glucose solution containing .25% glyceryl monostearate and blended immediately for 10 minutes in a Potter-Elvehjem homogenizer. For intraportal injections, .2 cc of these emulsions were used. This volume contained .5 mg of glyceryl monostearate, 10 mg glucose, .01 cc or less of ethanol, and the antioxidants in doses ranging from .0125 to 1.0 mg. Each substance was first screened with 2 animals at the 1 mg dose level. If reversion of respiratory decline resulted, 0.2 mg or less were tested. A more detailed dose-response curve was made with DPPD; levels between .0125 and 1 mg were assayed in order to establish an exact comparison with D,L-α-tocopherol.

TABLE I. Effect of Intraportal Antioxidants on Respiratory Decline of Liver Slices.

Dose in mg	No. of rats	Q_{O_2} (F100)† A — Pre-inj. control — 0-30 min.	A — Pre-inj. control — 100-120 min.	Q_{O_2} (F100)† B — 30-min. post-inj. — 0-30 min.	B — 30-min. post-inj. — 100-120 min.	% respiratory decline‡ dA	% respiratory decline‡ dB	Reversion of respiratory decline§ R
				None*				
—	4	335	92	344	86	72	75	0 (−6 ± 9.3)
				Antabuse (tetraethylthiuram disulfide)				
.2	4	328	108	295	95	71	69	0 (−2 ± 9.0)
1.0	4	346	107	340	230	66	31	55 ± 10
				DBPC (2,6-di-tert-butyl-4-methylphenol)				
.2	4	324	152	368	145	53	61	−17 ± 13
1.0	4	298	69	358	253	73	22	74 ± 12
				Hydroquinone‖				
1.0	2	365	207	322	114	44	66	−53 ± 29
				Amylhydroquinone (2,5-di-tert-amylhydroquinone)				
.1	4	292	96	272	91	68	68.5	0 (1 ± 9)
.2	4	332	89	350	299	73	13	84 ± 15
1.0	2	318	71	314	308	78	1	97 ± 5
				Santoquin (6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline)				
.1	4	338	99	368	178	70	51	27 ± 5
.2	4	314	151	351	285	53	18	65 ± 14
1.0	4	324	159	328	326	51	3	96 ± 11
				Santoflex B (6-phenyl-1,2-dihydro-2,2,4-trimethylquinoline)				
.1	6	280	107	324	273	63	18	71 ± 13
.2	4	281	113	293	272	61	7	87 ± 4
1.0	4	303	119	306	280	61	8	87 ± 5
				DPPD (N,N'-diphenyl-p-phenylenediamine)				
.0125	6	344	120	337	181	65	46	31 ± 9
.01875	6	314	96	322	270	68	16	75 ± 7
.025	7	306	95	303	273	69	10	88 ± 8
.05	6	322	61	353	339	81	5	94 ± 2
.1	2	324	129	306	293	59	4	92 ± 7
.2	6	273	115	320	307	62	3	97 ± 7
1.0	2	294	111	303	315	63	−4	107 ± 7
				D,L-α-tocopherol				
.025	4	297	126	298	158	57	45	19 ± 13
.05	6	311	56	335	202	82	41	50 ± 5
.075	4	320	88	327	264	73	19	73 ± 8
.2	4	315	150	322	310	54	3	94 ± 3

* Emulsifying medium without supplement.

† Q_{O_2} (F100) = oxygen consumption (in μl)/hr/100 mg of liver slices; values were calculated for the first 30 min. intervals, and the 100- to 120-min. intervals of incubation (averages, duplicate determinations).

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

§ R = dA minus dB, expressed in % of dA (mean ± stand. error).

‖ Toxic; see text.

Results. It is well established that normal liver slices maintain their initial respiratory activity over extended periods of Warburg experimentation‖. Slices from animals during the latent phase of necrotic liver degeneration, by comparison, show a rapid loss of respiratory activity (Table I). After 2 hours of incubation, O_2 consumption has on the average declined to ca 30% of the initial 30-minute value. Intraportal injection of the emulsion without supplement neither influenced initial respiration nor respiratory decline of post-injection slices, as compared to pre-injection controls. This indicated that there was no intrinsic difference between 2 lobes of the same

‖ Under prevailing experimental conditions, normal liver slices maintain respiration over 4-6 hrs of incubation at normal or only slightly altered levels.

liver with respect to respiratory decline, and that the emulsifying agents, by themselves, were without influence on the phenomenon¶.

In the dietary studies the compounds used were roughly classified into 3 groups(4). Group I contained substances which may directly or indirectly act as antioxidants, but which are better known for other properties (ascorbic acid, methylene blue, and Antabuse). Some of these showed limited activity when fed. Group II contained antioxidants of phenolic character which are widely used for stabilization of fats and other materials, but which were completely inactive when fed (NDGA, n-Propylgallate, DBPC, BHA, Propylparasept, and also hydroquinone). Group III contained those antioxidants which protected against liver necrosis (di-tert-amylhydroquinone, Santoquin, Santoflex B, and especially DPPD).

In the test against respiratory decline, ascorbic acid and methylene blue were ineffective when injected intraportally at the 1 mg level. Higher doses were not used.** Feeding of high levels of these 2 substances had afforded roughly 30% protection. Antabuse, on the other hand had shown no significant effect in dietary studies, but produced an effect on respiratory decline; approximately 55% reversion was obtained with 1 mg. It is possible that absorption from the gastrointestinal tract may be insufficient in cases where intraportal injection is relatively more effective than feeding.

Five antioxidants of Group II, inactive when fed in the diet were also ineffective after intraportal injection, with the exception of DBPC. The latter produced 74% reversion of respiratory decline when 1 mg was injected. This observation is of interest in connection with the finding by Bunnell, *et al.* that DBPC is comparatively effective in preventing exudative diathesis in chickens(6).

Unsubstituted hydroquinone was toxic; it appeared to enhance respiratory decline. Animals injected with 1 mg hydroquinone recovered poorly, slight convulsions were observed, and consciousness was not completely regained. After the 30-minute interval, the livers showed a deep-red color, with hemorrhagic spots of pin-point size. Conversely, a sterically hindered hydroquinone, di-tert-amylhydroquinone, was found to be quite effective. It reversed respiratory decline practically completely with 1 mg, whereas .2 mg and .1 mg doses caused 84, and 0% reversion, respectively. The effect is in agreement with the activity seen in the dietary assay; the substance prevented liver necrosis when added at .25% to the diet.

The activities of the 3 last substances of Group III are in accord with those of the dietary test: Santoquin at the 1.0, .2, and .1 mg dose level produced 96, 65 and 27% reversion, respectively. It was less active than Santoflex B. Chemically, the two compounds are closely related, Santoflex B containing a phenyl group instead of an ethoxy group. In the dietary assay, Santoflex B had demonstrated an anomaly in that 28% protection had been obtained with .05% in the diet, but dose levels up to .25% had not yielded increased effects. It is evident that this limitation of activity in the dietary assay was eliminated by intraportal application.

Of all antioxidants tested intraportally, DPPD had by far the strongest effect. With doses between .05 and 1 mg, practically 100% reversion of the respiratory lesion was obtained, and 25, 18.75, and 12.5 μg produced 88, 75 and 31% reversion, respectively. This activity was compared to that of D,L-α-tocopherol emulsions assayed concurrently. DPPD was more effective than Vit. E. Approximately 14.5 μg of DPPD were required for 50% reversion as compared to ca 50 μg of D,L-α-tocopherol for the same effect. On a molar basis, DPPD was 2 times as effective as Vit. E in this test system.

The results obtained with tocopherol are quite comparable to those reported for the

¶ Antioxidants did not influence O_2 consumption of normal liver slices, except for hydroquinone which induced a decline.

** Methylene blue changed the liver to a deep blue immediately after intraportal injection, but the color disappeared after a few minutes. After 30 minutes the liver had its normal appearance. In one instance, the dye remained unchanged in one lobe; in this lobe, complete reversion of the respiratory lesion was found.

Sprague-Dawley strain of rats(4), except that the vitamin shows a somewhat higher activity in the Fischer strain. This is in accordance with the fact that for dietary prevention of liver necrosis the Fischer rat requires only half as much Vit. E as the Sprague-Dawley strain. In chickens on a diet producing encephalomalacia, intramuscularly injected DPPD was reported to be approximately equivalent to α-tocopherol(6). In our assay system, it is clearly twice as potent as the vitamin.

The results show that the series of 13 antioxidants, tested previously by dietary supplementation, maintained with few exceptions the same relative order of activity when tested against respiratory decline by the intraportal route. It follows that antioxidants which protect against liver necrosis are not simply preventing rancidity of the diet, or autoxidation in the gut; they act within the organism. The effect is not merely an antioxidant effect in the general sense. More specific physical or chemical properties are necessary. On the basis of data on hand, it cannot be decided whether the active compounds ultimately substitute for Vit. E, *e.g.*, in enzyme reactions involving electron transfer, or whether they act through traces of tocopherol in the tissue. The fact that DPPD is twice as active as Vit. E speaks for the first possibility but does not entirely eliminate the second one. The observed effects seem to be analogous to those obtained with methylene blue and other dyes in enzymatic electron transfer systems.

Summary. The effects of intraportal injections of antioxidants on respiratory decline in dietary necrotic liver degeneration were investigated by comparing respiration of liver slices from rats before, and 30 minutes after injection. Several antioxidants (DPPD, Santoquin, Santoflex B, and di-tert-amlhydroquinone) were like α-tocopherol quite effective in the reversal of respiratory decline at doses of 1 mg or less, while others (NDGA, n-Propylgallate, BHA, Propylparasept, hydroquinone) were without any effect. Screening of 13 different compounds led with but few exceptions to the same scale of relative potencies as that obtained previously for protection against necrotic liver degeneration by feeding of the antioxidants in the necrogenic diet. Intraportal injection of 14.5 μg of DPPD was required to produce 50% reversal. On a molar basis, DPPD was twice as active as D, L-α-tocopherol. It is concluded that the protective effect against necrotic liver degeneration is not due to the prevention of autoxidation in the diet or in the gut; rather, it takes place within the organism itself.

Acknowledgement is made to Esther M. Hurley and Roswell A. Taylor, Jr. for efficient technical assistance.

1. Chernick, S. S., Moe, J. G., Rodnan, G. P., Schwarz, K., *J. Biol. Chem.*, 1955, v217, 829.

2. Rosecan, M., Rodnan, G. P., Chernick, S. S., Schwarz, K., *ibid.*, 1955, v217, 967.

3. Rodnan, G. P., Chernick, S. S., Schwarz, K., *ibid.*, 1956, v221, 231.

4. Schwarz, K., PROC. SOC. EXP. BIOL. AND MED., preceding paper. (For technical reasons this paper will appear in October 1958 issue.)

5. ———, *ibid.*, 1951, v77, 818.

6. Bunnell, R. H., Matteson, L. D., Singsen, L. P., Potter, L. M., Kozeff, A., Jungherr, E. L., *Poultry Sci.*, 1955, v34, 1068.

Received May 14, 1958. P.S.E.B.M., 1958, v98.