which have been opsonized in serum stored at −20 °C or in serum stored at +4 °C cause the same level of oxygen uptake when exposed to neutrophils but that only the zymosan opsonized in −20 °C serum initiates significant cytochrome C reduction[3]. Thus there appears to be a thermolabile substance in serum which stimulates neutrophils to reduce cytochrome C. That neutrophils could produce a cytochrome C-reducing agent without change in oxygen uptake suggests that the compound was not superoxide since, if it were, it would be expected that a variety of reductive and dismutative reactions would return oxygen to the system causing a reduction in net oxygen uptake. Preliminary studies to determine whether this unknown cytochrome C reducing agent was one of the well-known cellular antioxidants indicated that it was not ascorbic acid or glutathione since, although both reduced cytochrome C their reactions were not inhibited by superoxide dismutase (table 2). (It was noteworthy that glutathione was a much stronger cytochrome C-reducing agent when chelated with calcium or magnesium than in their absence.) The unknown compound could however be a protein with one or more reactive sulphydryl groups.

Present results indicate that the properties of the agent responsible for cytochrome C reduction in FMLP stimulated neutrophils are consistent with those expected of superoxide. Since FMLP is a chemotactic agent these results support a previous suggestion that superoxide output may have more to do with chemotaxis than with phagocytosis[3].

It has been accepted for many years that stimulation of neutrophils by a variety of particulate and soluble agents[4-8] gives rise to the production of superoxide radicals (cytochrome C reduction). The current work suggests that further examination of such cytochrome C-reducing activity is necessary before it may be unequivocally attributed to superoxide.

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0014-4754/83/010061-02$1.50 + 0.20/0
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Indication of reduced doxorubicin-induced cardiac toxicity by additional treatment with antioxidative substances

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Summary. The influence of antioxidative substances on doxorubicin-induced cardiac toxicity was studied in C 57 BL mice. Tocopherol (500 mg/kg), glutathione (1000 mg/kg), cysteamine (15 mg/kg) and L-cysteine (1000 mg/kg), injected i.p. 24 h before doxorubicin treatment (15 mg/kg i.p.) were able to reduce malonaldehyde production in cardiac tissues significantly. SH-containing substances with high reducing activity, such as vitamin E, could be a useful tool in clinical trials to prevent doxorubicin induced cardiac damage.

Doxorubicin is now part of the chemotherapeutic regimen for many hematopoetic malignancies as well as for a variety of solid tumors[1]. Until now treatment with this agent is still limited by a potentially lethal and dose-dependent congestive cardiomyopathy[2]. Although the etiology of doxorubicin-induced cardiomyopathy has not been clearly elucidated, there is some evidence for increased superoxide anion and hydrogen peroxide formation in mitochondria and sarcoplasmatic reticulum after doxorubicin treatment[3]. The accumulation of drug-induced reactive oxygen radicals in heart cells may explain the increased cardiac lipid membrane peroxidation[3], and the reduced cardiac glutathione pools that have been found after doxorubicin treatment[4]. Since sulfhydryl groups play an important role in the maintenance of muscular contractile function[5] and membrane integrity[6], and also promote the non-enzymatic detoxification of hydroxyl radicals[7] and lipid peroxides[8], it was previously suggested that augmenting sulfydryl group content in the heart might enhance the ability of heart muscle to withstand doxorubicin exposure[9].

In this study we investigated the activity of some antioxidative substances on doxorubicin induced lipid peroxidation in vivo.

Material and methods. Male C 57 BL mice had continuous access to food (Altromin 1324) and water and weighed 18–22 g when used. Animals were divided into 10 groups of 15 mice each. Animals in group 1–3 (6–8 weeks old) were treated with 15 mg/kg doxorubicin hydrochloride (Farmitalia) i.p. and killed by cervical dislocation at 24 h (group 1), 48 h (group 2) and 72 h (group 3) after i.p. injection of the anthracycline. Animals in group 4–6 were untreated, but of different ages; group 4: under 6 weeks old; group 5: 6–8 weeks old; group 6: more than 24 weeks

Table 1. Influence of doxorubicin treatment (15 mg/kg i.p.) on malondialdehyde production in C 57 Bl mice (6–8 weeks old)

Hours[a]	Malondialdehyde (nmole/g wet wt)[b] Heart	Liver
Controls	2.5- 3.1 (2.74± 0.33)	2.6- 3.5 (2.85±0.40)
24	9.9-14.2 (12.42± 1.59)	3.3- 5.1 (4.2 ±0.69)
48	12.4-21.5 (17.04± 3.67)	8.0-10.2 (8.8 ±0.96)
72	51.0- 157 (90.80±43.6)	4.0-13.5 (8.1 ±3.58)

[a]Time is calculated from doxorubicin i.p. injection. [b]Range and mean ± SD (n = 5; 3 mice in each group).

old. Animals in group 7–10 were pretreated by i.p. application of antioxidative substances 24 h before the i.p. injection of doxorubicin; group 7: 500 mg/kg tocopherol (specially prepared by Hoffmann-La Roche, Basel); group 8: 1000 mg/kg reduced glutathione (Merck); group 9: 150 mg/kg cysteamine (Fluka) and group 10: 1000 mg/kg L-cysteine (Merck). 72 h after the i.p. injection of the cytostatic mice were killed and prepared for the estimation of malonaldehyde content in heart and liver. After rapid removal of the organs, the tissues were washed in ice phosphate-buffered saline, pH 7.4, blotted dry and weighed in groups of 3 mice. Each group of hearts (each liver) was disrupted with a Polytron-homogenizer in 2 ml of 0.02 M potassium phosphate buffer, pH 7.4, containing butylated hydroxytoluene (0.5 mg/100 ml) to prevent futher oxidation of lipids. The mixture was transferred quantitatively into a Kjeldahl flask (25 ml) by washing with an additional 7.5 ml distilled water, and brought to pH 1.5 by adding 0.5 ml 2 N HCl. After the addition of a small amount of silicon-antifoam and a few saddle-stones to prevent bumping, the micro-distillation-apparatus was assembled and the flask heated in a glycerine bath at 120 °C. After distillation of 5 ml of the original volume (at 100 °C) the procedure was stopped. 2 ml of the distillate were mixed with 2 ml 2-thiobarbituric acid (TBA) (0.02 M in 90% glacial acetic acid) and incubated in a water bath at 95 °C for 35 min. A distilled water-TBA-mixture was treated the same way and used as a blank. After the incubation period, samples were cooled and transferred to a cuvette. The optical density was read against the blank at a wavelength of 538 nm. The identification of the distilled substance was carried out by application of an aliquot of 0.5 ml to a Sephadex G-10-column (1.0×40 cm), which was eluted with 0.05 M tris HCl buffer, pH 7.4, containing 0.1 M NaCl. Afterwards the TBA-test, described above, was performed with each 1 ml fraction. The results of these chromatograms were compared with the chromatograms of a malondialdehyde standard prepared from 1,1,3,3-tetraethoxypropane (Sigma) by acid hydrolysis as described elsewhere[10].

Results. The physical condition of the mice after application of 15 mg/kg doxorubicin was good until 72 h after the i.p. injection of the drug; neither diarrhoea nor vomiting nor signs of heart failure could be seen. Until sacrifice, no significant influence on body- and heart-weight could be detected in the treated group compared with the untreated animals. The TBA-reactive material of the distillates was identified by Sephadex G-10 column chromatography in the same fraction as the malondialdehyde standard produced by acid hydrolysis from 1,1,3,3,-tetra-ethoxypropane (fig. 1). UV-absorption spectra of distillates were identical

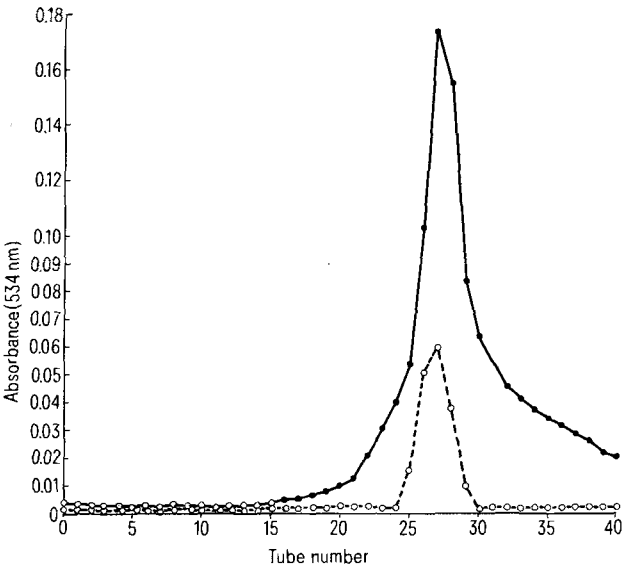

Figure 1. Sephadex G-10-chromatography of (●) malondialdehyde standard (0.5 ml: 2×10^{-5} M/l) and (○) distillate (0.5 ml) from cardiac tissue of doxorubicin treated B 57 BL mice. Samples were collected in 1 ml fractions and used for thiobarbituric acid assay.

Table 2. Influence of age on malondialdehyde concentration in cardiac tissues of C 57 Bl mice (untreated)

Age	Malondialdehyde (nmole/g wet wt)[a]
6 weeks	2.1- 4.2 (2.82±0.82)
6–8 weeks	2.5- 3.1 (2.74±0.33)
>24 weeks	17.2–31.1 (23.04±5.47)

[a] Range and mean ± SD (n = 5; 3 mice in each group).

Table 3. Influence of i.p. injection of SH-containing substances (24 h prior to doxorubicin application) on the malondialdehyde-content of cardiac and hepatic tissues in C 57 Bl mice. All tests were performed 72 h after i.p. doxorubicin injection (15 mg/kg)

Group[c]	Treatment	Malondialdehyde(nmole/g wet wt)[a] Heart	Liver
A	Controls[b]	2.74± 0.33	2.85±0.40
B	Doxorubicin	90.80±43.77	8.1 ±3.58
C	Tocopherol+ doxorubicin	11.56± 1.82	5.88±2.15
D	Glutathione+ doxorubicin	11.50± 2.55	5.86±1.49
E	Cysteamine+ doxorubicin	12.94± 2.47	5.86±1.59
F	Cysteine+ doxorubicin	8.54± 1.08	4.0 ±0.81

[a] Values are means ± SD (n = 5; 3 mice in each group). [b] Controls were not treated at all. [c] Statistical analysis were done by means of student's t-test: Heart: B/C: $p<0.01$; B/D: $p<0.01$; B/E: $p<0.01$; B/F: $p<0.01$. Liver: B/C: N.S.; B/D: N.S.; B/E: N.S.; B/F: N.S.

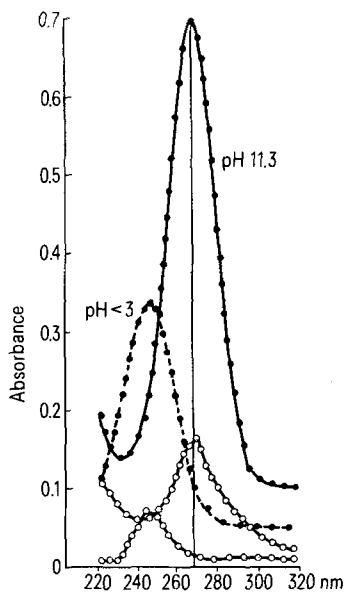

Figure 2. UV-absorption spectra of (●) malondialdehyde standard and (⊖) distillate of cardiac tissue of doxorubicin treated mice at pH < 3 and 11.3.

with those of malondialdehyde standard preparations in aqueous solution (fig. 2). The molar extinction coefficient for malondialdehyde was calculated from a standard curve at pH 11.3 ($\varepsilon = 6.68 \times 10^7$ cm/mole).

Malondialdehyde could be detected in normal, untreated murine cardiac and hepatic tissues in very small amounts using the method described above. 48 h after i.p. injection of 15 mg/kg doxorubicin malondialdehyde concentrations reached a peak level in the liver and at 72 h in the heart (table 1). There was distinct evidence for higher malodialdehyde concentrations in heart than in liver-tissues of treated animals. Untreated C 57 BL mice of different ages showed different malondialdehyde concentrations in heart tissue, indicating an increased lipid peroxidation in untreated older animals (table 2).

The process of doxorubicin induced malondialdehyde production in heart tissue could unequivocally be reduced by pretreatment with antioxidative substances (table 3).

Discussion. Up to now, only Myers et al.[3] have produced evidence of lipid peroxidation taking place in vivo; they found an increase in the malondialdehyde content of the hearts of doxorubicin-treated mice with respect to untreated controls. Their published method is difficult to perform and not easily reproducible because of great losses of malondialdehyde by the lyophilization process of the homogenized tissue extract (unpublished observation in our own laboratory). To investigate the effect of antioxidative substances on the potency of doxorubicin as a lipid peroxidation inducer we adapted a distillation method used in food research[11] to our requirements. With this micro-Kjeldahl-distillation apparatus we were able to detect malondialdehyde even in normal mice in very low concentrations and were able to investigate easily the protective activity of some SH-containing substances as possible inhibitors of doxorubicin-induced lipid peroxidation. Our findings that the malondialdehyde content of cardiac tissues is dependent on the age of untreated animals gives support to the observation that age-dependent accumulation of lipid peroxides follows as a consequence of increased radical formation in mitochondria[12]. Doxorubicin treatment can induce malondialdehyde production mainly in cardiac tissues of mice. This effect can be inhibited to a large extend by i.p. injection of tocopherol, glutathione, cysteamine and cysteine 24 h before doxorubicin-treatment. The smaller malondialdehyde content in hepatic tissues after doxorubicin-treatment could be the consequence of the existing higher content and turnover of reduced glutathione in the liver compared with the heart[13], which could be a protecting factor against this treatment. We conclude from our results that there is good evidence to justify clinical trials of the application of antioxidative substances in doxorubicin treatment in order to diminish drug-induced cardiac toxicity.

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0014-4754/83/010062-03$1.50+0.20/0
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On the failure to detect haemosiderin in the melano-macrophages of dogfish *Scyliorhinus canicula* (L.) after prolonged starvation

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Summary. When teleost fish are starved, the number of melano-macrophages increases markedly within the spleen and kidney. This increased pigment deposition is almost certainly a consequence of catabolic tissue breakdown. One of the pigments, haemosiderin, resulting from the breakdown of haemoglobin of red blood cells, accumulates almost exclusively in the melano-macrophages of the spleen but not within the kidney melano-macrophages. In contrast when elasmobranchs, as exemplified by the dogfish *Scyliorhinus canicula* are starved, melano-macrophages accumulate predominantly in the liver and to a lesser extent in the spleen. However no haemosiderin deposits could be detected in the melano-macrophages of either of these two organs. This is suggestive of functional differences between the melanomacrophages of elasmobranchs and teleosts.

Cells with a macrophage-like morphology and containing abundant amounts of pigments are a common feature of certain fish tissues. These pigments can be of the melanin, haemosiderin, lipofuscin or ceroid series and all 4 pigment types can occur in one and the same cell. While in cartilaginous fish and in primitive bony fishes these cells are found mainly in the liver, in the advanced bony fishes they are more abundant within the 2 main haemopoietic organs, the spleen and kidney. Also, whereas in all bony fishes (except the salmonids) these cells accumulate in large numbers to form discrete centres within which are also found leucocytes, in salmonids and cartilaginous fish they are randomly distributed throughout the tissues[2,3].

Starvation experiments with brown trout *Salmo trutta,* rainbow trout *Salmo gairdneri,* European eels *Anguilla anguilla,* plaice *Pleuronectes platessa,* swordtails *Xiphophorus helleri* and many *Tilapia* and *Sarotherodon* species have shown that prolonged periods of starvation result in considerably increased deposition of melano-macrophages within the spleen and kidney[4,5]. Concomitantly there is noted