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PARAQUAT-INDUCED ALTERATIONS OF PHOSPHOLIPIDS AND GSSG-RELEASE IN THE ISOLATED PERFUSED RAT LIVER, AND THE EFFECT OF SOD-ACTIVE COPPER COMPLEXES

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Abstract—The possibility that generation of active oxygen species and lipid peroxidation are involved in paraquat toxicity was examined through the use of the superoxide dismutase-active, low molecular weight copper complexes $Cu(tyr)_2$ and Cu–penicillamine. In addition, it was investigated whether the oxidation of glutathione is directly associated with paraquat-induced lipid peroxidation. An increase in diene absorption and a decrease in phospholipids of mitochondria and microsomes isolated after perfusion of rat livers with paraquat could be observed. These effects may be explained by lipid peroxidation. $Cu(tyr)_2$ abolished these effects of paraquat. In contrast, the increase of GSSG-release into the perfusate upon paraquat-treatment could not be influenced by Cu–penicillamine, and was only partially inhibited by $Cu(tyr)_2$. Therefore the GSSG-release cannot be the result of paraquat-induced generation of O_2^- or lipid peroxidation and seems more likely to be caused by the NADPH depleting action of paraquat. It is proposed that the alterations suggesting lipid peroxidation may only be observed if hepatic glutathione content decreased below a critical value.

Besides its toxicity against plants, the herbicide paraquat (1,1′-dimethyl-4,4′-bipyridylium dichloride, PQ) is also toxic for humans and animals, damaging lung, kidney and liver, where the lung is most severely affected.

In mammalian cells paraquat is readily reduced to the free radical by a one electron transfer. This reduction is NADPH dependent and enzyme catalyzed [1]. The paraquat radical may react with molecular oxygen forming the superoxide radical ($O_2^{\cdot}$) [2], which in turn dismutes spontaneously or catalyzed by superoxide dismutase (SOD) yielding H_2O_2 and O_2. H_2O_2 and $O_2^{\cdot}$ compounds may interact in a metal-catalyzed 'Haber–Weiss' reaction generating the reactive hydroxyl radical ($HO^{\cdot}$), followed by free radical chain reactions, e.g. lipid peroxidation.

The production of O_2^- and H_2O_2 in lung microsomes after addition of paraquat could be demonstrated by Montgomery [3]. It was also proposed that singlet oxygen ($^1O_2$) is another reactive oxygen species which may initiate lipid peroxidation [4].

Described by some investigators as the basis of the herbicidal action of paraquat [5], lipid peroxidation has been thought to be initiated by one or more of the above described oxygen species. Based on this concept experiments with antioxidants, radical- and $^1O_2$-scavengers and superoxide dismutase have been performed to demonstrate the involvement of O_2-radicals in paraquat toxicity [1, 6].

Despite several indications in favour of the above mechanism there are also many doubts about it (for a review see [7]). The NADPH dependent lipid peroxidation in microsomes from rat lung [8] and rat liver [9] was not stimulated but rather inhibited by paraquat. Shu *et al.* [10] showed that pretreatment of mice with an antioxidant prevented the stimulatory effects on lipid peroxidation but did not protect the animals against its lethal effects. Thus the question arose whether the mechanism of paraquat-induced lipid peroxidation *in vitro* could be related to its toxicity *in vivo*.

In a recent study [11] paraquat-induced alterations in lipid metabolism which may be explained by lipid peroxidation were observed in isolated perfused rat liver, representing a model closer to *in vivo* conditions as compared to isolated cell organelles. Cu–penicillamine, a stable, low molecular weight copper complex with superoxide dismutase activity [12], was able to diminish some of the PQ-induced effects indicative of lipid peroxidation, like diene conjugation and decrease in phospholipid content of mitochondria and microsomes. It was concluded that PQ causes generation of $O_2^{\cdot}$ which may be the precursor of an initiating oxygen species catalyzing lipid peroxidation.

In the present study besides Cu–penicillamine another 'artificial superoxide dismutase', $Cu(tyr)_2$, was used to investigate a possible involvement of O_2^- and lipid peroxidation in paraquat toxicity. $Cu(tyr)_2$, in pulse-radiolysis studies [13] was shown to exhibit SOD-activity comparable to the native enzyme. It has also been successfully used to study the generation of $O_2^{\cdot}$ in regions where the native SOD was unable to reach due to limited accessibility [14].

As paraquat reduced the GSH-content in mouse liver [15], and lung toxicity of paraquat was enhanced in selenium-deficient rats [16], we examined GSH-

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oxidation and GSSG-release from perfused livers to determine whether these are the consequences of PQ-induced lipid peroxidation and whether this reaction could be inhibited by SOD-active copper complexes.

METHODS

Perfusion procedure. Livers (7–9 g) from 3 months old (200–250 g), male Wistar rats were perfused in a hemoglobin-free non-circulating system [17]. To get a uniform metabolic basis we used 24-hr fasted rats. The perfusion medium was Krebs–Henseleit bicarbonate buffer, pH 7.6, containing 0.1% glucose, saturated with an oxygen–carbon dioxide mixture (95/5). The perfusion flow was 4 ml/min × g liver. All controls were obtained from livers perfused 3 hr with medium alone. In PQ and PQ + Cu-complex experiments after an initial perfusion period of 20 min with medium alone, the liver was subsequently perfused with the same medium containing 1 mmole/l PQ (Sigma) or 1 mmole/l PQ + 5×10^{-8} moles/l ($Cu(tyr)_2$ or Cu–penicillamine. The whole perfusion period was 3 hr. O_2-measurements were carried out polarographically with a Clark-type electrode.

The following viability criteria based on standard methods were used to monitor the metabolic state or the perfused livers: extracellular [K^+] (5 mmoles/l), loss of lactate dehydrogenase activity (15 mU/min/g liver), lactate/pyruvate ratio of 7–9, β-hydroxybutyrate/acetoacetate ratio (0.4–0.8), respiration (2–3 μmol O_2/min × g liver).

Preparation and analyses of lipids. After perfusion livers were homogenized in sucrose medium (0.25 mole/l sucrose, 2 mmoles/l EDTA, 0.02 mole/l triethanolamine hydrochloride, pH 7.6). Mitochondria and microsomes were isolated by differential centrifugation steps at 4°. Lipids were extracted according to the method of Folch [18]. Phospholipids were separated by two-dimensional thin-layer chromatography using silica gel 60 (Merck). First run was carried out in chloroform/methanol/ammonia (25 per cent) = 65/35/5 for 2 hr, second run after drying under nitrogen in chloroform/methanol/acetone/acetic/acid water = 30/10/40/5. Phospholipids were identified in preliminary experiments by comparing with standards. In the preparative thin layer plates PC and PE could be detected as oily spots with the naked eye. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were eluted from silica gel with chloroform/methanol (1:1) and used for further analyses.

Fatty acid methyl ester of total lipids, PC and PE were prepared by transmethylation with $ZnCl_2$ in methanol (1 g/25 ml) in sealed ampoules for 8 hr at 110° under nitrogen. Esters were purified by thin layer chromatography in 1,2-dichloroethane. Fatty acid methyl esters were analyzed using a Packard gaschromatograph, model 7200/7300 equipped with a dual flame ionization detector and a Waters integrator, model 730. The chromatograph was fitted with a glass column (3 m length, 3 mm diameter) coated with 5% butanediol succinate on Chromosorb W (AW-DMCS), 100–120 mesh (Packard). Detector and injection temperatures were 260°, the column oven was programmed from 100–225° at a rate of 5°/min and a final temperature hold for 30 min until the 22:6 peak appeared.

The peaks were identified by known standards and quantified by comparing the integrated peak areas.

Phosphate analysis was made by a modified method of Fiske-Subbarow [19], protein was estimated by a Biuret method [20].

GSSG-analysis. Perfusate probes were acidified with 0.6 N perchloric acid and neutralized with KOH after cooling for 30 min. GSSG-content was measured as described by Sies and Summer [21] using GSH-reductase (Sigma). In view of the low concentrations GSSG was determined by the dual wavelength method [22] at 334–400 nm in an Aminco DW-2 spectrophotometer.

Diene conjugation. For diene conjugation measurements, lipids were carefully dried under nitrogen and redissolved in chloroform/methanol (1:1). Absorption at 235 nm was taken from a spectrum from 210 to 300 nm in an Aminco DW-2 spectrophotometer in the split beam method [22]. Extinction was referred to mg of lipid.

Copper complexes. $Cu(tyr)_2$ was synthesized as previously described [13], (mol. wt. 425). Cu–penicillamine was prepared according to Lengfelder *et al.* [12] (mol. wt. 2,500).

RESULTS

In order to test whether $Cu(tyr)_2$ was able to penetrate liver cell membrane, copper analysis by atomic absorption was performed before and after the liver perfusion. When perfusion medium contained 10^{-4}, 10^{-5} or 10^{-6} mole/l $Cu(tyr)_2$, 50, 20 and 5 per cent of the initial copper concentration could be detected in the perfusate after the liver, respectively. Since the concentration in the perfusion medium was 5×10^{-8} mole/l $Cu(tyr)_2$ we can assume that nearly all $Cu(tyr)_2$ has been taken up by the liver. The uptake of Cu–penicillamine was shown by pulse radiolysis [11].

It is worth mentioning that the copper complexes are toxic *per se*. Viability criteria (see Methods) indicated a strong liver damage already after 20 min of perfusion with a concentration of 10^{-4} mole/l and after 100 min of perfusion with 10^{-6} mole/l of the copper complexes. The further used concentration of 5×10^{-8} mole/l, however, showed really no influence on viability criteria during the perfusion period of 3 hr.

Lipid and phospholipid content. Perfusion of livers with paraquat resulted in a decrease of the lipid/protein ratio in mitochondria and microsomes, indicating loss of lipids (Table 1). This loss was especially pronounced in the phospholipid fraction as shown by the phosphate analyses of isolated lipids. The decrease was about 35 per cent in mitochondria and nearly 50 per cent in microsomes. $Cu(tyr)_2$, but not $CuSO_4$, was able to inhibit the PQ-induced changes in phospholipid content. Perfusion with $Cu(tyr)_2$ alone had no effect (Table 1).

Diene absorption. Increased absorption at 235 nm of isolated and purified lipids of mitochondria and microsomes was measured and taken as an indication

Table 1. Lipid, phospholipid and protein analyses of mitochondria and microsomes obtained from livers after 3 hr perfusion with 0.1% glucose containing Krebs–Henseleit medium, pH 7.6, as control, medium + 1 mmole/l PQ, medium + 5×10^{-8} mole/l $Cu(tyr)_2$ and medium + 1 mmole/l PQ 5×10^{-8} mole/l $Cu(tyr)_2$

	Control	$Cu(tyr)_2$	PQ	PQ + $Cu(tyr)_2$
Mitochondria				
Lipid/protein ratio	0.34 ± 0.02	0.35 ± 0.07	0.26 ± 0.02*	0.38 ± 0.02†
Phospholipid-phosphate (μmoles/mg protein)	0.199 ± 0.020	0.184 ± 0.028	0.131 ± 0.015*	0.265 ± 0.028†
Microsomes				
Lipid/protein ratio	0.74 ± 0.06	0.84 ± 0.09	0.52 ± 0.09*	0.68 ± 0.05†
Phospholipid-phosphate (μmoles/mg protein)	0.560 ± 0.053	0.637 ± 0.074	0.296 ± 0.057*	0.481 ± 0.070

Phospholipid is expressed as phosphate content in purified lipids. Values are mean ± S.E.M. (n = 4–6).
* Significantly different from control ($P < 0.05$).
† Significantly different from PQ-values ($P < 0.05$).

of lipid peroxidation. As shown in Table 2, paraquat enhanced diene absorption from 0.24 to 0.3 E/mg lipid in both lipid fractions investigated. Presence of $Cu(tyr)_2$ reduced this absorption to control values. $Cu(tyr)_2$ alone had no effect. $CuSO_4$ (5×10^{-8} mole/l) could not influence the PQ-induced alterations (data not shown).

Fatty acid composition. Lipid peroxidation should result in an altered fatty acid composition, especially in a loss of polyunsaturated fatty acids. However, analysis of fatty acids in total lipids and major phospholipids as phosphatidylcholine (PC) and phosphatidylethanolamine (PE) of mitochondria (Table 3) and microsomes (Table 4) revealed no change in the fatty acid composition after paraquat treatment.

Thus, the over-all content of unsaturated fatty acids is not reduced by paraquat, as is indicated by the unsaturated/saturated ratio and the unsaturation index.

GSSG-release. A drastic elevation of GSSG-efflux from livers into the perfusate could be observed after paraquat addition to the perfusion medium (Fig. 1(a) and (b)). The maximum of GSSG-release from livers perfused with glucose (Fig. 1(a)) was reached after 40 min (20 min after PQ-addition), from livers perfused with glucose (Fig. 1(b)) after 60 min (40 min after PQ-addition). After 100 and 120 min, respectively, GSSG-release was markedly diminished, which suggests an exhaustion of GSH in the cell.

GSSG-release cannot be due to liver damage, since the sensitive parameters of liver function like extracellular potassium concentration and lactate dehydrogenase activity in the perfusate were not altered by paraquat. The same considerations apply for lactate/pyruvate and β-hydroxybutyrate/acetoacetate ratio which were <10.0 and <1.0, respectively.

O_2-consumption was only slightly lowered within 3 hr. At the end of the perfusion time it was 80 per cent of the initial value in the control perfusions (2–3 μmoles O_2/min × g liver). Addition of paraquat stimulated O_2-consumption by 20 per cent within the first hour, then it decreased to control values. So we can assume that livers were viable till the end of the perfusion period.

GSSG-release can be taken as an indication of the reaction of GSH-peroxidase with lipid hydroperoxides or with H_2O_2 [23]. If we assume this for our results and if superoxide radicals are involved in the formation of lipid-hydroperoxides, an SOD-active compound should show inhibitory effects. If GSSG-release is due to reaction of GSH-peroxidase with H_2O_2 additional SOD might stimulate GSSG-release by producing H_2O_2 from $O_2^{\cdot-}$. As shown in Fig. 1, Cu–penicillamine had no effect on GSSG-release, however, while $Cu(tyr)_2$ partially reduced GSSG-release. $CuSO_4$ showed a similar behavior as $Cu(tyr)_2$ on PQ-induced GSSG-efflux (data not shown).

The slight increase in GSSG-release by PQ + Cu–penicillamine (Fig. 1(a)) is statistically not sig-

Table 2. The effect of PQ, $Cu(tyr)_2$ and PQ + $Cu(tyr)_2$ on the diene absorption in mitochondrial and microsomal lipids. Absorption is taken from a UV spectrum from 210 to 300 nm of purified lipids dissolved in chloroform/methanol (1:1) and calculated as E_{235nm}/mg lipid

	Control	$Cu(tyr)_2$	PQ	PQ + $Cu(tyr)_2$
Mitochondria	0.237 ± 0.015	0.228 ± 0.027	0.302 ± 0.033*	0.212 ± 0.019†
Microsomes	0.207 ± 0.046	0.236 ± 0.008	0.300 ± 0.009*	0.213 ± 0.044

Values are means ± S.D. (n = 4–6).
* Significantly different from control ($P < 0.05$).
† Significantly different from PQ-values ($P < 0.05$).

Table 3. The influence of PQ and PQ + $Cu(tyr)_2$ on the composition of the main fatty acids of mitochondrial total lipids, phosphatidylcholine (PC) and phosphatidylethanolamine (PE)

Fatty acid	Control	PQ	PQ + $Cu(tyr)_2$
Total lipids			
16	15.9 ± 0.58	17.6 ± 0.74	16.2 ± 0.38
18	20.5 ± 0.42	22.7 ± 0.13	20.9 ± 0.21
18:1	5.8 ± 0.22	5.4 ± 0.20	6.3 ± 0.16
18:2	18.8 ± 1.06	16.9 ± 0.95	16.7 ± 0.83
20:4	27.1 ± 0.82	26.9 ± 0.59	27.7 ± 1.07
22:6	6.2 ± 0.28	6.2 ± 0.32	5.9 ± 0.73
Unsat/sat	1.65 ± 0.08	1.44 ± 0.04	1.59 ± 0.04
Index	200.8 ± 3.81	194.0 ± 2.04	197.0 ± 3.39
Phosphatidylcholine			
16	19.8 ± 0.85	23.1 ± 0.63	21.6 ± 0.67
18	21.6 ± 0.92	21.0 ± 0.31	19.2 ± 0.17
18:1	5.2 ± 0.04	5.1 ± 0.15	5.8 ± 0.24
18:2	13.3 ± 0.88	14.5 ± 0.36	13.7 ± 0.83
20:4	30.4 ± 0.75	27.4 ± 0.63	28.5 ± 0.61
22:6	4.4 ± 0.34	4.4 ± 0.08	4.4 ± 0.51
Unsat/sat	1.28 ± 0.03	1.16 ± 0.01	1.27 ± 0.05
Index	186.3 ± 3.5	175.4 ± 1.97	181.0 ± 4.87
Phosphatidylethanolamine			
16	15.8 ± 0.43	14.2 ± 0.68	14.7 ± 0.54
18	25.1 ± 0.39	24.7 ± 1.15	23.4 ± 0.88
18:1	5.4 ± 0.23	5.6 ± 0.30	6.0 ± 0.62
18:2	10.0 ± 0.42	11.0 ± 0.05	9.2 ± 0.83
20:4	28.6 ± 0.66	28.1 ± 0.51	30.1 ± 0.26
22:6	8.7 ± 0.29	7.3 ± 0.05	8.2 ± 0.65
Unsat/sat	1.36 ± 0.03	1.42 ± 0.02	1.45 ± 0.03
Index	206.6 ± 3.69	211.8 ± 3.02	207.9 ± 4.23

Data are expressed as per cent of total. Unsaturated/saturated fatty acid ratio is obtained by dividing the sum of per cent occurrence of unsaturated through the sum of per cent occurrence of saturated fatty acids. Unsaturation index is calculated as the sum of per cent occurrence multiplied with the number of double bonds of the corresponding fatty acid. Values are means ± S.E.M. (N = 4–6).

Table 4. Influence of PQ and PQ + $Cu(tyr)_2$ on the composition of the main fatty acids of microsomal lipid fractions. Other details are the same as described in legend to Table 3

Fatty acid	Control	PQ	PQ + $Cu(tyr)_2$
Total lipids			
16	17.9 ± 0.43	17.7 ± 0.47	16.9 ± 0.20
18	23.6 ± 0.50	25.8 ± 0.80	23.2 ± 0.50
18:1	4.5 ± 4.4	4.5 ± 0.11	4.6 ± 0.25
18:2	13.2 ± 0.61	13.5 ± 0.55	13.1 ± 0.39
20:4	30.0 ± 0.73	28.7 ± 0.51	30.4 ± 0.81
22:6	6.0 ± 0.27	5.9 ± 0.47	6.0 ± 0.33
Unsat/sat	1.33 ± 0.03	1.23 ± 0.02	1.37 ± 0.03
Index	195.9 ± 3.22	189.5 ± 3.18	200.6 ± 3.30
Phosphatidylcholine			
16	19.7 ± 0.24	21.8 ± 0.70	21.6 ± 0.24
18	24.5 ± 0.99	21.8 ± 0.70	21.6 ± 0.24
18:1	4.3 ± 0.20	4.7 ± 0.06	4.9 ± 0.18
18:2	12.8 ± 0.40	14.4 ± 0.51	13.0 ± 0.48
20:4	30.3 ± 0.30	27.7 ± 0.64	30.5 ± 0.57
22:6	4.4 ± 0.30	4.6 ± 0.16	5.0 ± 0.48
Unsat/sat	1.32 ± 0.02	1.13 ± 0.02	1.31 ± 0.03
Index	184.2 ± 3.16	173.8 ± 2.06	189.0 ± 2.64
Phosphatidylethanolamine			
16	16.5 ± 0.62	16.7 ± 0.26	15.2 ± 0.30
18	26.3 ± 0.16	28.1 ± 0.40	24.5 ± 0.29
18:1	4.6 ± 0.21	4.2 ± 0.11	5.3 ± 0.22
18:2	11.0 ± 1.08	10.7 ± 0.17	11.1 ± 0.20
20:4	27.6 ± 0.82	26.8 ± 0.47	28.8 ± 0.63
22:6	7.7 ± 0.20	9.6 ± 0.17	9.6 ± 0.35
Unsat/sat	1.30 ± 0.04	1.17 ± 0.02	1.38 ± 0.02
Index	203.3 ± 4.61	198.2 ± 1.60	209.2 ± 2.04

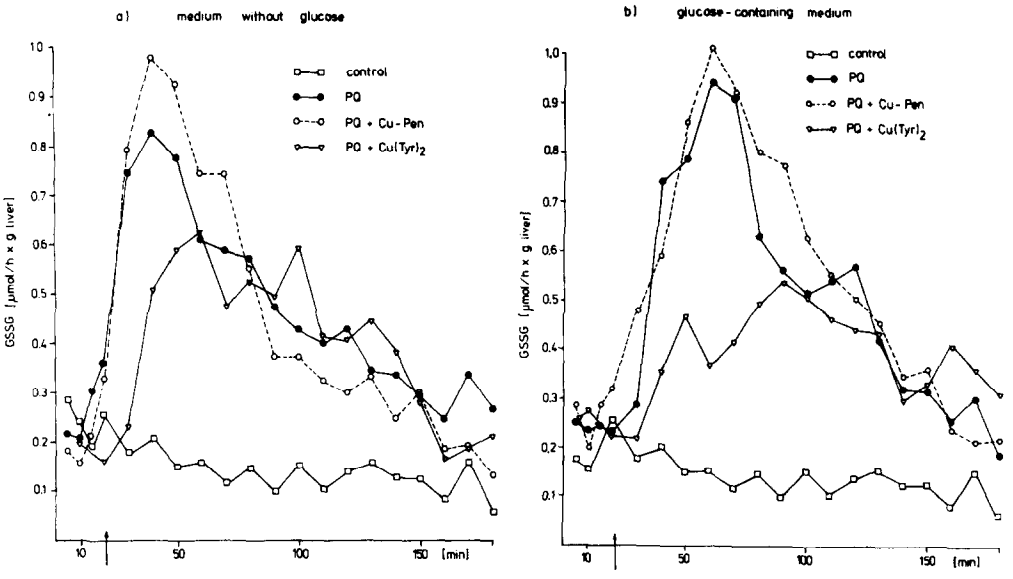

Fig. 1. GSSG-efflux from livers into the perfusate. The perfusion was carried out with Krebs–Henseleit medium, without glucose (1a) and containing 0.1% glucose (1b) as control □——□. After 20 min (arrow) to the medium were added the following compounds in a final concentration: 1 mmole/l paraquat ●——●, 1 mmole/l paraquat + 5 × 10^{-8} mole/l Cu–penicillamine ○---○, and 1 mmole/l paraquat + 5 × 10^{-8} mole/l Cu(tyr)$_2$ ▽——▽. Oxidized glutathione was estimated as described in Methods. Values are mean from 3–5 experiments. Coefficients of variation were 4–20 per cent.

nificant. Perfusion with copper complexes alone showed no difference in GSSG-release from controls (data not shown).

DISCUSSION

The intention of this study was (1) to examine the possibility that O_2^--generation and lipid peroxidation are associated paraquat toxicity through the use of the low molecular weight copper chelates, Cu–tyrosine and Cu–penicillamine which have superoxide dismutase-like activity and (2) to determine whether the oxidation of glutathione is directly associated with paraquat-induced lipid peroxidation.

Perfusion of isolated rat livers with paraquat resulted in an increase in diene conjugation, loss of phospholipids but not in a decrease of polyunsaturated fatty acids. In the presence of the superoxide dismutase-active copper complex Cu(tyr)$_2$ the PQ-induced alterations were not observed. Therefore one conclusion, though not a compelling one, may be that these alterations are due to generation of O_2^--radicals in the autoxidation of paraquat radicals.

The results of GSSG-measurements are not necessarily in agreement with the hypothesis of lipid peroxidation as a primary effect of paraquat, too.

Since Cu–penicillamine does not influence the release of GSSG, it might be produced by paraquat-induced GSH-peroxidase action on lipidhydroperoxides or on H_2O_2. Instead, one might envisage another mechanism for GSSG-release: Paraquat is reduced by NADPH-dependent reductases [1]. Electrons are removed from physiological pathways and NADPH is oxidized continuously resulting in a loss of reduced pyridine dinucleotides as observed by Witschi *et al.* (24). Furthermore by organ absorbance spectrophotometry [22] in isolated perfused rat liver the decrease in reduced pyridine dinucleotides could be detected (Brigelius and Sies, unpublished).

Thus, if no NADPH is available, endogeneously produced GSSG will accumulate in the cell and then be released in the medium. Inhibition of fatty acid synthesis due to lack of NADPH [25] may be an explanation for the decrease in lipid and phospholipid content as well as lipid peroxidation. The increase in diene conjugation is the same for mitochondria and for microsomes. As the enzymes that reduce paraquat are not the same in both cell compartments this may not be a specific effect of paraquat but an unspecific lipid peroxidation due to lack of antioxidants as it will be discussed.

The results discussed so far were that obtained with perfusion medium containing glucose. There was no difference compared to the effects resulting from perfusion with medium without glucose [11]. But the earlier observed [11] increase of arachidonic acid (20:4) in microsomal PE could not be detected in this study. An enhanced content of arachidonic acid was taken as expression of antioxidant deficiency [26]. Glucose delays the time of antioxidant leakage (Fig. 1) and it seems that in experiments with glucose liver has not yet reacted to the decreased GSH-content by stimulating the synthesis of polyunsaturated fatty acids in the three hours of the experimental period. Furthermore glucose is the substrate for the pentose phosphate pathway and it may be that the effect of glucose is the result of enhanced NADPH synthesis.

In a recent publication Wendel *et al.* [27] showed that paracetamol could only induce lipid peroxidation in mice if liver glutathione content was extremely lowered by starvation.

Based on this concept, Younes and Siegers [28] could demonstrate that depletion of GSH in rat and mouse livers leads to lipid peroxidation without any initiating agent. So we conclude from our results that the observed lipid peroxidation is a consequence of the GSH-depleting action of paraquat. In the isolated perfused organ liver cannot synthesize glutathione due to the lack of sulphur containing amino acids in the perfusion medium. In the intact organism the corresponding amino acids are available. This may be the reason why increased lipid peroxidation by paraquat could not be observed *in vivo* [10, 29].

$Cu(tyr)_2$ in contrast to Cu–penicillamine, is able to reduce GSSG-release to some extent. Perhaps this is not a result of its superoxide dismutating activity, since perfusion with $CuSO_4$ in similar concentrations showed the same effect on GSSG-release. But Cu^{2+} ($CuSO_4$) alone could not suppress diene conjugation and decrease in phospholipid content caused by paraquat. It may be that copper interacts in some way with the paraquat molecule reducing the consumption of NADPH during its redox cycle. A reaction of Cu^{2+} with disulfides and formation of complexes which are not able to be released may be possible, too. The copper complex of tyrosine may not be stable enough in biological systems. For its superoxide dismutating activity, however, it is not necessary for copper to be in a strongly chelated form. It is sufficient that both compounds are present in satisfactory concentration and close enough together (L. Schubotz, personal information). It seems that inhibition of lipid peroxidation by $Cu(tyr)_2$, unlike Cu–penicillamine, does not proceed exclusively by its SOD-activity.

Our results do not support the view that generation of superoxide radicals followed by lipid peroxidation is the only and/or basic mechanism of paraquat toxicity. It seems more likely that the removal of reducing equivalents by interfering with electron transport systems leads to an inhibition of biochemical mechanisms dependent on NADPH. So lipid peroxidation seems to be a secondary effect initiated by paraquat and can only be detected if GSH is no longer available.

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