Biochemical Pharmacology, Vol. 34, No. 2, pp. 161–166, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

THE INVOLVEMENT OF IRON AND LIPID PEROXIDATION IN THE PATHOGENESIS OF HCB INDUCED PORPHYRIA

M. A. ALLEMAN,* J. F. KOSTER,† J. H. P. WILSON,* A. EDIXHOVEN-BOSDIJK,*
R. G. SLEE,† M. J. KROOS‡ and H. G. v. EIJK‡

* Department of Internal Medicine II, † Department of Biochemistry I, ‡ Department of Chemical Pathology, Medical Faculty and University Hospital, Erasmus University, Dr. Molewaterplein 40, 3015 GD Rotterdam, The Netherlands

(*Received* 4 *April* 1984; *accepted* 2 *July* 1984)

Abstract—Hexachlorobenzene (HCB) induces a porphyria characterized by a diminished activity of the enzyme uroporphyrinogen decarboxylase (URO-D), presumably due to inactivation by reactive metabolites of HCB.

We studied the effect of iron on HCB porphyria in female rats, to determine whether the iron dependent process of lipid peroxidation was involved in the pathogenesis of porphyria. We showed that malondialdehyde formation is increased in rat liver tissue of porphyric rats and that high molecular weight proteins due to cross-linking are formed. We also showed that the induction of porphyria by HCB is dependent on the presence of iron. Our findings suggest that lipid peroxidation is involved in the toxicity of HCB and that the aggravating effects of iron on HCB are mediated by lipid peroxidation.

It is well known that hexachlorobenzene (HCB)§ is a potent inducer of porphyria in man as well as in rats [1]. This type of porphyria is similar to the disease porphyria cutanea tarda and is characterized by photosensitivity and an increased urinary excretion of uro- and heptacarboxyl porphyrins [2, 3]. In porphyria cutanea tarda a decreased activity of the enzyme uroporphyrinogen decarboxylase (URO-D) has been observed; in the inherited form both in erythrocytes and in liver tissue, in the acquired form only in liver tissue [4].

In HCB-induced porphyria in the rat this decrease in activity of URO-D also has been found [5, 6]. The exact mechanism by which HCB exerts its toxicity is not known. Recently this subject was extensively reviewed by Debets and Strik [7]. It is thought that electrophilic products appearing during the metabolism of HCB react with the catalytic site of URO-D, this leading to the inactivation of the enzyme [7, 8]. This is supported by the finding of Elder that immunoreactive URO-D is unchanged in HCB induced porphyria [9].

Administration of iron together with HCB gives an enhancement of the toxicity of HCB [10, 11]. This is further substantiated by the finding that 2,3,7,8-tetrachlorodibenzodioxine (TCDD), another polyhaloginated aromatic compound (PHA) is not toxic in iron deficient mice [12, 13]. Controls with a normal iron status develop porphyria. Furthermore Maines found evidence for the involvement of endogenous iron in lipid peroxidative destruction of heme by allylisopropylacetamide, which induces acute porphyria [14]. Lipid peroxidation is a process in which the unsaturated fatty acids, mainly localized in the phospholipids of the membranes are peroxidized [15]. In this process the fatty acids are broken down to aldehydes and carbonyl compounds, resulting in membrane damage. During this process cross-linking of membrane proteins also occurs. This leads to the formation of high molecular weight proteins (HMW proteins). Iron catalyzes this process of lipid peroxidation according to the Haber Weiss reaction and is therefore of great importance [16]. It is also claimed that iron performs its catalysation of lipid peroxidation through perferryl iron [17]. The role of iron in the process of lipid peroxidation has been the subject of an excellent review by Aust and Svingen [18]. Considering these findings in relation to iron we decided to investigate whether lipid peroxidation is involved in the development of HCB porphyria.

§ Abbreviations used: G-6-P-ase, glucose-6-phosphatase; HCB, hexachlorobenzene; HMWP high molecular weight proteins; MDA, malondialdehyde; SDS, sodium dodecyl sulphate; TBA, thiobarbituric acid; TCA, trichloro acetic acid; TCDD, 2,3,7,8-tetrachlorodioxine; URO-D, uroporphyrinogen decarboxylase.

MATERIALS AND METHODS

Animal procedures

Female Wistar rats with an initial weight of about 75 g were obtained from TNO (Zeist). Hexachlorobenzene was suspended in olive oil (100 mg HCB/ml). Six groups of rats under various conditions were studied. Three groups of rats (groups III, V, VI) received 100 mg HCB intraperitoneally on day 8. One of these groups (III) was fed iron deficient food from the first day during the whole experiment (Hope Farms, Alphen a/d Rijn, Holland) and were bled three times by cardiac puncture. One ml was taken on day 1, 2 and 3. Another group (V) received 25 mg iron dextran s.c. on day 1 and received normal food. The third group received no special treatment. Three control groups, normal chow (group I), iron deficient group (group II) and iron overload (group IV) received no HCB. The rats were sacrified on day 60.

Chromatographic quantitation and separation of porphyrins in rat liver tissue. For the determination of porphyrin concentration 1 g of rat liver was homogenized in 9 ml NaCl 154 mM/l with a Potter–Elvehjem homogenizer at 4°. The homogenate was freeze dried. The material was methylated with sulphuric acid methanol and extracted with chloroform as described before [19, 20] HPLC was performed on a siliconic acid column. Merkosorb S.I. 100 (20 μm particle diameter) was obtained from Merck (Darmstadt, Germany). Detection of porphyrins was done with a fluorometer (Perkin Elmer, L2000, Beaconsfield, U.K.). Instrument parameters were as follows: excitation wavelength 400 nm emission wavelength 625 nm, band width 8 mm at half peak height. As reference material the methylesters of protoporphyrin, coproporphyrin I and uroporphyrin I were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Their purity was checked by measuring the molar absorptivity in chloroform and also by high pressure liquid chromatography. Because methyl ester standards of hepta-, hexa- and pentacarboxyl porphyrins were not available to us, we standardized pentacarboxyl porphyrin against the coproporphyrin ester and the hepta- and hexacarboxyl porphyrins against uroporphyrin methyl ester.

Determination of liver iron

This method has been described previously [21].

Lipid peroxidation

Malondialdehyde (MDA) measurement. A 20% liver homogenate was prepared in 0.15 M KCl–5 mM Tris maleate, pH 7.4.

The amount of malondialdehyde was determined as described previously [22].

In short: to 0.5 ml liver homogenate 0.3 ml 20% TCA, 0.6 ml TBA (0.05 M), 0.1 ml butylated hydroxy-toluene (0.2%) and 0.5 ml homogenate buffer was added. After centrifugation the supernatant was heated at 100° for 8 min, cooled and the absorbance was read at 535 nM. The MDA content was expressed as nmole/mg protein using the extinction coefficient of 156.000. Protein was determined according to Lowry [23].

High molecular weight proteins (HMWP). High molecular weight proteins were detected with S.D.S. polyacrylamide gel electrophoresis as described previously [24].

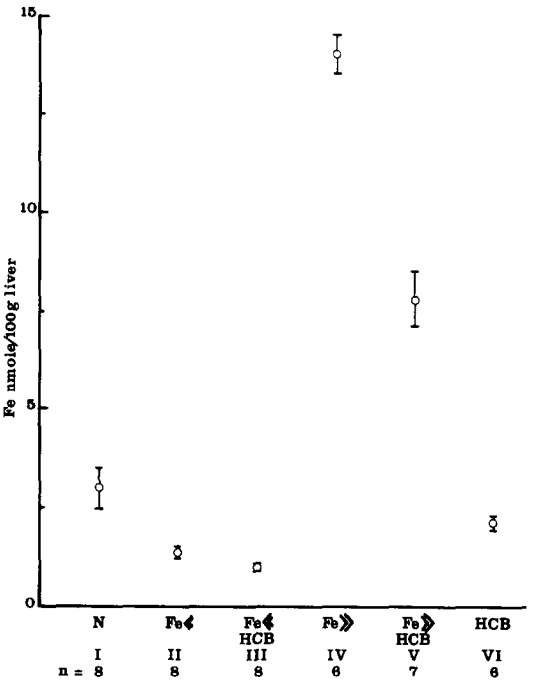

Fig. 1. Liver iron content of the various groups; as tabulated in Table 1. Statistics (Student *t*-test); I ~ II, $P < 0.009$; I ~ III, $P < 0.003$; I ~ IV, $P < 0.0005$; I ~ V, $P < 0.0005$; I ~ VI, P < N.S.; IV ~ V, $P < 0.0005$; V ~ VI, $P < 0.0005$.

Glucose-6-phosphatase (G-6-P-ase). Glucose-6-phosphatase activity was measured according to Harper [25].

RESULTS

Figure 1 shows the various iron contents of the livers obtained from iron deficient, iron overloaded and normal rats. It can be seen that the iron overloaded rats have a significantly higher content in the liver. In agreement with Schaeffer [26] rats overloaded with iron and also given HCB had a significantly lower iron content in the liver compared to the iron overload control. Schaeffer suggests that this is due to reduced absorption of iron from the small intestine following HCB administration. In contrast to iron deficient rats, the iron overloaded rats developed HCB porphyria. The liver porphyrin

Table 1. Mean uroporphyrin content of the liver measured by HPLC

Group	nmol/g net weight $\bar{X}$	S.E.M.	*	†	‡
I Normal chow	0.03	0.02			
II Iron deficient	0.00	0.00	ns	—	
III Iron deficient + HCB	0.00	0.00	ns	—	
IV Iron excess	0.07	0.01	ns	0.005	
V Iron excess + HCB	368.14	55.54	0.005	ns	
VI Normal chow + HCB	17.93	13.50	ns		0.05

* P compared to I.
† Compared to VI.
‡ V Compared to VI.

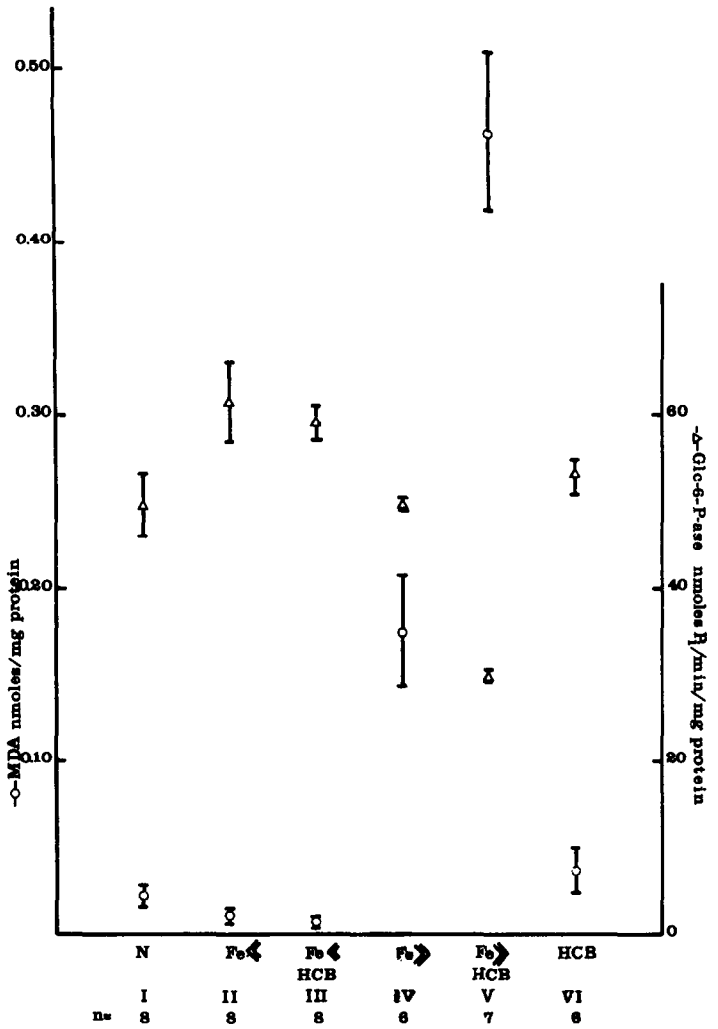

Fig. 2. Malondialdehyde formation and glucose-6-phosphatase activity in liver homogenates of the various groups; as tabulated in Table I. Statistics (Student *t*-test): M.D.A.: I ~ II, $P < 0.05$; I ~ III, $P < 0.0005$; I ~ IV, $P < 0.005$; I ~ V; $P < 0.0005$; I ~ VI, $P <$ N.S.; IV ~ V, $P < 0.002$; V ~ VI, $P < 0.0005$. G-6-P-ase: I ~ II. N.S.; I ~ III, $P < 0.05$; I ~ IV, $P <$ N.S.; I ~ V, $P < 0.0005$; I ~ VI, P N.S.; IV ~ V, $P < 0.0005$; V ~ VI, $P < 0.0005$.

content was significantly increased in the HCB-iron overloaded group (V) compared to the HCB control (VI) ($P \leq 0.05$) (Table 1).

To investigate if lipid peroxidation had occurred in the liver, the MDA content and the glucose-6-phosphatase activity was measured. MDA is the end product of the breakdown of peroxidized polyunsaturated fatty acids. During the process of lipid peroxidation the enzyme G-6-P-ase is inactivated. We found that iron overloading resulted in an increase of MDA formation (group IV), but the microsomal enzyme G-6-P-ase remained in the control range (Fig. 2). For the HCB-iron overloaded rats (group V) a tremendous increase in the MDA level occurred with a concomitant decrease in G-6-P-ase activity. The rise in MDA and the fall of G-6-P-ase activity are considered to be the result of lipid peroxidation.

Lipid peroxidation of rat liver microsomes leads not only to the MDA formation, but also to cross-linking of membrane proteins. Figures 3 and 4 shows the SDS polyacrylamide electrophoresis of the liver homogenates of groups I, IV, V and VI. All the animals of group V (HCB plus iron) showed high molecular weight protein formation, but the control groups did not. It should be mentioned that in group VI (HCB control) one rat showed formation of cross-linking of proteins while two other rats showed some formation of HMWP. It was not found in the other rat livers of this group.

DISCUSSION

It has been known for several years that iron aggravates experimental HCB-induced porphyria and it has also been described that the activity of URO-D is inhibited in the presence of iron in HCB porphyria. Our experiments show that iron is a necessary factor for the porphyrinogenic properties of HCB. This substantiates the results of Sweeney and Jones, who have found that iron deficient mice, who received TCDD, another polyhalogenated

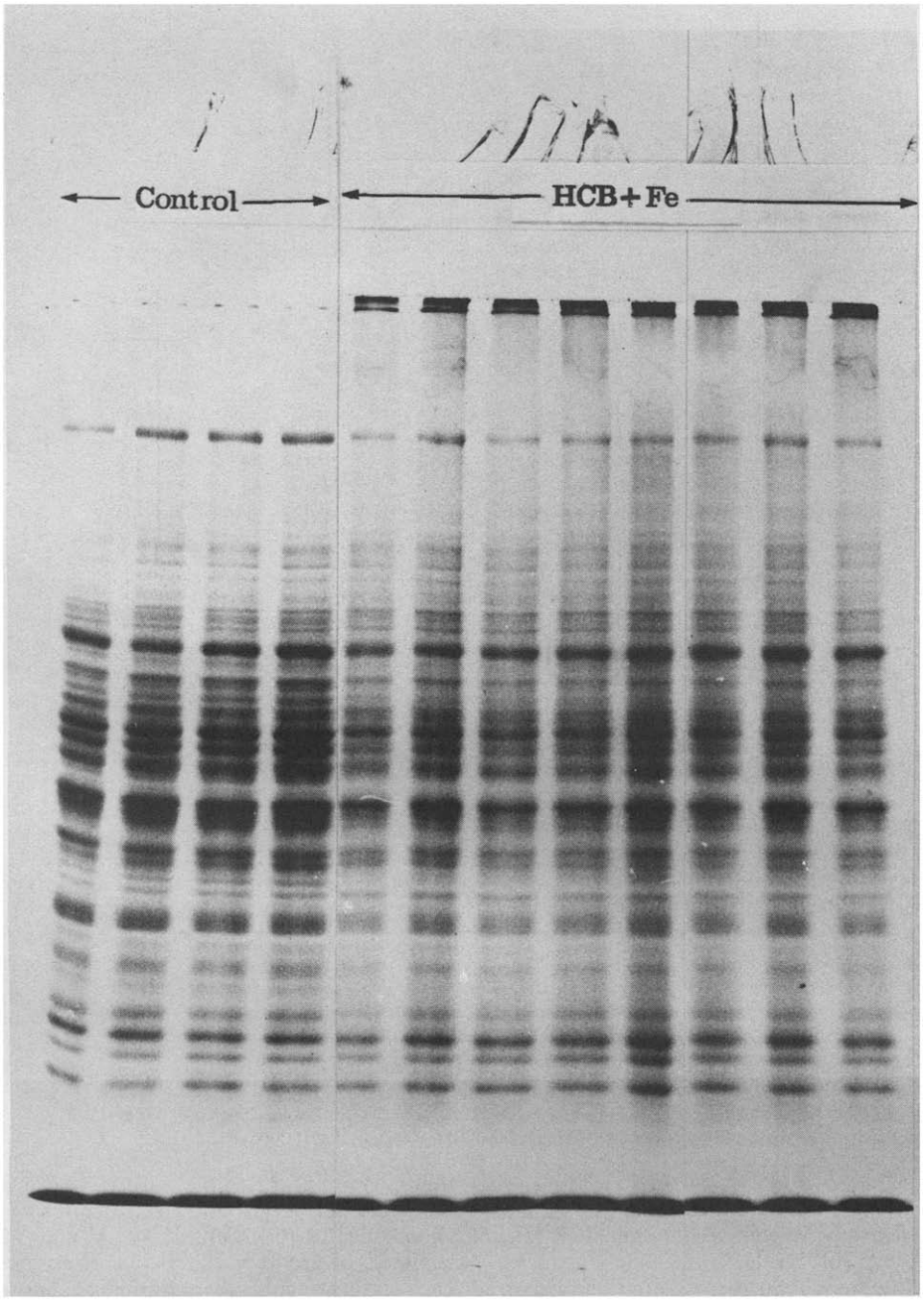

Fig. 3. The SDS polyacrylamide gel electrophoresis of the liver homogenates from control group (I) and the HCB plus iron group (V). The upper band in group V represents the high molecular weight proteins.

compound, did not develop porphyria, in contrast to the mice on a normal diet [12, 13].

Although it was known that iron plays an important role to both the induction of HCB porphyria and the process of lipid peroxidation, these two phenomena have not been associated. In the process of lipid peroxidation MDA is formed as the end product of the breakdown of peroxidized polyunsaturated fatty acids, located in the phospholipid fraction of biomembranes. During this process also cross-linking of proteins occurs leading to the formation of high molecular weight proteins. It has previously been shown that in the process of lipid peroxidation the activity of the enzyme G-6-P-ase is diminished [24, 27]. Our data show that all these phenomena are present in HCB porphyria in the rat. Thus the concurrence of porphyria and lipid peroxidation appears to be dependent on the presence of iron.

Electron microscopic studies [7] of rat liver tissue have shown that damage of membranes takes place during HCB intoxication. In the cytoplasm "whorl"-

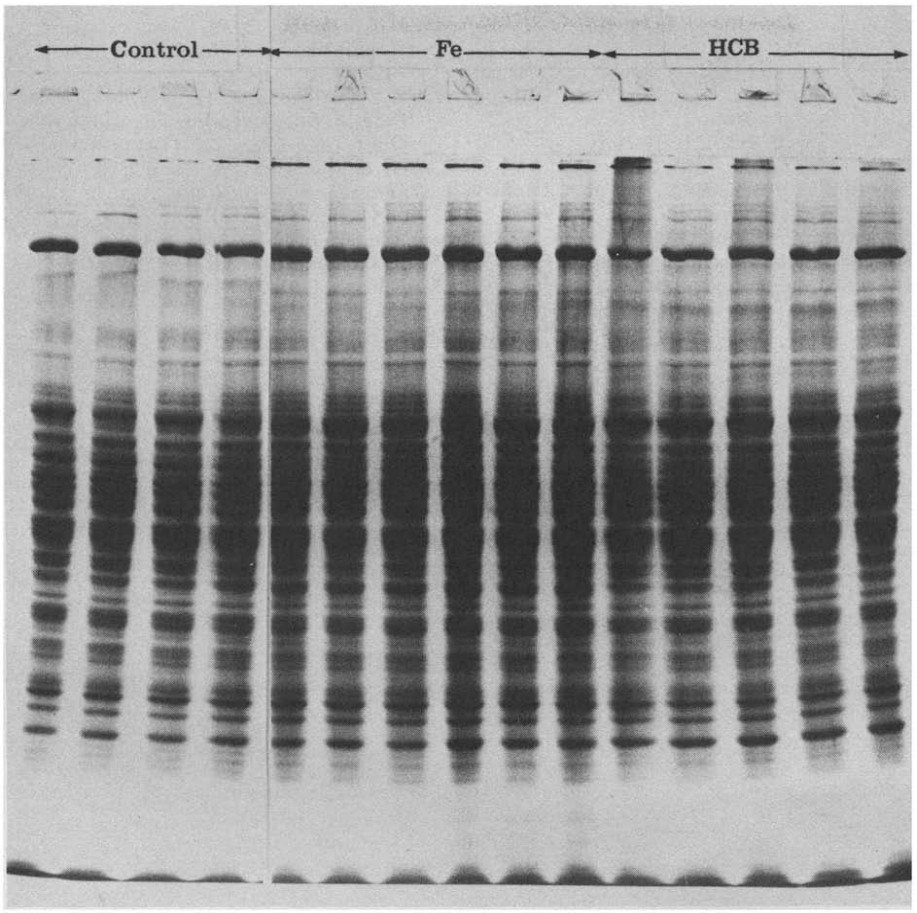

Fig. 4. The SDS polyacrylamide gel electrophoresis of the liver homogenates from control group (I), the iron overloaded group (IV) and the HCB group (VI).

like structures are found which appear to have been built up concentrically from membrane-like structures. In the centre of these structures fat drops are often found. It is possible that these electron microscopical changes are the result of membrane damage by lipid peroxidation.

It is known, that with the induction of HCB porphyria the activity of the enzyme URO-D is inhibited. This inhibition is enhanced by the presence of iron [5]. It seems reasonable to assume, as suggested by Debets and Strik, that destruction of the enzyme URO-D by HCB radicals is involved in the pathogenesis of HCB induced porphyria [7, 8]. Our experiments suggest that these radicals can also be responsible for the initiation of lipid peroxidation. Although its mechanism is still in debate [18], it is known that iron catalyses lipid peroxidation. This will lead to an increased destruction of cytochrome P-450, which results in an increase of haem synthesis by induction of the enzyme ALA synthetase [28]. Thus the interplay between inactivated URO-D and the effects of an increased lipid peroxidation aggravates the porphyria induced by HCB. If radical formation does play an important role in the pathogenesis of HCB induced porphyria, antioxidants could theoretically modulate the disease process. Debets *et al.* [29] found in *in vitro* experiments with chick embryo liver cell cultures, a protective effect of vitamin E (DL-alpha-tocopherol) and other antioxidants against the porphyrinogenic action of HCB. However, vitamin E given orally did not prevent toxicity of HCB in Agus rats. In patients with porphyria cutanea tarda, however, remissions have been described after administration of vitamin E [30].

Possibly these conflicting results can be explained by the erratic absorption of vitamin E after oral administration. This subject warrants further study because the iron dependency of toxicity of PHA compounds and the possible protective effect of vitamin E can have important implications for prevention of toxicity of PHA compounds, which increasingly pollute our environment.

Acknowledgement—We thank Prof. Van Strik from the Department of Biostatistics for his valuable advice.

REFERENCES

1. K. D. Courtney, *Environ. Res.* **20**, 225 (1979).
2. C. Cam and G. Nigogosyan, JAMA **183**, 88 (1963).
3. D. J. Cripps, A. Gocmen and H. A. Peters, *Arch. Dermatol.* **116**, 46 (1980).
4. H. Verneuil, Y. Nordman and H. Phung, *Int. J. Biochem.* **9**, 927 (1978).
5. J. J. F. Taljaard, B. C. Shanley and S. M. Joubert, *Life Sci.* **10**, 887 (1971).

6. R. Tiepermann, G. Koss and M. Doss, *Hoppe-Seyler's Z. physiol. Chem.* **361**, 1217 (1980).
7. J. J. T. W. A. Strik and H. J. Koeman, in *Chemical Porphyria in Man.* p. 181. Elsevier/North Holland Biomedical Press, Amsterdam (1979).
8. F. M. H. Debets, W. J. H. M. B. Hamers and J. J. T. W. A. Strik, *Int. J. Biochem.* **12**, 1019 (1980).
9. G. H. Elder and D. H. Sheppard, *Biochim. biophys. Res. Commun.* **109**, 113 (1980).
10. J. J. F. Taljaard, B. C. Shanley, W. M. Deppe and S. M. Joubert, *Br. J. Haematol.* **23**, 587 (1972).
11. J. J. F. Taljaard, B. C. Shanley, W. M. Deppe and S. M. Joubert, *Br. J. Haematol.* **23**, 513 (1972).
12. G. D. Sweeney, K. G. Jones and F. M. Cole, *Science* **204**, 332 (1979).
13. K. G. Jones, F. M. Cole and G. D. Sweeney, *Toxic. appl. Pharmac.* **61**, 74 (1981).
14. M. D. Maines, *Int. J. Biochem.* **12**, 781 (1980).
15. J. M. Mead, in *Free Radicals in Biology*, Vol. 1 (Ed. William Pryor), p. 52. Academic Press, New York (1980).
16. B. A. Freeman and J. D. Crapo, *Lab. Invest.* **47**, 412 (1982).
17. B. A. Svingen, J. A. Buege, F. O. O'Neal and S. D. Aust, *J. biol. Chem.* **254**, 5892 (1979).
18. S. D. Aust and B. A. Svingen, in *Free Radicals in Biology*, Vol. 5 (Ed. William Pryor), p. 1 (1982).
19. J. H. P. Wilson, J. W. O. van den Berg, A. Edixhoven-Bosdijk and L. H. M. van Gastel-Quist, *Clinica. Chim. Acta* **89**, 165 (1978).
20. M. A. Alleman, J. H. P. Wilson, J. W. O. van den Berg, A. Edixhoven-Bos-dijk and L. H. M. van Gastel-Quist, *Clin. Chem.* **28**, 1144 (1982).
21. H. G. van Eijk, W. F. Wiltink, G. Bos and J. P. Goossens, *Clinica. Chim. Acta* **50**, 275 (1974).
22. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
23. D. J. Kornbrust and R. D. Mavis, *Lipids* **15**, 315 (1980).
24. J. F. Koster and R. G. Slee, *Biochim. biophys. Acta* **620**, 489 (1980).
25. A. E. Harper, in *Methoden der enzymatischen Analyse* (Ed. H. U. Bergmeyer), 2nd Edn., p. 788. Verlag-Chemie, Weinheim (1965).
26. S. G. Schaeffer, E. Richter and H. D. Meyer, *Arch. Dermatol. Res.* **274**, 349 (1982).
27. E. D. Wills, *Biochem. J.* **123**, 983 (1971).
28. F. Matteis and W. Norman Aldridge, in *Heme and Heme Proteins*, p. 110. Springer, Berlin (1975).
29. F. Debets, J.-H. Reinders, G. Koss, J. Seidel and A. Strik, *Chem. Biol. Interactions* **37**, 77 (1981).
30. P. P. Nair, E. Mezey, H. S. Murty, J. Quartner and A. I. Mendeloff, *Arch. Int. Med.* **128**, 411 (1971).