Biochemical Pharmacology, Vol. 21, pp. 239–247. Pergamon Press, 1972. Printed in Great Britain

EFFECTS OF IRON OVERLOAD ON LIPID PEROXIDE FORMATION AND OXIDATIVE DEMETHYLATION BY THE LIVER ENDOPLASMIC RETICULUM

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(*Received* 8 *April* 1971; *accepted* 14 *July* 1971)

Abstract—Injections of large doses of iron in the form of Imferon into mice (0·125 mg/g body weight) is followed by an increase in the non-haem iron and total iron content of the liver microsomal fraction over a few days.

As a result of iron injection, the rate of lipid peroxidation in microsomal suspensions incubated in presence of NADPH increased by approximately 14 per cent. Much larger increases in the rate of peroxidation, of about 75 per cent, occurred however if the microsomes were incubated in presence of ascorbate.

Rates of oxidative demethylation of aminopyrine or of *p*-chloro-*N*-methyl aniline were reduced 1 day after iron injection by 6–11 per cent and the reduced rate was maintained for 12 days.

Non-haem iron in the liver microsomal fraction is believed to be present in at least two forms, as ferritin and as a component of an electron transport chain catalysing lipid peroxide formation in presence of NADPH. A small proportion of the injected iron is converted into the electron transport component which causes a small increase in the rate of NADPH induced peroxidation. Peroxidation in presence of ascorbate is increased to a much larger extent because ascorbate can utilise iron in the normally stored form, ferritin, to catalyse lipid peroxidation.

Lipid peroxidation, enhanced by iron overload, is believed to lead to a breakdown of membranes of the endoplasmic reticulum which causes a decrease in the capacity to carry out oxidative demethylation and related oxidative metabolism dependent on cytochrome P-450 and its associated electron transport chain.

IRON overload, or haemochromatosis, is characterised by the deposition of large quantities of iron in many tissues of the body and especially the liver, spleen and pancreas. The causes of the condition in man may be primary and possibly caused by a genetic defect which results in excessive synthesis of the apo-ferritin in liver, or secondary, and caused by repeated blood transfusions, excessive haemolysis or by a diet containing a very high iron content such as is taken by the Bantu subjects of South Africa.[1,2] Serious pathological manifestations, such as diabetes, liver cirrhosis, skin pigmentation and disturbances in endocrine metabolism are caused by this excess deposition of iron but the reasons for these pathological changes are not understood. Iron overload may also be a factor contributing to radiation sickness because Yendell *et al.*[3] showed that levels of iron greatly increased in liver and spleen following whole body irradiation of mice.

In studies on lipid peroxide formation in preparations of liver endoplasmic reticulum, Wills[4] showed that the rate of formation of lipid peroxide in incubated microsomal suspensions depended on a non-haem iron component. Peroxidation was

inhibited by iron chelating agents and increased by addition of inorganic iron. Furthermore, lipid peroxide formation resulted in partial disintegration of the membranes of the endoplasmic reticulum which was accompanied by a loss of capacity to carry out hydroxylation reactions.[5] Impairment of this oxidative capacity in the liver, on which a great deal of detoxication, drug and steroid metabolism depend could clearly cause serious disturbances in whole body metabolism. In the present investigation the effects of overloading animals with iron on the rate of lipid peroxidation and drug hydroxylation in preparations rich in endoplasmic reticulum have been studied in an attempt to establish whether some of the pathological consequences of iron overload could be ascribed to lipid peroxidation.

MATERIALS AND METHODS

Animals. Male mice, 12–16 weeks old, (Scientific Animal Supplies), average weight 43 g were used for all experiments.

Diet. The stock diet (MRC 41B) contained 87 μg iron/g.

Materials. $NADP^+$, sodium isocitrate and batho-phenanthroline were obtained from Sigma Chemical Co. (St. Louis Mo. U.S.A.), thiobarbituric acid and malonaldehyde tetracetal from Kodak Ltd., (Kirby, Lancs.), aminopyrine from John Bell and Croyden, London and *p*-chloro-*N*-methyl aniline from Cal. Biochem. Cal. U.S.A.

Iron was injected as "iron-dextran" complex–Imferon containing 50 mg iron/ml and obtained from Fisons Ltd., who also supplied Dextran C used for control experiments. All buffer solutions were prepared as described by Dawson *et al.*[6] Tris-HCl or phosphate buffers were used in most experiments.

Methods. All methods used have been described previously. Preparation of the liver microsomal fraction, NADPH, measurement of the rates of lipid peroxide formation in microsome suspensions in presence of NADPH or ascorbate have been described by Wills,[7] non-haem iron and total iron were determined by methods described by Wills,[4] and determination of the rate of oxidative demethylation of *p*-chloro-*N*-methyl aniline as described by Wills and Wilkinson.[8]

RESULTS

General experimental plan

Groups of 24 male mice, 12–16 weeks old (average weight 43 g) were injected intraperitoneally with 5 mg iron as 0·1 ml iron dextran complex "Imferon". Similar groups of 24 untreated mice were kept as controls. Most experiments were continued for 12 days but a few were allowed to continue for 96 days. In some experiments further 0·1 ml injections were given at intervals to increase the total quantity of iron injected to 15 mg. Unless stated otherwise, however, the experiments described are based on a single 5 mg injection.

At intervals over the 12-day period, injected mice were killed and livers from groups of three mice pooled. The microsome fraction was prepared as described[7] and suspended in 125 mM KCl (1 ml/g liver used for the preparation). Similar preparations were made at the same time for untreated animals.

A sample (0·2 ml) of each microsomal suspension was incubated with phosphate buffer (pH 7·0) with NADPH (40 μM) in a total volume of 2·0 ml and the rate of lipid peroxide formation was measured as previously described.[7] The rate of lipid peroxida-

tion was also measured in phosphate buffer (pH 6·0) in presence of 0·1 mM or 0·2 mM ascorbate in place of NADPH.

Portions (1·0 ml) of microsomal suspensions were used for the determination of the rate of oxidative demethylation of aminopyrine and of *p*-chloro-*N*-methyl aniline as described.[5,8]

Non-haem iron, total iron and protein were determined as previously described.[4]

Liver weight and protein content of microsomal fraction

The mean protein content of microsomal preparations, expressed as milligrams protein/gram liver used, showed a small increase over the control after iron injection but it was not significant. The total liver weight did however increase significantly so that the total liver microsomal protein was significantly increased after iron injection (Table 1).

TABLE 1. LIVER WEIGHTS AND PROTEIN CONTENTS OF LIVER MICROSOMAL FRACTIONS AFTER IRON INJECTION

	Number of animals	Mean liver weight (g)	Mean microsomal protein (mg/g liver used in preparation)	Total liver microsomal protein (mg)
Controls	85	2·06 ± 0·36	10·63 ± 2·04	21·90 ± 4·20
Injected with iron	101	2·29 ± 0·32	10·92 ± 2·25	25·00 ± 4·95
Significance (P value)		< 0·01	N.S.	< 0·01

At daily intervals, after injection of 5 mg iron, as the iron dextran complex, Imferon, livers were removed, weighed, and the microsome fraction prepared and protein content determined as described previously.[7] Values given for iron injected animals are means for 1st–12th day after injection.

Iron content of microsomal fractions

The non-haem iron content of the microsomal fractions expressed in terms of protein increased after a single injection of "Imferon" (5 mg iron) to reach a maximum after 3–4 days. It then decreased a little on the 4th day and remained nearly constant at a level which was approximately four times that of the controls, for the 12-day duration of the experiment, 27·48 ± 7·99 as compared with 6·80 ± 3·59 nmoles iron/mg protein for control animals (Fig. 1).

The total microsomal iron did not reach a maximum until 5 days after injection, but like non-haem iron, the mean total iron for all injected animals was approximately four times that of the controls, 73·05 ± 24·27 as compared with 19·78 ± 7·61 nmoles iron/mg protein (Fig. 1). A few experiments were allowed to proceed for 96 days. No significant changes in total or non-haem iron in the microsomal fraction were observed during this prolonged period.

Lipid peroxidation

Immediately after preparation of the microsomal suspensions from both control and iron injected groups the rate of peroxidation was measured during incubation at 37°.

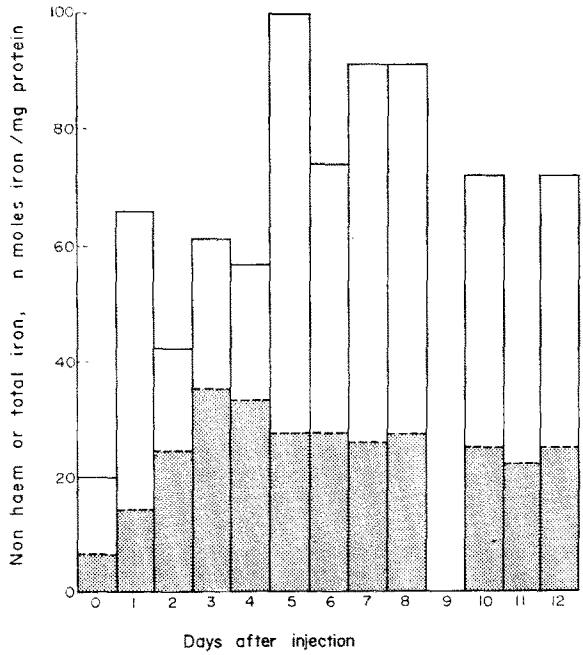

FIG. 1. Non-haem and total iron content of mouse liver microsomes after iron injection. Microsomes were prepared from mouse livers at daily intervals after a single intraperitoneal injection of 5 mg iron as the iron dextran complex, Imferon. Non-haem iron and protein were determined as described in the text. The control is a mean of 26 separate animals and each other value is a mean of three injected animals.

Non-Haem Iron (Shaded blocks). Mean control value = 6·80 ± 3·59 nmoles iron/mg microsomal protein. Mean of injected animals (2nd–12th day) = 27·48 ± 7·99 nmoles iron/mg microsomal protein (P value < 0·001).

Total Iron (clear blocks). Mean control = 19·78 ± 7·61 nmoles/mg microsomal protein. Mean of injected animals = 73·05 ± 24·27 nmoles/mg microsomal protein (P value < 0·001).

In presence of NADPH (40 μM) at pH 7·0 the rate of peroxidation increased on the first day after injection and was maintained at a slightly higher rate than the mean of the control suspensions. The mean for all injected animals was 1·70 ± 0·50 nmoles malonaldehyde/mg protein/min as compared with 1·47 ± 0·36 for the control suspensions (Fig. 2).

When ascorbate was added to microsomal suspensions to give a concentration of 0·1 mM the rate of peroxidation was greater than controls on the first day after injection and was maintained at a much higher value, the mean for all injected animals being 1·67 ± 0·57 nmoles malonaldehyde/mg protein/min as compared with 1·01 ± 0·44 for control suspensions. When the concentration of ascorbate was 0·2 mM injections of iron caused an even greater increase of rate, the mean for the whole experiment being 1·83 × mean control values (Fig. 2).

Oxygen uptake in microsomal suspensions

May and McKay[9] and Wills[7] showed that lipid peroxidation in incubated suspensions of microsomes was accompanied by uptake of oxygen which could be quantitatively related to the lipid peroxide, or malonaldehyde formed. Measurement of the rate

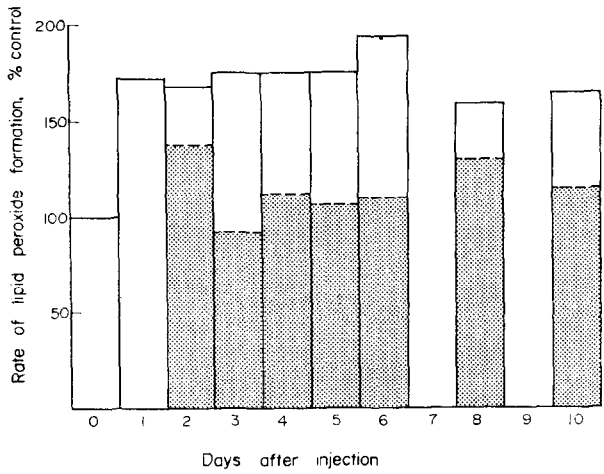

FIG. 2. Rate of lipid peroxide formation in mouse liver microsomes in presence of NADPH or ascorbate after iron injection. Suspensions of microsomes, prepared from mice treated as described for Fig. 1, and containing 1·00 ± 0·20 mg protein/ml, were incubated at 37° in a total volume of 2·0 ml, containing pH 7·0 phosphate buffer (50 mM) and NADPH (40 μM) or pH 6·0 phosphate buffer and ascorbate (0·2 mM). The rate of lipid peroxide formation was determined as previously described.[7] Results after iron injection are means of three animals and are expressed as percentages of the mean control values.

NADPH system (shaded blocks). Control rate (mean of 12 animals) = 1·47 ± 0·36 nmoles malonaldehyde/mg microsomal protein/min. The mean value for all the injected animals was 1·70 ± 0·50 nmoles malonaldehyde/mg microsomal protein/min (P value < 0·2).

Ascorbate system (clear blocks). Control value (mean of 15 animals) = 0·83 ± 0·54 nmoles malonaldehyde/mg microsomal protein/min. Rate after injection (mean of 1st–10th day) = 1·52 ± 0·44 nmoles malonaldehyde/mg microsomal protein/min (P value < 0·01).

of oxygen uptake of microsomal suspensions in the oxygen electrode in presence of NADPH showed that injection of iron into the animal caused a small increase in rate (Table 2) but after addition of ascorbate the iron loaded microsomal suspensions took up oxygen at a much faster rate than controls (Table 2). The effect of iron loading on oxygen uptake of microsomal suspensions was therefore similar to the effect on peroxidation (Fig. 2).

Rate of oxidative demethylation

A consequence of the induction of lipid peroxidation in microsomal suspensions is that the rate of drug oxidation, such as oxidative demethylation of aminopyrine, is reduced. This is believed to be a result of the loss of membrane structure essential for the function of cytochrome P-450 and other components of the electron transport chain involved in the oxidation.[5]

In iron overloaded animals the rate of lipid peroxidation in the microsomal fraction is increased, and if peroxidation were increased *in vivo* the capacity of the microsomes to carry out oxidative drug metabolism might be decreased. Studies were therefore made of the rates of oxidative demethylation of aminopyrine and of *p*-chloro-*N*-methyl aniline for 12 days after iron injection. Using either substrate a decrease in the rate of

TABLE 2. THE RATE OF OXYGEN UPTAKE OF SUSPENSIONS OF MOUSE LIVER MICROSOMES AFTER IRON INJECTION IN PRESENCE OF ASCORBATE OR OF NADPH

Days after injection	Rates of oxygen uptake (nmoles oxygen/mg protein/min)	
	Ascorbate	NADPH
0 (Control)	4·00 ± 1·67	14·47 ± 5·00
3	10·84	18·80
4	10·08	16·90
5	8·56	19·8
7	12·96	15·4
10	8·48	18·9
Mean after iron injection (30 experiments)	9·48 ± 4·10	17·69 ± 4·93
P Value	< 0·001	< 0·2

Suspensions (2·0 ml) of microsomes containing 1·00 ± 0·2 mg protein were incubated at 37° in presence of ascorbate (0·1 mM) or of NADPH (40 μM). Rates of oxygen uptake were measured as described.[7] The control rate is a mean of 30 animals and each value after injection is a mean of three animals.

oxidation resulted on the first day after injection and the decreased rate was maintained for the 12-day period. The rate of oxidative demethylation of aminopyrine was more affected by iron injection than was that of *p*-chloro-*N*-methyl aniline (Fig. 3).

The important effect that excess iron loading had on oxidative demethylation was shown more clearly if the microsomal suspensions prepared from control and iron treated animals were incubated with NADPH or ascorbate before determination of the rate of oxidative demethylation of aminopyrine. A much greater loss of activity was observed using microsomes prepared from iron injected animals which was a consequence of peroxidation (Table 3).

TABLE 3. EFFECT OF INCUBATING SUSPENSIONS OF MICROSOMES ON THE RATE OF OXIDATIVE DEMETHYLATION OF AMINOPYRINE

Incubation	Rate of oxidative demethylation of aminopyrine (nmoles formaldehyde/mg protein/min)	
	Controls	Iron injected
None	2·46	2·17
NADPH	2·32	1·75
Ascorbate	2·22	1·40

Suspensions of microsomes (5 mg protein/ml) prepared from livers of normal mice 3 days after injection with Imferon (5 mg iron) were incubated at 37° with NADPH (40 μM) or ascorbate (0·1 mM) for 15 min before determination of the rate of oxidative demethylation of aminopyrine. Rates are means of three determinations.

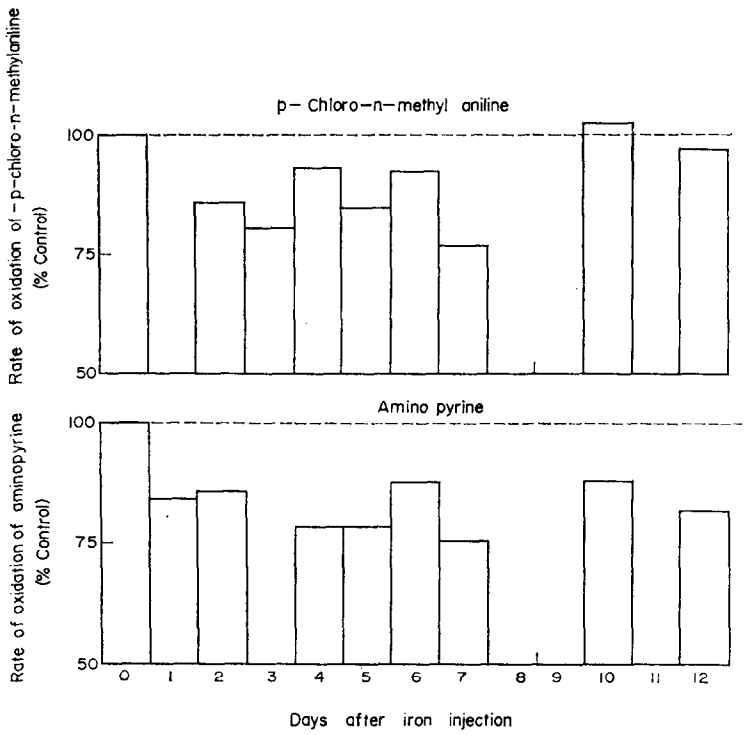

FIG. 3. Rate of oxidation of aminopyrine and of *p*-chloro-*N*-methyl aniline by mouse liver microsomes after iron injection. Suspensions of microsomes prepared from normal animals and after injection of 5 mg iron and containing 10·0 ± 1·0 mg protein were incubated with NADPH, nicotinamide, aminopyrine, or *p*-chloro-*N*-methyl aniline and the rate of oxidative demethylation measured as described.[5,8] Each point after injection is a mean value for three animals.

Aminopyrine. The control rate (mean of 47 animals) was 2·54 ± 0·61 (nmoles formaldehyde/mg microsomal protein/min) and the mean rate after iron injection was 2·22 ± 0·62 nmoles formaldehyde/mg microsomal protein/min. The difference is significant (P value < 0·05).

p-Chloro-*N*-methyl aniline. The control rate (mean of 41 animals) was 5·10 ± 1·9 nmoles *p*-chloroaniline/mg microsomal protein/min and the mean rate for all iron injected animals was 4·55 ± 1·2 nmoles *p*-chloroaniline/mg microsomal protein/min. The difference between control and injected animals is not significant.

High doses of iron

Most experiments were carried out on animals which had been injected with 0·1 ml Imferon containing 5 mg iron. In a few experiments further injections were given to bring the total iron injection per mouse to 10 or 15 mg. These additional injections caused further increases in the non-haem and total iron levels in the microsome fraction. Peroxidation in presence of NADPH was little affected by increased iron load but marked stimulation of peroxidation in presence of ascorbate resulted (Table 4).

Injection of dextran

Although it is not possible to inject dextran free of iron which is identical to the iron–dextran complex, preparations of "Dextran C" containing negligible quantities of iron, were supplied as a 20% sterile solution by Fisons Ltd. for comparison.

TABLE 4. RATE OF LIPID PEROXIDE FORMATION IN INCUBATED MICROSOMAL SUSPENSIONS PREPARED FROM MICE INJECTED WITH TWO DIFFERENT AMOUNTS OF IRON

Days after injection 0 (Control)	Rate of lipid peroxidation (nmoles malonaldehyde/mg protein/min) 1·22	
	5 mg iron injection	10 mg iron injection
2	1·82	2·90
5	1·77	2·77
6	1·95	3·12
7	1·96	2·85

Experimental details are as described for Fig. 2 but 0·1 mM ascorbate was used in place of 0·2 mM. Control value is a mean for 15 animals and each experimental value is a mean of three animals.

After injection of 0·1 ml "Dextran C" determinations of non-haem iron, total iron, rates of lipid peroxidation and oxidative demethylation were made in microsome suspensions as described. No significant differences from control values were observed.

DISCUSSION

Previous studies[4] provided strong evidence that iron, probably in a non-haem form, is a vital component of the system catalysing lipid peroxide formation in suspensions of liver microsomes incubated with either NADPH or ascorbate. These experiments indicated that the non-haem iron involved in peroxidation was not a normal storage form of iron, but an active component, possibly containing iron in a ferredoxin-like combination. However, the normal stored form of iron, ferritin, can catalyse lipid peroxidation in presence of ascorbate[10] and thus more than one form of iron may be utilised in ascorbate catalysed peroxidation.

The present series of experiments provide additional evidence for the role of two forms of non-haem iron in peroxidation. When microsomes are incubated in presence of NADPH the rate of lipid peroxidation is little stimulated by iron overloading during a period of 12 days (Fig. 2). Peroxidation therefore appears to depend on an iron component which is relatively constant in concentration and which is not readily increased in concentration by a greatly increased supply of iron. This was despite the fact that, as a result of iron injection, the non-haem iron content of the liver increased approximately four times (Fig. 1). On the other hand, peroxidation and oxygen uptake in presence of ascorbate is markedly stimulated by iron loading (Fig. 2, Table 2). This is almost certainly a result of the ability of ascorbate to utilize, in addition to active non-haem iron components of the electron transport system, iron stored in the ferritin form. It is of interest that peroxidation in presence of ascorbate increased to a maximum on the first day after injection (Fig. 2) whilst the maximum values for non-haem iron were not attained until 4 days after injection (Fig. 1). Fed on the normal stock diet, containing 87 μg iron/g, mice do not deposit sufficient iron in their livers to give a maximum rate of peroxidation and excess iron can be deposited which causes substantial increases in the rate of peroxidation (up to 180 per cent). Further increases in peroxidation rate result from injection of larger doses of iron (Table 4). Small

percentage decreases in this capacity of the endoplasmic reticulum to carry out oxidative demethylation, occur as a result of iron overload (Fig. 3). This fall in activity, approximately 10 per cent, is comparable with the increased rate peroxidation of approximately 14 per cent, (Fig. 2) which occurs in presence of NADPH and may be a result of excess peroxidation *in vivo* leading to membrane instability which is known to result in loss of oxidative capacity.[5] Ascorbate may also play a role by increasing the rate of peroxidation to cause loss of membrane structure but it is difficult to be certain of the significance of the ascorbate system *in vivo*. On the other hand, NADPH is well known to be constantly involved in oxidative demethylation and steroid metabolism, and only small changes in membrane iron content of the system may be necessary to induce extensive lipid peroxidation, with associated membrane damage. In an interesting complementary study to the present investigation Catz *et al.*[11] showed that the rate of oxidative demethylation of aminopyrine was significantly increased after a period of iron deficiency.

It is difficult to assess the precise physiological importance of peroxidation in naturally occurring human cases of iron overload. It is clear, however, that in such patients, the endoplasmic reticulum, heavily loaded with iron, is much more vulnerable to the formation of lipid peroxide than in normal subjects, especially if the membranes are exposed to ascorbate. This situation is not a hypothetical one because recently Wapnick *et al.*[12] have treated iron overload in Bantu subjects with high doses of ascorbate to release the bound iron. As a consequence, iron overloaded patients may be less capable of efficient drug and steroid oxidation than normal and therefore more vulnerable to some of their effects. It may be significant that physiological changes in steroid metabolism is one of the characteristic symptoms observed in human patients suffering from iron overload.[1]

Acknowledgements—The author expresses his thanks to the Cancer Research Campaign for grants that helped to defray the cost of the investigation, to Mrs. S. Collisson for valuable technical assistance and to Professor E. M. Crook, for useful advice in the preparation of this paper.

REFERENCES

1. W. Boyd, *A Textbook of Pathology*, 8th Edn, pp. 457, 928. Kempton, London (1970).
2. R. W. Charlton and T. H. Bothwell, *Biochemical Disorders in Human Disease* (Eds. R. H. S. Thompson and I. D. P. Wootton) p. 201. Churchill, London (1970).
3. A. Yendell, R. Tupper and E. D. Wills, *Biochem. J.* **102,** 23 p. (1966).
4. E. D. Wills, *Biochem. J.* **113,** 325 (1969).
5. E. D. Wills, *Biochem. J.* **113,** 333 (1969).
6. R. M. C. Dawson, D. C. Elliott, W. H. Elliott and K. M. Jones, *Data for Biochemica Research*, p. 195. Oxford Clarendon Press (1959).
7. E. D. Wills, *Biochem. J.* **113,** 315 (1969).
8. E. D. Wills and A. E. Wilkinson, *Int. J. Radiat. Biol.* **17,** 229 (1970).
9. H. E. May and P. B. McCay, *J. biol. Chem.* **243,** 2288 (1968).
10. E. D. Wills, *Biochem. J.* **99,** 667 (1966).
11. C. S. Catz, M. R. Juchan and S. J. Yaffe, *J. Pharmac. exp. Ther.* **174,** 197 (1970).
12. A. A. Wapnick, S. R. Lynch, R. W. Charlton, H. C. Seftel and T. H. Bothwell, *Br. J. Haemat.* **17,** 563 (1969).