# CHANGES ASSOCIATED WITH THE ACCUMULATION OF EXCESSIVE AMOUNTS OF IRON IN CERTAIN ORGANS OF THE RAT

L. GOLBERG AND J. P. SMITH

*From Benger Laboratories Ltd., Holmes Chapel, Cheshire, and the Department of Pathology, University of Manchester*

Received for publication October 17, 1957

THERAPY with parenteral iron has always been assumed to entail the risk that administration of excessive amounts of iron may lead to the development of haemochromatosis and other pathological sequelae. Direct evidence in support of this contention has never been forthcoming: indeed from observations on man and experimental animals it would appear highly unlikely that haemochromatosis can result from severe and protracted haemosiderosis (Golberg, 1957). Published data concerning the effects on animals of gross overloading with iron provide no evidence of an intrinsic toxic effect which may be exercised by prolonged tissue siderosis. The lesions which have been produced in animals treated parenterally with large amounts of colloidal iron have been the consequence of (i) instability of the colloid in plasma, (ii) liberation of free ionic iron in amounts which greatly exceed the serum iron-binding capacity, (iii) very high concentrations of undissociated iron complex in the blood, and especially in the glomeruli (Golberg, Smith and Martin, 1957).

The recent development of iron-dextran complexes which can be given to animals parenterally in huge quantities relative to the therapeutic dose has enabled the study of gross overloading with iron to be carried further. Some of the findings arising from work previously reported (Golberg *et al.*, 1957) suggested that there is a definite similarity between the changes in certain organs of the iron-loaded rat, notably the kidney and genital tract, and the pathological manifestations of vitamin E deficiency in this species (Martin and Moore, 1939). An effort has been made to establish the link between iron accumulation in tissues and the changes which are known to take place in those tissues when the whole animal, in this case the rat, is deprived of vitamin E over a long period.

## METHODS

*Original experiments*

Details of these experiments were provided in a previous publication (Golberg *et al.*, 1957). Altogether about 200 albino rats of both sexes were used. They were maintained on diet 41 (Bruce and Parkes, 1949, 1950) which contains 1 per cent of cod liver oil as the only added fat. The iron treatment consisted of intramuscular injections of an iron-dextran complex in doses of 25 and 75 mg. Fe/kg., given 2 and 3 times a week respectively to a total of 300–1650 mg. Fe/kg., *i.e.*, up to levels which correspond to about 100 times the total therapeutic dose. Control rats given equivalent amounts of similar low molecular dextran (without iron) were maintained on the same diet over the same period, *i.e.*, up to 60 weeks after the start of injections.

*Further experiments*

The new series (Table I) was carried out on similar lines to the earlier experiment, using diet 41 (Oxo) in which the cod liver oil is replaced by stabilised vitamins A + D. Its iron

content was found to be 0·044 per cent. Its tocopherol content was estimated by Dr. J. Green and found to be (in mg./100 g. diet): α 0·52; β 0·16; ζ 0·31; ε 0·35. One or other or both of two supplements were added: cod liver oil B.P. (soaked into the pellets to give a final concentration of 10 per cent w/w) and vitamin E (1 ml. of 10 per cent w/v solution of DL-α-tocopheryl acetate in oil given orally once a week). Iron to a total of 1650 mg. Fe/kg. was given by thrice weekly injections of 75 mg. Fe/kg. during the first 8 weeks of the experiment. The group symbols in the table denote the nature and duration of treatments, as well as the order in which they were given: Fe—iron-loading; E—vitamin E; O—cod liver oil. Treatments commencing on the seventeenth week and ending at 32 weeks are marked " 17–32 ". Animals were killed at the end of the week in which their treatment stopped.

At autopsy the liver was weighed and stored at −20° until its vitamin A content was determined, using antimony trichloride. The change in renal non-protein nitrogen level was measured before and after incubation at 37° for 24 hr. Specimens of adipose tissue were collected from various sites, a part of each fixed in formalin and tested histochemically for the presence of peroxides (Hartmann and Glavind, 1949) and the remainder stored at −20° until estimation of the peroxide level could be carried out. For this purpose the method of Glavind, Granados, Hartmann and Dam (1949) was modified by the use of purified *n*-butanol as solvent.

*Histological procedures*

Sections were prepared from most organs in the body and were stained with haemalum and eosin, Perls' method for iron, Mallory's basic fuchsin, long Ziehl-Neelsen's stain for ceroid periodic acid-Schiff, acetyl Sudan black B and neutral red-tartrazine (Harris' haematoxylin, followed by 0·5 per cent neutral red at 60° for 30 min.; rinse well in water and differentiate with a saturated solution of tartrazine in cellosolve for 15–20 sec. Dehydrate, clear in xylene and mount in Xam. Renal ceroid stains red). Unstained sections mounted in Distrene 80 were examined for fluorescence in ultraviolet light. Significant findings reported here are seen in the kidneys, liver, uterus, testis, small intestine, lymph nodes and intra-abdominal and subcutaneous fat.

TABLE I.—*Experimental Details*

| Groups | Number of rats ♂ | Number of rats ♀ | Iron loading | Duration of treatment (weeks): Vitamin E | Duration of treatment (weeks): Cod liver oil |
|---|---|---|---|---|---|
| $FeE_4$; $FeE_8$; $FeE_{16}$; $FeE_{32}$ | 5 | 5 | + | 4, 8, 16, 32 | — |
| $FeE_{32}O_{17-32}$ | 2 | 3 | + | 32 | 16 (weeks 17–32) |
| $O_4$; $O_8$; $O_{16}$; $O_{32}$ | 4 | 4 | — | — | 4, 8, 16, 32 |
| $O_{32}E_{17-32}$ | 3 | 3 | — | 16 (weeks 17–32) | 32 |
| $FeO_4$; $FeO_8$; $FeO_{16}$; $FeO_{32}$ | 5 | 5 | + | — | 4, 8, 16, 32 |
| $FeO_{32}E_{17-32}$ | 2 | 3 | + | 16 (weeks 17–32) | 32 |
| $E_8$; $E_{16}$ | 1 | 1 | — | 8, 16 | — |
| $Fe_9$*; $Fe_{19}$*; $Fe_{32}$* | 9 | 4 | + | — | — |
| $Fe_{16}$ (continuing) | 2 | 2 | + | — | — |

* Original experiments, which continued up to 60 weeks.

## RESULTS

### *Original Experiments*

*Observations on the kidney*

In the original experiments, sections of kidneys of one of two rats killed at 9 weeks, and of all rats killed more than 9 weeks after the start of the injections show a remarkable picture of apparent necrosis of the proximal convoluted tubules. The affected tubules show complete disintegration of both cytoplasm and nucleus leaving only amorphous, lightly staining material within an apparently intact

basement membrane (Fig. 1). The acute nature of this renal lesion and the complete absence of tissue reaction are not in keeping with the duration of the experiment, and good health and normal renal function of the animals. We concluded that the tubular changes had developed after death. This has been confirmed in the present experiments where the right kidney, fixed immediately, invariably shows a normal histological appearance whereas the left kidney, fixed after an interval, shows extensive tubular autolysis. A study of the time relationships involved has shown that autolysis begins in isolated proximal tubules at all depths in the cortex 1 to 1½ hr. after death (Fig. 2); the changes are more extensive at 2 hr. and by 3 hr. most of the proximal and many of the distal tubules have disintegrated (Fig. 1). Little further change is seen 6 and 24 hr. later.

Kidneys from normal animals or control rats injected with dextran do not display this pattern of autolysis even after 24 hr. at room temperature. The only change occurring in these animals during the first 3 hr., is a surface effect involving shrinkage, early cytolysis and loss of eosinophilia in the outer 2 or 3 rows of proximal tubules (Fig. 3). This could be eliminated by anaerobiosis, by immersion of the slice of kidney in tris-(hydroxymethyl)-aminomethane buffer pH 7·2 under anaerobic conditions or in the presence of a reducing agent. None of these modifications had the slightest influence on the course of renal autolysis in the iron-loaded rat kidneys.

In vitamin E deficiency a similar accelerated autolysis is seen; Emmel (1955, 1957) has detected a rise in renal autolytic NPN level as early as the sixth week and he has reported that it becomes more striking with time. In the iron-loaded rats no significant difference from autolysing normal control kidneys was found when the autolysis was carried out in air at 18° or 37° for 6 hr. Houchin (1946) reported that the kidneys of hamsters deficient in vitamin E had a distinctly raised $Q_{O_2}$. This did not prove to be the case with iron-loaded rat kidneys.

*Uterus and testis*

The hypertrophied uterus, brown as a result of accumulation of ceroid pigment is a well-recognised feature of vitamin E deficiency (Martin and Moore, 1936). We first encountered it in fully developed form in rats killed 50 weeks after the start of iron-dextran injections. At 32 weeks the effect was macroscopically only a slight one, but ceroid was histologically demonstrable in myometrial fibres and macrophages.

Testicular atrophy was observed only in two instances, in rats killed at 47 and 52 weeks from the start of injections. The testes of 18 other iron-loaded male rats, killed at the same time or earlier in the experiment, were normal, as were those of 27 male rats of other groups given smaller total doses of iron and 14 dextran-treated controls. In the two affected animals the testes were obviously shrunken and show marked tubular atrophy, suppression of spermatogenesis and apparent hyperplasia of interstitial cells (Fig. 4). The interstitial cells are heavily loaded with haemosiderin but ceroid-like polymers are not conclusively demonstrated.

*Formation of polymeric pigments*

At least three different polymeric pigments have formed in our animals in various viscera. The more important histochemical features of the pigments are summarised in Table II.

TABLE II.—*Histochemical Characteristics of Polymeric Pigments*

| | H. & E. | Fe | Mallory haemo-fuscin | Ziehl-Neelsen | Periodic acid-Schiff | Acetyl Sudan Black B | U.V. fluores-cence | Neutral red tartra-zine |
|---|---|---|---|---|---|---|---|---|
| Pigment 1 (true ceroid) | Light grey | 0 | + | + | + | + | + | 0 |
| Pigment 2 (in kidney) | Light brown | 0 | ± | + | ± | 0 | ± | + |
| Pigment 3 (iron-containing polymer) | Orange | + | 0 | 0 | 0 | 0 | 0 | 0 |
| Haemosiderin | Brown | + | 0 | + | + | 0 | 0 | 0 |

Type 1 polymer, classical ceroid, is seen in the cytoplasm of myometrial cells and in macrophages among these cells (Fig. 5), imparting to the uterus a dark brown colour which is a most striking feature at autopsy. A similar polymeric material is seen in smooth muscle of oviduct, vagina and small intestine, and in siderophages in most other viscera.

Type 2 polymer occurs in the epithelial cells of the proximal tubules of the kidney. This pigment is seen in very small amounts in some of our normal animals as fine granules within the tubular epithelial cells. It is present in far greater amounts in siderotic animals, appearing as large globules, frequently up to 15$\mu$ across, and lying apparently within the lumen of the tubule (Fig. 6). These globules are probably attached to the free border of the epithelial cells as they do not appear in the lumen of more distal tubules nor have we identified them in urinary deposits. The cortical tubular cells which contain this pigment usually contain small or moderate amounts of iron, but there is no correlation between the presence of polymer and development of autolysis : many necrotic tubules do not contain the polymer. Apart from small quantities of haemosiderin in tubular epithelium and stromal macrophages no pigment is found elsewhere in the kidneys.

Type 3 polymer is present chiefly in the liver. In iron-loaded animals, large siderophages appear in the hepatic sinusoids and in the portal tracts. Nearly all the animals killed 19 weeks or more following the start of injections show in many of these siderophages oval or irregular hyaline bodies, which vary greatly in size and stain bright orange with eosin (Fig. 7). This polymer is very occasionally present in siderophages in other viscera. Unlike the polymers listed above, this stains very heavily with the Prussian blue stain and is referred to in subsequent descriptions as " iron-containing polymer " (I.C.P.). It differs from haemosiderin in its failure to stain by the periodic acid-Schiff and Ziehl-Neelsen's techniques.

*Comparison with the vitamin E deficient rat*

Rapid post mortem renal autolysis, brown uterus, testicular atrophy and ceroid pigment formation are elements of the syndrome of vitamin E deficiency in the rat. A direct comparison was made with tissues of a rat maintained by Dr. T. Moore on a vitamin E deficient diet for 651 days. The following conclusions emerge :

1. There is a striking similarity in the picture of renal autolysis, which is rather more extensive and uniform in the vitamin deficient kidney.

2. There is much less ceroid in the kidney of the vitamin deficient rat. The globules are smaller, more disperse and irregular in outline.

3. The brown uterus of vitamin E deprivation shows a higher proportion of pigment in macrophages.

The suggestion of a relationship between the consequences of iron loading and vitamin E deficiency in the rat prompted a detailed search for confirmatory phenomena. The other features whose presence might have been anticipated in rats developing vitamin E deficiency on a low fat diet are: degeneration of muscles and nervous tissue, incisor depigmentation and susceptibility to haemolysis by dialuric acid (Dam, 1957).

Even before the vitamin E-deficient rat develops paralysis and neuromuscular disturbances, severe biochemical lesions are present in the muscles and histologically occasional damage to the fibres is seen (Mackenzie, 1949). In the iron-loaded rats a most careful scrutiny of the muscles likely to be affected failed to show any kind of degeneration. Biochemically, excessive creatinuria, phosphaturia and amino-aciduria were sought for, but all the results lay within the normal range. It should be recalled that the rats used in the experiments described here were well grown, whereas vitamin E depletion is usually undertaken in very young animals.

Methylene blue, when included in vitamin E-deficient diets, has proved capable of preventing for several months the development of brown discoloration of the uterus and renal autolysis (Moore, Sharman and Ward, 1953). Rats which had begun the course of Imferon injections 55 weeks before were given methylene blue (0·1 per cent) in the diet for 4 weeks. At the end of that time a definite retardation of renal autolysis was discernible. The uteri were only very pale brown in contrast to the dark colour in iron-loaded rats not given methylene blue. The thickening and dilatation which usually accompanies the brown colour were absent. Histologically there was diminution of ceroid in the uterus and some reduction also in the amounts of polymeric pigment present in liver and kidney.

### *Further Experiments*

Up to this point the experiments were carried out with rats which had been given iron up to a year or more before. It was desirable to study the relationship to vitamin E deficiency from the start of iron loading, and to accentuate the need for vitamin E by incorporating unsaturated fat in the diet. For this reason, in the present experiments, a moderate concentration of cod liver oil (10 per cent w/w) was incorporated in the diet. Polymer formation in various tissues of the original experiment was carefully reviewed.

The growth curves and liver weights of the experimental animals were very similar to those previously reported (Golberg *et al.*, 1957) for iron-loaded and control rats with initial weights in the range 100–120 g. The supplements given in the course of these experiments did not materially influence the basic effect of iron loading.

#### *Haemolysis tests*

Christensen *et al.* (1955) demonstrated that spontaneous haemolysis in isotonic saline at 37° was a more consistent and reliable test of vitamin E deficiency than the dialuric acid haemolysis test of Rose and György (1950). Our own findings (Table III) support these observations. By using the saline technique,

the effects of siderosis and of cod liver oil in promoting haemolysis are clearly shown, as is the protective part played by vitamin E.

TABLE III.—*Haemolysis of Red Cells from Individual Rats*

| Group | Degree of haemolysis* | |
|---|---|---|
| | Dialuric acid | Saline |
| Normal | 0, 0, 0, 0, 0, 0 | 0, 0, 0, 0, 0, 0 |
| $Fe_{16}$ | 0, 0, 1, 0, 0, 0 | 1, 1, 2, 0, 1, 0 |
| $FeE_{32}$ | 0, 0, 0, 2 | 1, 1, 0, 0 |
| $FeE_{32}O_{17-32}$ | 0, 0, 0, 0, 0 | 0, 1, 0, 0, 0 |
| $O_{32}$ | 0, 0, 0, 1, 0 | 1, 1, 1, 2 |
| $O_{32}E_{17-32}$ | 0, 0, 0, 0, 0 | 0, 0, 0, 0, 0 |
| $FeO_{32}$ | 0, 0, 0, 2, 0 | 2, 0, 1, 3, 2 |
| $FeO_{32}E_{17-32}$ | 1, 0, 0, 0 | 1, 0, 0, 0 |

* 0—absent; 1—faint but distinct; 2—moderate; 3—strong.

*Kidney*

The synergistic effect of iron loading and cod liver oil on the development of the propensity to renal autolysis could hardly have been demonstrated more clearly than in these experiments (Table IV). With cod liver oil alone renal autolysis was not observed even after 32 weeks. Iron loading alone produces only a slight degree of post mortem autolysis before 16–19 weeks. The two combined induced consistent and striking autolytic change at 8 weeks. Vitamin E, given from the start of the experiment, completely prevented autolysis resulting from iron-loading alone. In 4 out of 5 animals it prevented autolysis from developing in iron-loaded rats after 16 weeks of cod liver oil diet ($FeE_{32}O_{17-32}$). On the other hand when vitamin E was given in the latter half of the experiment ($FeO_{32}E_{17-32}$), it was able to restore to normal only one out of four of the rats' kidneys.

TABLE IV.—*Renal Autolysis and the Formation of Type 2 Polymer*

| Groups | Autolysis | Type 2 polymer* |
|---|---|---|
| Normal | 0, 0, 0, 0, 0, 0 | 0, +, ±, ±, ±, 0 |
| $Fe_9$; $Fe_{19}$ | 0, ±; +, +, +, + | 0, ±; +, ±, ++, ++ |
| $Fe_{32}$ | +, +, +, +, +, +, + | ++, ±, +, ++, ++, ++, ++ |
| $E_8$; $E_{16}$ | 0; 0 | 0; + |
| $FeE_4$; $FeE_8$; $FeE_{16}$ | 0, 0; 0, 0; 0, 0 | ±, 0; +, 0; ++, + |
| $FeE_{32}$ | 0, 0, 0, 0 | ±, +, ++, +++ |
| $FeE_{32}O_{17-32}$ | 0, 0, +, 0, 0 | ±, ++, +, ++, ++ |
| $O_4$; $O_8$; $O_{16}$; $O_{32}$ | 0; 0; 0; 0, 0, 0, 0, 0 | 0; ±; ±; ++, ±, +, +, ± |
| $O_{32}E_{17-32}$ | 0, 0, 0, 0, 0, 0 | ±, +, +, ±, ±, ± |
| $FeO_4$; $FeO_8$; $FeO_{16}$ | 0, 0; +, +; +, + | ±, ±; ±, ±; +, ++ |
| $FeO_{32}$ | +, +, +, +, + | +++, +++, +++, +++, +++ |
| $FeO_{32}E_{17-32}$ | 0, +, +, + | +++, +++, +++, +++ |

* The symbols indicate increasing amounts from traces (±) to heavy deposits (+++).

Despite the failure of our earlier efforts to demonstrate an abnormal increase in NPN during autolysis we tried again in the new series, with longer periods of incubation *in vitro*. Now a rise in NPN beyond the normal range could be shown to occur, but several kidneys which did not show autolysis histologically gave high values and conversely in a few instances striking autolysis was not

accompanied by very large NPN increments. Although it would be anticipated that the widespread autolysis would be associated with some rise in NPN, it may well be that the greater proportion of the autolytic products are peptides precipitable by trichloroacetic acid, which thus escape notice. In other words a strict parallel between autolysis and rise of NPN need not necessarily be expected.

The question of renal tubular type 2 polymer is most complex. Small amounts of this pigment are present as small granules in the proximal convoluted tubules of many normal rats in our colony. They were found even in one rat which had had vitamin E supplement to its normal diet for 16 weeks. All that can be said is that the amount of pigment present undergoes a great increase from 16 weeks of iron loading onwards, especially if there is added cod liver oil in the diet. There is a suggestion that tocopherol slightly retards its formation ; the vitamin certainly has no apparent effect on type 2 pigment once it has formed.

*Liver*

The effects of vitamin E and cod liver oil upon polymer formation and iron deposition in the liver are summarised in Table V. There is no significant influence on the amounts of I.C.P. in the siderophages ; the presence of this material appears to be associated only with prolonged accumulation of iron in the cell. Type 1 polymer occurs for the most part in small amounts in liver siderophages and parenchyma of iron-treated animals at 32 weeks. When vitamin E is given simultaneously with the iron no type 1 polymer is seen. With cod liver oil and iron, far greater amounts of polymer develop much earlier in the experiments. Even in the iron-vitamin E-treated group, supplementation of the diet with cod liver oil during the latter half of the period of study overcomes the protective action of the vitamin.

TABLE V.—*Summary of Polymer Formation and Iron Deposition in the Liver*

| Groups | I.C.P. in siderophages | Type 1 polymer | | Iron deposition | |
|---|---|---|---|---|---|
| | | Siderophages | Parenchyma | Siderophages | Parenchyma |
| $Fe_9$; $Fe_{19}$; $Fe_{32}$ | 0; +; ++ | 0; 0; ++ | 0; 0; ± | ++; ++; ++ | ±; +; ++ |
| $E_8$; $E_{16}$ | — | — | 0; 0 | — | — |
| $FeE_4$; $FeE_8$; $FeE_{16}$ | 0; 0; ± | 0; 0; 0 | 0; 0; 0 | +; +; ++ | +; ++; ++++ |
| $FeE_{32}$ | ++ | 0 | 0 | ++ | ++++ |
| $FeE_{32}O_{17-32}$ | ++ | + | + | ++* | +++ |
| $O_4$; $O_8$; $O_{16}$ | — | — | 0; 0; 0 | — | — |
| $O_{32}$ | — | — | 0 | — | — |
| $O_{32}E_{17-32}$ | — | — | 0 | — | — |
| $FeO_4$; $FeO_8$; $FeO_{16}$ | 0; 0; + | 0; +++; +++ | 0; +; + | +; ++; +++ | ±; ±; + |
| $FeO_{32}$ | +++(+) | +++ | + | +++* | ++ |
| $FeO_{32}E_{17-32}$ | ++ | +++ | + | ++* | ++++ |

* Tendency for siderophages to aggregate around central veins and portal tracts.

The distribution of iron between liver siderophages and parenchymal cells is not altered by the presence of cod liver oil in the diet. By contrast, vitamin E brings about a remarkable increase in the parenchymal iron. The only noticeable effect of cod liver oil is to enhance the time taken for the siderophages to be cleared from the sinusoids in order to form aggregations around the portal tracts and central viens. We had not previously noted migration of this kind in iron-loaded animals until about the fortieth week (Golberg *et al.*, 1957).

*Vitamin A storage in the liver*

Estimations were carried out on untreated control rats and on rats killed after 32 weeks of treatment. The normal rats have a relatively poor reserve of vitamin A in the liver (Table VI). At such low levels the differences between groups are rendered insignificant by the error in the estimation. In animals given cod liver oil the stores of vitamin A are naturally considerably increased. The effect of simultaneous iron-loading is distinctly to reduce these stores, despite the larger size of the liver (Golberg *et al.*, 1957). The protective action of vitamin E is clearly demonstrated and again iron loading reduces the vitamin A level. Equally clear-cut differences are seen if the results are expressed as total vitamin A in the whole liver or as total vitamin A per 100 g. body weight.

TABLE VI.—*The Vitamin A Content of Rat Livers*

| Group | Number of rats | Vitamin A, in i.u. per g. of liver* | | |
|---|---|---|---|---|
| | | Range | Mean | S.D. |
| Normal | 10 | 21–55 | 31 | 11 |
| $Fe_{32}$ | 4 | 18–38 | 28 | 9 |
| $FeE_{32}$ | 4 | 13–75 | 45 | 26 |
| $O_{32}$ | 5 | 700–1750 | 1340 | 418 |
| $FeO_{32}$ | 5 | 190–900 | 463 | 275 |
| $O_{32}E_{17-32}$ | 6 | 2050–4250 | 3025 | 797 |
| $FeO_{32}E_{17-32}$ | 4 | 100–350 | 207 | 106 |

* Measured at 32 weeks after the start of the experiment.

*Uterus and testis*

In iron-loaded rats on diet 41 alone the uteri appeared normal at 19 weeks and even at 32 weeks were only faintly brown. With cod liver oil as the sole supplement the uteri appeared either normal or pale yellow-brown at 32 weeks; only in one instance was there definite thickening and dilatation. Iron-loading in conjunction with dietary cod liver oil induced, from 8 weeks onwards, distinctly brown uteri. Besides the thickened dilated walls there was hydrometra in many cases and type 1 polymer was present in the uterine wall, both in myometrium and macrophages.

EXPLANATION OF PLATE.

FIG. 1.—Complete autolysis of proximal tubules of the kidney of an iron-laden rat after keeping in air at room temperature for 3 hours prior to fixation. P.A.S. × 340.

FIG. 2.—Less advanced autolysis of renal tubules after keeping for 2 hours. P.A.S. × 450.

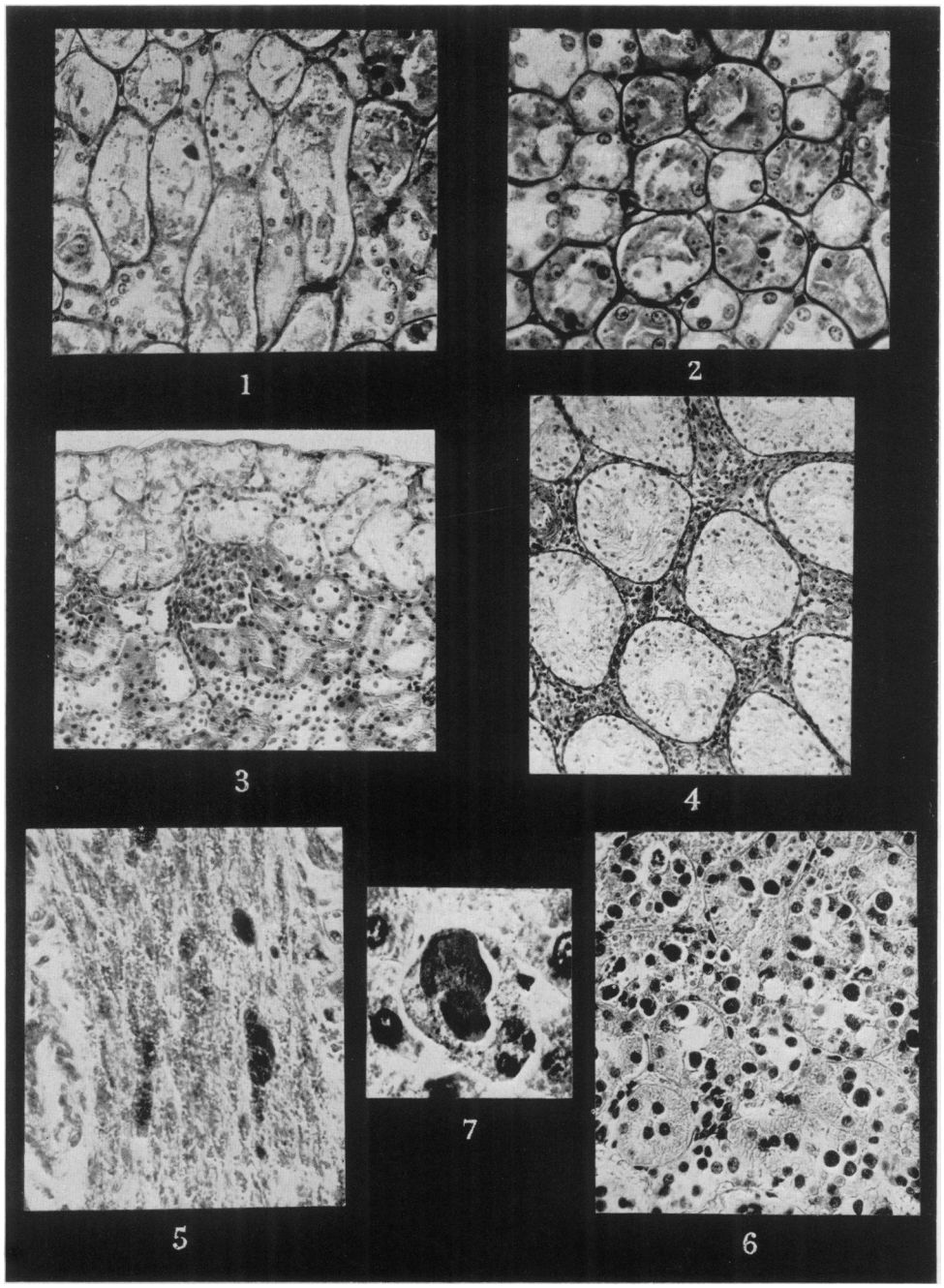
FIG. 3.—Surface shrinkage and minor cytolysis in outer rows of tubules only of the kidney from a normal rat kept in air for 3 hours prior to fixation. H. & E. × 190.

FIG. 4.—Testis from a rat 52 weeks following a course of iron injections. The tubules are shrunken and spermatogenesis is absent; there is hyperplasia of interstitial cells. H. & E. × 110.

FIG. 5.—Fine granules of ceroid polymer in myometrial cells and interstitial macrophages of an iron-laden rat. P.A.S. × 500.

FIG. 6.—Rounded globules of ceroid polymer in renal tubular epithelium of an iron-laden rat. Neutral red-Tartrazine. × 400.

FIG. 7.—An irregular mass of iron-containing polymer (I.C.P.) in a siderophage in the liver of an iron-laden rat. H. & E. × 810.




Golberg and Smith.

Demole (1941) has claimed that prolonged administration of tocopheryl acetate will completely restore the brown uterus to normal. In general, however, vitamin E is believed to have a prophylactic rather than a curative action. In these experiments vitamin E neither prevented nor completely cured the degenerative uterine changes, though it did serve to reduce the amount of ceroid present (Table VII).

TABLE VII.—*Summary of the Occurrence of Type* 1 *Polymer in Various Organs of the Rat*

| Group | Uterus | Body fat | Small intestine | | Lymph nodes |
|---|---|---|---|---|---|
| | | | Villi | Circular muscle | |
| $Fe_{32}$ | + | 0 | ± | 0 | ± |
| $FeE_{32}$ | 0 | 0 | 0 | 0 | 0 |
| $FeE_{32}O_{17-32}$ | 0 | ± | ++ | ± | ± |
| $O_{32}$ | ± | + | ++ | 0 | ++ |
| $O_{32}E_{17-32}$ | 0 | 0 | + | 0 | ± |
| $FeO_{32}$ | ++ | ++ | +++ | + | ++ |
| $FeO_{32}E_{17-32}$ | + | ++ | ++ | + | ++ |

Further evidence of the synergistic effect of cod liver oil and tissue siderosis was forthcoming from observations on the testis. Atrophy of the seminiferous tubules developed *only* in the rats of group $FeO_{32}$. The complete absence of atrophy in the testes of rats of other iron-loaded groups is particularly striking in view of the equal deposition of iron in interstitial cells. The presence of vitamin E in adequate amounts in diet 41 may have contributed to this clear-cut result.

*Body fat*

The body fat of our original iron-loaded rats which consumed a diet of low lipid content showed no abnormality. On a diet incorporating 10 per cent cod liver oil there developed, after 32 weeks, distinctly brown fat in 2 out of 5 rats. Histologically, type 1 polymer was present in the subcutaneous fat of only one, but in the lumbar fat of all 5. These changes were absent in animals which had had vitamin E over the last 16 weeks ($O_{32}E_{17-32}$).

Even though there is practically no histologically demonstrable iron in the intra-abdominal fat, iron-loading greatly intensified both the brown colour and the amount of type 1 ceroid present in both lumbar and subcutaneous fat (Table VII). In $FeO_{32}$ rats, dosing with vitamin E during the last 16 weeks produced fat which did not appear distinctly brown ; but histologically ceroid was present in all specimens of subcutaneous and lumbar fat. Group $FeE_{32}$ had normal fat ; of the 5 rats in group $FeE_{32}O_{17-32}$, one showed pale brown fat, containing type 1 ceroid.

It is clear that when unsaturated fat is present in the diet iron-loading accentuates the antagonism to vitamin E ; and that treatment with additional vitamin E eliminates the effects of the unsaturated lipid and of the iron. These phenomena were demonstrated by chemical and histochemical tests for lipoperoxides. Chemical analyses of peroxides in subcutaneous, lumbar and periovarial or paraepididymal fat showed that occasionally there was a considerable variation from one sample to another of fat from the same region of the same animal. Thus in one rat from group $FeO_{32}$ five specimens of subcutaneous fat gave the following values (in

m. equiv. peroxide/kg. fat): 1·8, 2·1, 2·8, 9·7, 42·2. Much of the fat was greatly altered, as described by Mason, Dam and Granados (1946). For lumbar fat of the same animal the values were 5·7 and 6·2, for perigonadal fat 4·0. The normal range by our method was 0·3–2·2. Each of the animals in the groups which had had cod liver oil showed at least one value beyond the normal range. Vitamin E had only a slight effect in reducing the peroxide content of the fat. In the case of the $FeO_{32}$ group the peroxide figures for subcutaneous, lumbar and perigonadal fat were respectively (range and mean of whole group): 1·8–42·2 (6·0), 1·4–17·7 (6·3) and 1·6–7·8 (3·8); whereas for the $FeO_{32}E_{17-32}$ group the figures were: 1·5–2·2 (1·9), 1·4–6·8 (2·9), 1·1–3·0 (1·9).

Histologically a positive peroxide reaction could be elicited in formalin-fixed material. The intensity of the pink colour produced was greatest in the $FeO_{32}$ group but no regular correlation was found between the histochemical test and chemical estimations. This is hardly surprising in view of the transient existence of peroxides in tissues and is in accordance with the findings of Dam and Granados (1945). It should be noted that haemosiderin granules do not give a positive reaction, nor are they associated with the presence of peroxides in their immediate neighbourhood.

*Type* 1 *polymer in other organs*

Table VII shows the effects of vitamin E and cod liver oil on the development of type 1 polymer in the villi of the small intestine and lymph nodes. Ceroid polymer is seen in macrophages and in smooth muscle cells in the cores of the villi of the small intestine where small accumulations of haemosiderin also occur. Less commonly the polymer is seen in the cells of the circular muscle of the intestinal wall. Lymph nodes throughout the body contain much iron in large siderophages: these cells, especially in intra-abdominal lymph nodes frequently show small and sometimes large accumulations of ceroid. As in the liver, vitamin E inhibits whilst cod liver oil enhances the formation of polymer.

## DISCUSSION

*Intracellular iron*

The belief has gained credence that haemosiderin is a relatively inert material, incapable of contributing to, or participating in, the body's iron pool. Heilmeyer (1957) and others have asserted that the iron of haemosiderin is not available to the body in the same way as that of ferritin. In fact it was demonstrated by Shoden, Gabrio and Finch (1953) that " these two types of storage iron are intimately associated and functionally indistinguishable ". Haemosiderin consists of large agglomerations of iron micelles and ferritin itself may be a component of haemosiderin granules (Richter, 1957*a* and *b*). The iron granules are set in a matrix composed of mucopolysaccharide, lipid and protein (Goessner, 1953; Gedigk and Strauss, 1953, 1954). Ferritin molecules themselves are composed of micelles of ferric hydroxide and phosphate (Granick, 1945). According to Laurell (1957) there exists a " diffusion pressure " of ionic iron in tissues, maintaining an equilibrium between different cells of the body. A dynamic relationship exists between the iron ions within the ferritin micelle, on the surface of the micelle, in solution in the intracellular fluid and bound to transferrin in the plasma.

Abnormally heavy accumulations of ferritin and haemosiderin within cells may bring about effects mediated by high localised concentrations of unbound ionic iron. An effect of this kind is the influence, direct or indirect, on vitamin E. Despite adequate dietary levels of this vitamin, the pathological and biochemical consequences of deficiency appear in those organs and at those sites where iron accumulates preferentially, while other organs and tissues which would be affected by an overall deficiency of vitamin E remain unscathed.

*Localisation of lesions resulting from iron-loading*

The high uptake of $Fe^{59}$ in the kidney, more particularly in the proximal convoluted tubules, was first noted by Finch *et al.* (1950). Bergström, Magnusson, Odeblad and Ziliotto (1955) demonstrated with injected $Fe^{59}$, not only that the proximal convoluted tubules always took up a great deal of iron, but that the amount increased until the conclusion of the experiment 9 days later. Our own observations that iron accumulates in the same site during iron-loading would suggest the presence in the tubular epithelium of some iron-binding material from which, perhaps under favourable circumstances, the large amounts of ceroid can develop.

Organs which are known to accumulate iron both as a result of reticulo-endothelial activity or as part of the normal ageing process were similarly affected by iron-loading ; again ceroid formation was a prominent feature. Although the muscular changes of vitamin E deficiency do not develop in the adult rat until an advanced age, some evidence might have been expected in our rats if the effect of iron-loading were a generalised one, *i.e.* induction of an overall vitamin E deficiency. The fact that a careful search for muscle lesions did not reveal any does suggest that lack of iron deposition in muscle was responsible. Even the earliest sign of an effect on muscle, namely creatinuria, was not present.

*Iron and vitamin E*

Evidence for a connection between iron and vitamin E has existed for many years. The oxidation of tocopherols with ferric chloride, giving rise to ferrous ions, is the basis of the Emmerie-Engel method of estimating vitamin E. Under carefully controlled conditions $\alpha$-tocopherol is converted by ferric chloride *in vitro* to the unstable intermediate $\alpha$-tocopheroxide, further oxidation of which leads to inactivation (Boyer, 1951 ; Harrison, Gander, Blakley and Boyer, 1956).

The biological consequences of the interaction of iron with vitamin E were demonstrated by Waddell and Steenbock (1928, 1931) who added ferric chloride in solution in ether to a stock ration and subsequently aerated it. This diet rendered male and female rats infertile, while no such effect was manifested when the iron-treated diet was supplemented by daily doses of wheat-germ oil or when the iron was added to the diet in aqueous solution without aeration. Goettsch and Pappenheimer (1931) used a similar method for inducing nutritional muscular dystrophy in the guinea-pig and rabbit.

King, Lee and Visscher (1955*a*) found that mouse paralysis attributable to lack of vitamin E developed on a diet containing ferrous sulphate, but did not do so when no iron was provided or when the iron was in the form of " ferric phosphate soluble ". The importance of ionised as opposed to non-ionised iron was demonstrated in a further paper (1955*b*) in which cardiac lesions due to vitamin E deficiency

were seen in mice given a diet whose salt mixture contained ferrous sulphate, while no such lesions were obtained with " ferric phosphate soluble " or on a normal diet. The influence which ionic iron and copper have on oxidative processes is well-known and, as Cummings and Mattill (1931) have said, " the efficiency of a given source of vitamin E depends in part on the auto-oxidizable materials and anti-oxidants associated with it ".

Despite its extensive use and satisfactory performance under standard laboratory conditions, diet 41 may have shortcomings as revealed by Bruce (1950). He found that an increase in the content of cod liver oil from 1 to 2 per cent served to induce vitamin E deficiency in mice fed on this diet. The tocopherol content of diet 41 used in the present experiments is (in mg./100 g. food) $\alpha$ 0·52 ; $\beta$ 0·16 ; $\zeta$ 0·31 ; $\epsilon$ 0·35. The total falls short of the value of 3 mg./100 g. food assessed by Brown and Sturtevant (1949) to be the vitamin E requirement of the growing rat. Our rats were somewhat older than those usually employed in vitamin E studies ; also some of the published values place the tocopherol requirement much lower—for instance 0·75 mg. $\alpha$-tocopherol/100 g. food protects the male and female rat from sterility (Mason, 1940). It follows that diet 41 may be regarded as adequate with respect to vitamin E under ordinary circumstances, but not perhaps when tissue siderosis is present.

*Iron and vitamin A*

Why vitamin E is more sensitive than vitamin A to the influence of ionic iron is uncertain but it is a fact established by Waddell and Steenbock (1928) and others. The interesting story of " salt ophthalmia " or vitamin A deficiency resulting from the presence of ferrous sulphate in the diet (Jones, 1927) cannot be recounted here. Suffice it to say that the ophthalmia could be prevented or cured, by replacing ferrous sulphate by the relatively unionised salt ferric citrate, by giving wheat-germ oil, or by making up fresh ferrous sulphate diet each day, thus avoiding prolonged contact of oxidisable fats with ferric ions (McCollum, Simmonds and Becker, 1927).

Vitamin E has a protective effect on vitamin A in the intestinal tract (Hickman, Harris and Woodside, 1942) and in the liver (Davies and Moore, 1941). Thus our finding of decreased liver stores of vitamin A in siderotic animals could be interpreted either in terms of a direct action of intracellular iron on vitamin A or as an indirect effect of destruction or antagonism of vitamin E. This question cannot be resolved with the data available but the fact that the $FeO_{32}E_{17-32}$ groups had no higher levels of liver vitamin A than the $FeO_{32}$ group does suggest a direct and continuing action of iron on the vitamin A in the liver. It may be that vitamin E administered orally cannot accumulate in the liver in sufficient amounts to combat the very high localised intracellular concentrations of iron. Reticulo-endothelial blockade lowers the vitamin A content of the liver (Krishnamurthy and Ganguly, 1956), but there is little likelihood that such blockade would be manifested six months after the injections of iron-dextran had ceased.

*Evidence contributed by the present experiments*

The effects which have been described here could be exercised by the large accumulations of intracellular iron through one or more of the following

mechanisms: oxidative destruction of vitamin E (and, possibly, vitamin A); direct or indirect (catalytic) participation in the formation of peroxidised lipids; direct oxidation of sulphydryl groups; a generalised disturbance of oxidation-reduction potential in the affected cells.

Like many others, Haurowitz, Schwerin and Yenson (1941) demonstrated the action of haemoglobin and haemin on autoxidation of unsaturated fatty acids. Attempts to repeat these observations with ferritin have shown only a slight catalytic effect (Golberg and Pickering, 1957). However the experiments described here point to the pro-oxidant activity of iron by demonstrating the presence of lipid peroxides and decreased liver stores of vitamin A. Even if the iron-laden tissues were found to contain less tocopherol than normal, it would scarcely resolve the problem. Hove (1955) has pointed out that " reliance on pathologic similarity as an indication of vitamin E deficiency or antagonism may be misleading ". He defines a " stress factor " as " any substance that precipitates a pathological condition curable or preventable by vitamin E ". Iron under these circumstances could presumably be acting as a " stress factor ", having this property in common with tri-*o*-cresyl phosphate, pyridine and a host of other compounds. This is unlikely to be the complete explanation since it fails to take account of ceroid formation—the large accumulations of various polymers at sites of iron deposition. In the case of the kidney these accumulations greatly exceeded any hitherto described.

The mode of formation of ceroid and its significance are as yet little understood. Pigments corresponding to haemosiderin and our types 1 and 2 polymers were described by Edwards and White (1941) in rats fed butter yellow. These authors suggested that the renal ceroid was the same as the pigment reported by Fairhall and Miller (1941) in the kidneys of rats given lead compounds. Hepatic (type 1) ceroid was also reported in rats subjected to chronic copper poisoning (Mallory, Parker and Nye, 1921) and in mice in whom necrosis was induced by ligation of the left lower lobe of the liver (Lee, 1951). The latter author showed striking chronological alterations in the histochemical reactions of the ceroid. Initially it was iron-positive but by the time the characteristic staining properties of the polymer were fully developed the iron reaction had become variable.

Is there a common basis underlying all these methods of ceroid production? It would appear that the union of iron and lipid will, under appropriate circumstances, lead to ceroid formation. The iron may be present as a result of haemorrhage, necrosis (with resulting liberation of iron from intracellular enzymes) or accumulation of ferritin and haemosiderin beyond physiologically normal limits. Perhaps the heavy metals copper and lead can assume the role of iron in stimulating the production of ceroid by acting as pro-oxidants; or they may liberate free iron indirectly by damaging the mitochondria or other cellular components. Unsaturated lipid could arise from pathological accumulations, as suggested by Casselman (1951) or, more simply, from damaged cellular components. The circumstances appropriate for ceroid formation have been defined by Casselman as a " relative, if not concurrent absolute, deficiency of biological antioxidants ". A preponderance of heavy metal, particularly iron or copper, would fill the bill.

The hypothesis that tissue siderosis exercises pathological effects in parenchymal and other cells by changing the intracellular oxidation-reduction potential opens up many avenues for further investigation.

SUMMARY

A gross degree of iron overload has been induced in rats on M.R.C. diet 41 by the administration of a total of 1·65 g. Fe/kg. body weight, given intramuscularly in doses of 75 mg. Fe/kg. three times a week. The sequelae closely resemble many of the characteristic pathological and biochemical changes observed in rats deficient in vitamin E.

Many of these changes are accentuated by the incorporation of cod liver oil in the diet, while vitamin E exercises a protective action. The absence of evidence of total body deficiency of vitamin E is attributed to the preferential accumulation of iron in certain sites.

Three types of polymeric pigment are described and their mode of formation briefly discussed.

Our thanks are due to the staffs of our respective departments for technical assistance, to Dr. T. Moore for providing tissues from a vitamin E deficient rat and to Dr. J. Green for carrying out the analysis of tocopherols in diet 41. We are indebted to Professor A. C. P. Campbell for his interest and advice and to the Directors of Benger Laboratories Ltd. for permission to publish the results of our investigations.

## REFERENCES

BERGSTRÖM, I., MAGNUSSON, G., ODEBLAD, E. AND ZILIOTTO, D.—(1955) *Acta physiol. scand.*, **35**, 36.

BOYER, P. D.—(1951) *J. Amer. chem. Soc.*, **73**, 733.

BROWN, R. A. AND STURTEVANT, M.—(1949) *Vitam. & Horm.*, **7**, 171.

BRUCE, H. M.—(1950) *J. Hyg., Camb.*, **48**, 171.

*Idem* AND PARKES, A. S.—(1949) *Ibid.*, **47**, 209.

CASSELMAN, W. B.—(1951) *J. exp. Med.*, **94**, 549.

CHRISTENSEN, F., DAM, H., GÖRTNER, R. A. Jr. AND SONDERGAARD, E.—(1955) *Acta physiol. scand.*, **35**, 215.

CUMMINGS, M. J. AND MATTILL, H. A.—(1931) *J. Nutr.*, **3**, 421.

DAM, H.—(1957) *Pharmacol. Rev.*, **9**, 1.

*Idem* AND GRANADOS, H.—(1945) *Acta physiol. scand.*, **10**, 162.

DAVIES, A. W. AND MOORE, T.—(1941) *Nature, Lond.*, **147**, 794.

DEMOLE, V.—(1941) *Schweiz. med. Wschr.*, **22**, 1251.

EDWARDS, J. E. AND WHITE, J.—(1941) *J. nat. Cancer Inst.*, **11**, 339.

EMMEL, V. M.—(1955) '3rd Int. Congr. Vitamin E, Abstr.', p. 31.—(1957) *J. Nutr.*, **61**, 51.

FAIRHALL, L. T. AND MILLER, J. W.—(1941) *Publ. Hlth Rep., Wash.*, **56**, 1610.

FINCH, C. A., HEPSTED, M., KINNEY, T. D., THOMAS, E. D., RATH, C. E., HASKINS, D., FINCH, S. AND FLUHARTY, R. G.—(1950) *Blood*, **5**, 983.

GEDIGK, P. AND STRAUSS, G.—(1953) *Virchows Arch.*, **324**, 240.—(1954) *Ibid.*, **326**, 172.

GLAVIND, J., GRANADOS, H., HARTMANN, S. AND DAM, H.—(1949) *Experientia*, **5**, 84.

GOESSNER, W.—(1953) *Virchows Arch.*, **323**, 685.

GOETTSCH, M. AND PAPPENHEIMER, A. M.—(1931) *J. exp. Med.*, **54**, 145.

GOLBERG, L.—(1957) *Postgrad. Med.*, **22**, 382.

*Idem*, SMITH, J. P. AND MARTIN, L. E.—(1957) *Brit. J. exp. Path.*, **38**, 297.

GRANICK, S.—(1945) *Chem. Rev.*, **38**, 379.

HARRISON, W. H., GANDER, J. E., BLAKLEY, E. R. AND BOYER, P. D.—(1956) *Biochim. biophys. Acta*, **21**, 150.

HARTMANN, S. AND GLAVIND, J.—(1949) *Acta chem. scand.*, **3**, 954.
HAUROWITZ, F., SCHWERIN, P. AND YENSON, M. M.—(1941) *J. biol. Chem.*, **140**, 353.
HEILMEYER, L.—(1957) *Clin. Chim. Acta*, **2**, 159.
HICKMANN, K. C. D., HARRIS, P. L. AND WOODSIDE, M. R.—(1942) *Nature, Lond.*, **150**, 91.
HOUCHIN, O. B.—(1946) 'Biol. Antioxidants Trans. 1st Conference', 60. Josiah Macy Foundation, New York.
HOVE, E. L.—(1955) *Amer. J. clin. Nutr.*, **3**, 328.
JONES, J. H.—(1927) *J. biol. Chem.*, **75**, 139.
KING, J. T., LEE, Y. C. P. AND VISSCHER, M. B.—(1955*a*) *Proc. Soc. exp. Biol., N.Y.*, **88**, 406.—(1955*b*) *J. Nutr.*, **57**, 111.
KRISHNAMURTHY, S. AND GANGULY, J.—(1956) *Nature, Lond.*, **177**, 575.
LAURELL, C.-B.—(1957) *Mem. Univ. Calif.*, in press.
LEE, C. S.—(1951) *J. nat. Cancer Inst.*, **11**, 339.
MACKENZIE, C. G.—(1949) *Ann. N.Y. Acad. Sci.*, **52**, 202.
MALLORY, F. B., PARKER, F. Jr. AND NYE, R. N.—(1921) *J. med. Res.*, **42**, 461.
MARTIN, A. J. P. AND MOORE, T.—(1936) *Chem. & Ind.*, **55**, 236.—(1939) *J. Hyg., Camb.*, **39**, 643.
MASON, K. E.—(1940) *Amer. J. Physiol.*, **131**, 268.
*Idem*, DAM, H. AND GRANADOS, H.—(1946) *Anat. Rec.*, **94**, 265.
MCCOLLUM, E. V., SIMMONDS, N. AND BECKER, J. E.—(1927) *Proc. Soc. exp. Biol., N.Y.*, **24**, 952.
MOORE, T., SHARMAN, I. M. AND WARD, R. J.—(1953) *Biochem. J.*, **53**, xxxi.
RICHTER, G. W.—(1957*a*) *Amer. J. Path.*, **33**, 590.—(1957*b*) *J. exp. Med.*, **106**, 203.
ROSE, C. S., GYÖRGY, P.—(1950) *Proc. Soc. exp. Biol., N.Y.*, **74**, 411.
SHODEN, A., GABRIO, B. W. AND FINCH, C. A.—(1953) *J. biol. Chem.*, **204**, 823.
WADDELL, J. AND STEENBOCK, H.—(1928) *Ibid.*, **80**, 431.—(1931) *J. Nutr.*, **4**, 79.