large-granule fraction. However, increasing the sucrose concentration to 0·88M (Fig. 2) did not appear to decrease the catalase activity found in the extraparticulate fraction immediately after the preparation of the homogenate.

SUMMARY

1. The addition of cations to suspensions of liver large granules in 0·25M-sucrose decreases the catalase migration from them during subsequent incubation at 38°.

2. The addition of polyvinylpyrrolidone to such suspensions also decreases the catalase migration.

3. Catalase migration from granules is increased as the sucrose concentration in the medium is increased.

4. The addition of respiratory cofactors to granule preparations in 0·25M-sucrose has little or no effect on catalase migration.

5. When granules are incubated in either a phosphate medium or in 0·25M-sucrose, 30–50% of their protein rapidly appears in the medium. This is probably due to an elution of material from the granule surface, whereas the catalase loss appears to occur by migration through the granule membranes.

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Biochemical Changes in the Tissues of Animals Injected with Iron: Acid Phosphatase and other Enzymes

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Iron occupies a unique place among heavy metals, both in the large amount normally present in the body and in the special mechanism available for its storage in tissues as ferritin and haemosiderin. Syndromes associated with the storage of excessive quantities of iron are of clinical importance but have not been studied biochemically. The availability of iron for parenteral administration, in the form of an iron–dextran complex which displays low toxicity, makes it possible to increase by 100 times or more the total body iron of mice, rats and other animals. Apart from some reduction in growth rate there is no obvious adverse effect on the health and well-being of the animal, which can live its normal span (Golberg, Smith & Martin, 1957*a*). In the course of time, however, the rat which is rendered siderotic in this way develops many of the manifestations of vitamin E deficiency and there is strong evidence that the stored iron reacts with, or otherwise renders inadequate, the tissue reserves of antioxidant (Golberg, Smith & Martin, 1957*b*; Golberg & Smith, 1958). It has also been shown that the siderotic liver, although histologically intact, is particularly susceptible to toxic and adverse nutritional influences (Golberg & Smith, 1960).

Biochemical study of the grossly siderotic liver has revealed enhanced activities of some enzymes, with acid phosphatase showing an outstandingly large increase of an apparently permanent nature. An increase of this kind was observed (van Duijn, Willighagen & Meyer, 1958; van Duijn & Willighagen, 1959) for animals injected with polymers such as high-molecular-weight dextran or polyvinylpyrrolidone. With iron–dextran, enhanced liver acid-phosphatase activity appears to be attributable to excessive iron deposition, since moderate doses of iron, still well above the therapeutic doses in man, have no such effect.

METHODS

Iron dosage. Injections were given to albino mice, rats, guinea pigs and hamsters by the routes and in the doses indicated below. Two iron preparations were used: (*a*) iron–dextran complex (Imferon) containing 50 mg. of ferric iron/ml.; this stably chelated colloidal-iron preparation was the subject of British Patent Specification 748,024; (*b*) saccharated oxide of iron (Ferrivenin) containing 20 mg. of ferric iron/ml., which was mixed with an equal volume of horse serum (Evans Medical Supplies) to lower the pH and maintain the stability of the colloid. The low-molecular-weight dextran has a weight-average molecular weight of 6500–7600.

For organ analysis the animals were killed by stunning and exsanguination. The organs were quickly dissected out, washed to remove blood, blotted and weighed. They were homogenized in ice-cold water, in a cooled Potter–Elvehjem homogenizer fitted with a stainless-steel pestle which had been previously cooled to 0°. The homogenate was then macerated at room temperature without overheating, in a Nelco blender (MSE) for 3 min. to liberate all the enzyme activity (Wattiaux & de Duve, 1956). The volume of homogenate was adjusted to a tissue concentration of 20 mg./ml.

Liver analysis. For protein estimation 0·1–0·2 ml. of the homogenate was diluted with water to 5 ml. in a centrifuge tube, 1 ml. of 20% trichloroacetic acid was added and, after centrifuging at 1500 *g*, the precipitated protein was dissolved in 2 ml. of 15% sodium hydroxide with warming if necessary. Water was added until the volume was 9 ml. With tissues from iron-injected animals a precipitate of iron hydroxide was formed and was removed by centrifuging at 1500 *g*. To 8 ml. of the alkaline solution of the protein, 1 ml. of 5% copper sulphate was added. The suspension was kept for 10 min. with occasional mixing, shaken vigorously, centrifuged at 1500 *g* and the extinction of the supernatant was read at 555 mμ against a blank carried through the same procedure. A calibration curve was prepared from dried bovine albumin (fraction V, The Armour Laboratories). The results obtained by the method described were checked in selected cases by the Markham (1942) distillation method.

Ash was estimated by heating the tissue at 700–800° in a muffle furnace. Two or three drops of nitric acid were added to the ash, the crucible was reheated and the ash weighed. Moisture was determined by drying at 110°, and carbohydrate by the anthrone method (Bloom & Willcox, 1951). For nucleic acids the procedure of Schmidt & Thannhauser (1945) was used. After wet-ashing of the tissues with nitric acid and sulphuric acid, iron was determined colorimetrically (Ventura & Klopper, 1951).

Estimation of tissue-enzyme activities. The terms 'enzyme activity' and 'enzyme concentration' are applied to the results of various measurements, without prejudice to the question whether, when changes occurred, the amount of enzyme protein had varied or whether the activity of the enzyme/mg. of protein had altered.

Acid-phosphatase activity was measured in 0·2 ml. samples of tissue homogenate, with citrate buffer, pH 4·9, containing 0·01 M-disodium phenyl phosphate (King & Wootton, 1956*a*). For measuring alkaline-phosphatase activity at pH 10 the method of King & Wootton (1956*b*) was used with the same substrate. The unit of phosphatase activity was μg. of P released/100 mg. (wet wt.) of tissue/min. Since all experiments involved comparisons of normal and siderotic livers having substantially equal N concentrations, the specific activities of the enzymes measured were not calculated and all units are expressed per 100 mg. wet wt. of tissue.

For estimation of catalase activity the homogenate was diluted 100–200 times with 1% gum arabic; 1 ml. was taken for assay by the iodometric method, with hydrogen peroxide–phosphate buffer (pH 6·8) as the substrate (Sumner, 1941).

Catheptic and β-glucuronidase activities were estimated as described by Gianetto & de Duve (1955). For cathepsin, 0·39 mM-haemoglobin in 0·2 M-sodium acetate–acetic acid buffer, pH 3·6, was used, and the tyrosine released was measured according to Anson (1937). For β-glucuronidase the substrate was 1·5 mM-sodium phenolphthaleinglucuronide. With xanthine oxidase, 1 g. of liver was homogenized in 10 ml. of 0·1 M-sodium pyrophosphate buffer, pH 8·0, with a cold stainless-steel pestle and Potter–Elvehjem smooth-glass homogenizer. The homogenate was centrifuged at 1500 *g* at 3–6°. The supernatant layer was incubated at 37° for 1 hr., centrifuged again for 30 min. at 20 000 *g* at 3–6° and the clear supernatant layer was removed for assay. A portion (4 ml.) of 0·26 mM-xanthine solution in 0·1 M-buffer was placed in a temperature-controlled cell at 25°, 0·1 ml. of the supernatant was added and uric acid measured by the increase in extinction at 290 mμ at 1 min. intervals for 10 min.

The procedure described by Swanson (1955) was used for measuring glucose 6-phosphatase. Liver microsomes as a source of glucose 6-phosphatase were prepared by the method of Beaufay & de Duve (1954) and the concentration of microsomes was assessed by protein N. Adenosine triphosphatase was estimated in accordance with directions given by Kielley (1955), except that the final incubation was carried out at 38° instead of 28°. For non-specific esterase the procedure of Nachlas & Seligman (1949) was followed, with Fast Blue Salt B (Gurr). The method of Mahler, Sarkar, Vernon & Alberty (1952), as modified for crude tissue extracts, was applied to the estimation of reduced diphosphopyridine nucleotide (DPNH)–cytochrome *c* reductase. The arylsulphatase assays were performed according to Viala & Gianetto (1955). The manometric assay was used for uricase (Leone, 1955) because the brown colour of the siderotic-liver homogenates prevented the use of ultraviolet spectroscopy. The method of de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955) was used in determining acid deoxyribonuclease and ribonuclease; in the latter instance total phosphorus was measured rather than ultraviolet absorption.

RESULTS

Hepatomegaly

In the mouse, as in the rat and rabbit, a prompt and permanent increase in liver weight is an invariable response to heavy doses of parenteral iron. Histologically, the enlargement is seen to be due to a hyperplasia of all the liver elements. By chemical methods, comparisons have been made between the livers of untreated mice and those of mice given a series of daily intravenous injections of iron–

Table 1. *Composition of livers of untreated mice and of mice injected with iron*

Each value is the mean ± S.D. for a group of 10 male mice. The periods stated refer to the number of days after the last of nine daily intravenous injections of 0·1 ml. of iron–dextran.

	Mice injected with iron		Untreated mice	
	9 days	17 days	9 days	17 days
Body wt. (g.)	22·00 ± 4·14	26·25 ± 3·28	29·55 ± 2·73	37·40 ± 3·60
Liver wt. (g.)	1·96 ± 0·28	2·16 ± 0·39	1·37 ± 0·17	1·65 ± 0·26
Liver wt. (as % of body wt.)	9·00 ± 1·06	8·21 ± 1·08	4·64 ± 0·43	4·41 ± 0·42
Carbohydrate (mg./g.)	21·37 ± 8·31	6·79 ± 2·37	1·46 ± 0·77	3·27 ± 2·59
Protein (mg./g.)	—	224 ± 12·0	—	249 ± 15·4
Total solids (mg./g.)	—	269 ± 9·9	—	286 ± 5·2
Ash (mg./g.)	—	23·00 ± 0·82	—	21·10 ± 1·84

dextran. The liver content of water, ash, iron, carbohydrate, protein and nucleic acids has been measured. A few of the results are given in Tables 1 and 2.

The following generalizations may be made. There is stunting of growth in mice injected with heavy doses of iron, with an increase in the ratio of liver weight to body weight of 50–100% or more. On an average the liver contained 20–30% of the iron administered. Taking the groups overall, hepatomegaly in mice injected with large doses of iron is not reflected in differences of water or protein content. The additional ash content was the only constant feature. In animals killed at 9 days the carbohydrate content of the liver was 21·37 ± 8·31 mg./g. Included in this figure would be glycogen, residual dextran and possibly the carbohydrate component of haemosiderin. By 17 days the carbohydrate content of the siderotic livers had decreased to 6·79 ± 2·37 mg./g. This change may be accounted for by metabolism of the residual dextran.

A comparison of liver nucleic acids (Table 2) reveals that in the siderotic mice there is a decrease of ribonucleic acid P (RNA-P) and an increase of deoxyribonucleic acid P (DNA-P).

Liver acid-phosphatase activity

In mice the effects were first studied of factors such as age, sex, body weight and liver weight, as well as of multiple injections of 0·9% sodium chloride solution, low-molecular-weight dextran, clinical dextran and horse serum. Fig. 1 shows the relationship between liver weight and total liver acid-phosphatase activity in mice of different ages. In males the liver acid phosphatase increases moderately with increasing liver weight and age, but in old mice the liver phosphatase tends to fall. In females, increasing age and liver weight raise the enzyme level to a higher maximum than in the males.

In parallel experiments with groups of 10 male mice 6 weeks of age, the intravenous injection of iron, as iron–dextran complex or as saccharated oxide of iron, brought about striking increases in liver acid phosphatase (Table 3). Six hours after a single very heavy dose of iron there was a distinct increase in activity. Repeated injections progressively elevate the acid phosphatase, whether the iron is given as the dextran complex or as the saccharated oxide.

For a more accurate appraisal it is necessary to take into account the increase in liver weight which follows iron injections. A moderate excess of iron (5 mg. of iron/kg.), given daily, does induce hepatomegaly but any rise in acid phosphatase is small; given at weekly intervals, it increases neither the liver weight nor the enzyme activity. Twenty to forty times this dose produces a striking elevation of acid phosphatase (Table 4).

The untreated rat has a higher acid-phosphatase concentration per gram of liver than does the mouse. On injection of massive doses of iron the rise in acid phosphatase is less spectacular but still quite definite (Table 5). In the guinea pig the liver phosphatase remained unchanged. In both species anaphylactoid reactions were in evidence after multiple intraperitoneal injections of iron–dextran complex. Hamsters, however, tolerated the injections well and showed a sharp rise in liver acid phosphatase.

In Fig. 2 are shown some observations on the rate of increase in acid-phosphatase activity with time and the maintenance of the enhanced level over the ensuing weeks. After a single injection of saccharated oxide of iron the rise is more gradual than with iron–dextran. The high levels of acid phosphatase were maintained for 21 days but there was a suggestion that in time they might return to pretreatment levels. Accordingly, the long-term changes in liver phosphatase after massive doses of iron–dextran complex were studied in male and female mice given from 2 to 15 weekly subcutaneous injections and killed at various times after the last injection. Fig. 3 shows that even after only two injections of this kind a significant rise of liver phosphatase is present 6 months later. With 12 or 15 injections a large and persistent increase in enzyme activity has occurred.

Table 2. *Nucleic acids in livers of untreated mice and of mice injected with iron*

The periods stated refer to the number of days after the last daily intravenous injection.

Treatment	No. in group	Body wt. (g.)		Liver wt. (g.)		Liver nucleic acids: RNA-P (mg. of P/100 g.)		Liver nucleic acids: DNA-P (mg. of P/100 g.)		Liver nucleic acids: μg. of P/mg. of protein N		Liver nucleic acids: Ratio: RNA-P/DNA-P	
		Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	RNA-P	DNA-P	Mean	S.D.
Untreated	10	22·7	1·5	1·08	0·10	80·3	6·8	23·6	2·4	22·5	6·61	3·42	0·23
Iron–dextran (3 × 0·1 ml.; 5 days)	6	23·2	3·1	1·59	0·77	57·2	8·0	35·7	4·1	16·4	10·2	1·66	0·24
Iron–dextran (3 × 0·2 ml.; 5 days)	10	22·9	3·4	1·68	0·70	59·6	6·2	34·9	3·7	17·9	10·50	1·72	0·21
Untreated	8	29·1	1·8	1·67	0·30	77·2	15·1	24·9	3·5	22·9	7·28	3·16	0·78
Iron–dextran (1 × 0·2 ml.; 1 day)	6	26·3	2·6	1·70	0·12	51·6	8·9	20·2	2·2	17·7	6·94	2·47	0·21
Iron–dextran (3 × 0·2 ml.; 1 day)	6	28·7	3·2	2·35	0·51	62·4	8·4	31·6	5·3	18·5	9·37	2·01	0·36

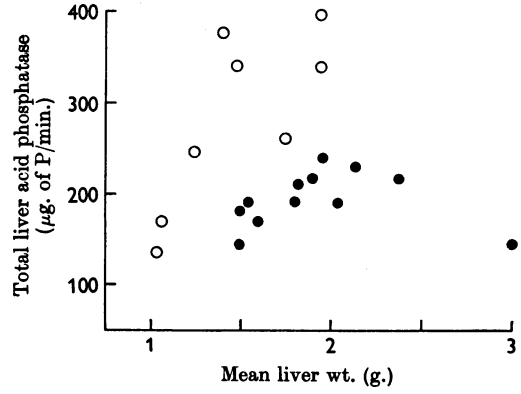

Fig. 1. Relationship between mean liver weight and total liver acid-phosphatase activity. ●, Groups of ten male mice; ○, groups of six female mice.

Acid-phosphatase activity in injected tissues

Repeated intramuscular or subcutaneous administration of iron–dextran in large doses leads to the accumulation of iron at the injection sites, as shown in Table 6. Two days after the last injection 25 % of the total dose of iron given remained locally in the injected leg and 0·5 % in the opposite leg; at 21 days there was 15 % of residual iron. The thigh muscles and overlying skin and subcutaneous tissues showed a concomitant rise in acid-phosphatase concentration, rendered even more distinct than in the liver by the initial low values found in these tissues in the untreated animals. Two days after injection the iron content of the skin of the uninjected leg confirmed the naked-eye observation that there was an initial wide distribution of iron–dextran in the subcutaneous tissue throughout the body. By 21 days lymphatic clearance of the iron complex had occurred, and the residual iron was localized in macrophages as ferritin and haemosiderin (unpublished observations). The acid-phosphatase concentration of the tissue had increased by this time.

It was of interest to ascertain whether the rise in acid phosphatase at the injections sites is a permanent change, as it appears to be in heavily siderotic liver. The results in Table 6 show that this is so, in both mice and rats.

Acid-phosphatase activity of other organs

In view of the striking rise in liver acid phosphatase it was of interest to assess the corresponding enzyme levels in other organs of the mouse. Serum acid phosphatase was extremely variable and was apparently unaffected by the injection of large doses of iron. Significant changes were observed in the spleen, where high doses of iron–dextran complex, saccharated oxide of iron and

even low-molecular-weight dextran ($P < 0{\cdot}01$) increased the acid phosphatase (Table 7). The 20% (w/v) dextran solution achieved this effect by increasing the size of the spleen from $156 \pm 37{\cdot}5$ mg. in the group injected with 0·9% sodium chloride to 289 ± 99 mg. Saccharated iron oxide had a striking effect in one-quarter of the dose, as iron, as compared with iron–dextran. Again in the kidney, the saccharated oxide brought about a striking rise in acid phosphatase; iron–dextran in high dosage also raised the acid-phosphatase activity significantly ($P < 0{\cdot}01$).

The results in Table 7 reveal some correlation between acid-phosphatase activity and the extent of iron deposition in these organs as judged histochemically by the method of Pearse (1953). When

Table 3. *Effect of treatment with iron on the acid-phosphatase activity of mouse liver*

Values are the mean results for 10 male mice. Injections were given intravenously.

Material injected	Dose per injection (ml.)	Dose per injection (mg. of Fe/kg.)	No. of daily injections	Time after last injection (hr.)	Acid-phosphatase activity (μg. of P/100 mg. of fresh liver/min.) Mean	S.D.
None	—	—	—	—	11·5	2·6
Sodium chloride (0·85%)	0·1	—	9	24	12·3	4·2
Low-molecular-weight dextran (20%, w/v)	0·1	—	9	24	12·4	3·3
Clinical dextran (6%, w/v)	0·1	—	9	24	11·4	2·7
Iron–dextran complex	—	450	1	6	25·2	4·6
Iron–dextran complex	—	220	1	24	24·7	7·0
Iron–dextran complex	—	220	2	24	33·9	5·1
Iron–dextran complex	—	220	3	24	39·8	3·9
Iron–dextran complex	—	220	9	24	58·0	13·4
Saccharated oxide of iron	—	182	9	24	50·3	11·6

Table 4. *Total acid-phosphatase activity of mouse livers*

Values are the mean results for 10 male mice, injected intravenously (i.v.) or intramuscularly (i.m.).

Material injected	Dose per injection (ml.)	Dose per injection (mg. of Fe/kg.)	No. of injections Daily i.v.	No. of injections Weekly i.m.	Liver wt. (g.) Mean	Liver wt. (g.) S.D.	Total liver acid-phosphatase activity (μg. of P/min./whole liver) Mean	S.D.
Sodium chloride (0·85%)	0·1	—	9	—	1·55	0·17	191	70
Low-molecular-weight dextran (20%, w/v)	0·002	—	9	—	1·60	0·43	169	56
Low-molecular-weight dextran (20%, w/v)	0·1	—	9	—	1·96	0·43	240	66
Iron–dextran complex	—	5	9	—	2·42	0·15	408	51
Iron–dextran complex	—	220	9	—	2·70	0·75	1532	139
Saccharated oxide of iron	—	90	10	—	2·06	0·34	1054	397
Saccharated oxide of iron	—	180	10	—	2·12	0·39	1015	269
Sodium chloride (0·85%)	0·002	—	—	10	2·38	0·32	216	26
Iron–dextran complex	—	5	—	10	2·57	0·32	237	35

Table 5. *Comparison of effects of treatment with iron in female rats, guinea pigs and hamsters*

Five daily intraperitoneal injections of 0·2 ml. of iron–dextran were given; animals were killed 48 hr. after last injection.

Species	Group	No. of animals	Mean body wt. (g.)	Mean liver wt. (g.)	Total liver acid-phosphatase activity (μg. of P/min.)
Rat	Untreated	10	158 ± 34	$6{\cdot}58 \pm 0{\cdot}75$	763 ± 94
Rat	Iron-treated	10	129 ± 14	$6{\cdot}70 \pm 0{\cdot}97$	1500 ± 388
Guinea pig	Untreated	5	283 ± 16	$13{\cdot}06 \pm 2{\cdot}14$	1267 ± 98
Guinea pig	Iron-treated	5	256 ± 25	$14{\cdot}64 \pm 2{\cdot}52$	1193 ± 204
Hamster	Untreated	5	97 ± 12	$4{\cdot}31 \pm 0{\cdot}86$	484 ± 181
Hamster	Iron-treated	5	107 ± 13	$5{\cdot}24 \pm 0{\cdot}47$	1104 ± 251

iron was given as the saccharated oxide, deposition in the kidney was greater than with 2·5 times the dose of iron–dextran. In a further experiment on the lines of group E, there were again clear-cut increases in acid-phosphatase activity of liver, kidney, spleen and small intestine. Heart, brain, lung, pancreas and testis were not significantly different in acid-phosphatase concentration from the untreated group of mice.

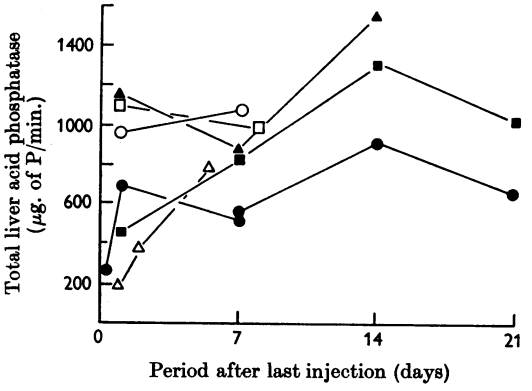

Fig. 2. Total liver acid-phosphatase activity of groups of ten male (M) or six female (F) mice at various times after the termination of daily intravenous injections of iron–dextran complex: ●, one injection of 0·1 ml. (F); ○, two injections of 0·1 ml. (M); ■, one injection of 0·2 ml. (F); □, three injections of 0·1 ml. (M); ▲, three injections of 0·2 ml. (M); △, one injection of 0·2 ml. of saccharated iron oxide (M).

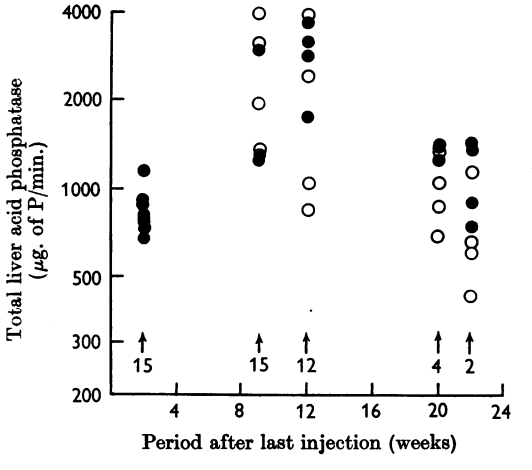

Fig. 3. Total liver acid-phosphatase activity in mice killed at various times after the termination of a series of weekly subcutaneous injections of 0·2 ml. of iron–dextran complex. The number of injections is shown under the arrow for each group. ●, Male mice; ○, female mice.

Table 6. *Effect of iron–dextran injections on acid-phosphatase activity at the injection site*

Results are expressed in terms of μg. of iron/100 mg. of fresh tissue and μg. of P/100 mg. of fresh tissue/min. Statistics refer to means ± S.D. Muscle refers to thigh muscles except in the mouse group marked *, where muscle from the lower anterior abdominal wall was used. In both groups marked * the data in the 'uninjected' columns refer to control untreated animals; in the two other groups injected with iron into one hind limb 'uninjected' tissues were derived from the other hind limb. 'Skin' includes subcutaneous tissues. Animals were injected intramuscularly (i.m.) or subcutaneously (s.c.).

Treatment	Species	No. in group	Period after last injection (days)	Uninjected				Injected			
				Muscle		'Skin'		Muscle		'Skin'	
				Iron	Phosphatase	Iron	Phosphatase	Iron	Phosphatase	Iron	Phosphatase
Untreated	Mouse	10	—	4·58 ±1·04	1·91 ±0·39	10·65 ±3·16	2·81 ±0·75	—	—	—	—
Iron–dextran (8 × 0·1 ml., i.m.)	Mouse	6	2	26·9 ±7·42	2·54 ±0·52	131·5 ±39·6	3·81 ±0·91	468 ±61·9	7·35 ±2·65	982 ±161	9·87 ±2·44
Iron–dextran (8 × 0·1 ml., i.m.)	Mouse	6	21	21·1 ±27·5	2·68 ±0·68	32·9 ±8·6	5·37 ±0·92	281 ±51·3	8·12 ±2·15	596 ±327	10·57 ±5·07
Iron–dextran (15 × 0·2 ml., s.c.)	Mouse*	6	400	—	1·91 ±0·38	—	2·10 ±1·00	—	12·76 ±4·45	—	15·87 ±8·73
Iron–dextran (22 × 75 mg. of iron/kg., i.m.)	Rat*	6	110	—	2·32 ±0·79	—	1·88 ±0·75	—	13·65 ±5·16	—	21·37 ±10·19

Table 7. *Acid-phosphatase activity of mouse organs*

Values are the mean results for groups of 10 male mice killed 24 hr. after the last injection. Group G is included here as a key to Fig. 4.

Group	Material injected	Dose: Vol. (ml.)	Dose: Dilution	Dose: (mg. of iron/kg.)	No. of daily intravenous injections	Acid-phosphatase activity (μg. of P/min.): Kidney: Activity/100 mg.: Mean	Kidney: Activity/100 mg.: S.D.	Kidney: Total: Mean	Kidney: Total: S.D.	Spleen: Activity/100 mg.: Mean	Spleen: Activity/100 mg.: S.D.	Spleen: Total: Mean	Spleen: Total: S.D.
O	Untreated	—	—	—	—	7·7	1·6	28·0	4·5	26·2	3·8	52·0	27·4
A	Sodium chloride (0·85%)	0·1	—	—	9	10·1	1·9	38·7	6·8	46·6	6·8	72·4	19·6
B	Low-molecular-weight dextran (20%, w/v)	0·1	1:50	—	9	11·7	0·7	43·9	8·8	59·8	13·4	75·6	19·8
C	Low-molecular-weight dextran (20%, w/v)	0·1	—	—	9	11·0	0·6	45·9	7·7	46·8	12·6	127·3	35·1
D	Iron–dextran complex	0·1	1:50	5	9	9·7	2·1	45·4	5·9	46·6	10·3	90·9	18·9
E	Iron–dextran complex	0·1	—	250	9	18·8	4·0	57·6	10·1	100·8	35·1	194·7	51·3
F	Saccharated oxide of iron	0·1	1:1	100	10	28·4	8·1	91·1	20·5	104·2	28·4	270·6	51·1
G	Saccharated oxide of iron	0·2	1:1	200	10	—	—	—	—	—	—	—	—

Other enzymes in siderotic mouse liver

Three further enzymes were selected for detailed study in the liver of groups A–G (as listed in Table 7): alkaline phosphatase, β-glucuronidase and cathepsin. These activities are compared in Fig. 4 with the corresponding values for the sodium chloride-treated control group A. All three enzymes were significantly increased in group G, more particularly cathepsin, which showed enhanced levels in groups E and F as well. Moderate dosage with iron, as in group D, had no such effects.

Finally, groups A and E were selected for further study and a number of other enzymes likely to be affected by hepatic siderosis were measured (Table 8). Significant increases were found in arylsulphatase, acid ribonuclease, acid deoxyribonuclease and adenosine triphosphatase. The remainder were unchanged or reduced. Attempts were also made to measure hepatic cytochrome oxidase; these failed because the dark-brown colour of the blank, due to dissolved iron, rendered spectrophotometry impossible.

DISCUSSION

Liver enlargement and iron overdosage

The hepatomegaly which develops in mice after treatment with very large doses of iron–dextran or saccharated oxide of iron is a non-specific response to colloidal particles and macromolecules like colloidal carbon, thorotrast, dextran (low-molecular-weight and clinical types) and polyvinylpyrrolidone. It must be stressed that the doses used are high. With iron–dextran, the standard injection routine (9 × 0·1 ml.) introduces over 20 times the total body iron of the mouse and exceeds the total clinical dose (20 mg. of iron/kg.) by over 100 times. When smaller doses are given, still well in excess of the total body iron, no hepatomegaly can be detected. It is clear that an overloading effect is present and the analysis of liver RNA-P and DNA-P is suggestive of hyperplasia rather than hypertrophy. Thus in hypertrophied rabbit hearts Nowy, Frings & Rey (1959) found a fall in DNA-P, a rise in RNA-P and a rise in the RNA-P:DNA-P ratio from 2·9 to 4·0. In the siderotic mice, liver DNA-P rose and RNA-P fell, so that the ratio RNA-P:DNA-P fell from over 3 to 2 or less. The ratio reported by Schneider, Hogeboom & Ross (1950) for normal mouse-liver homogenate was 3·33; for rat liver Goodlad & Mills (1957) found a ratio of 4.

Significance of acid-phosphatase activity

To assess the importance of acid-phosphatase activity in siderotic liver it is necessary to consider the possible source of the increased activity.

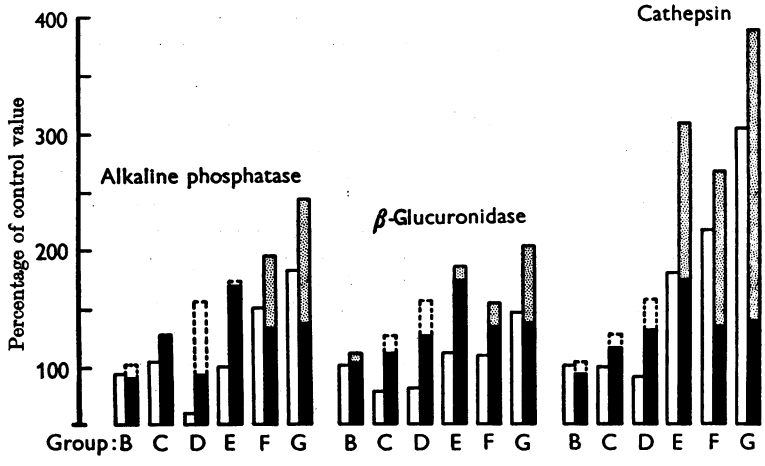

Fig. 4. Mean enzyme activities of mouse livers of groups B–G (for details see Table 7) in relation to those of the control group A (injected with 0·9% NaCl). Activities are compared on a 100 mg. basis in the left-hand columns and on a total liver basis in the right-hand columns. The ratio of mean liver weights for each group is marked on the right-hand column to indicate the extent to which the observed activity ratio exceeds (▤), or falls short of (□), what would be expected (■) merely from the mean changes in liver weight.

Table 8. *Enzyme contents of mouse livers*

Dosage of iron–dextran: five daily intravenous injections of 0·2 ml.; the mice were killed 24 hr. after the last injection. The groups marked *, † and ‡ received nine, three and two injections of 0·1 ml. respectively. Control groups received 0·9% NaCl in place of iron–dextran. Units are μmoles of substrate/100 mg. of liver/min., except in the following cases: uricase, μmoles of O_2; acid ribonuclease and deoxyribonuclease, E_{280}; DPNH–cytochrome *c* reductase, units defined by Mahler, Sarkar, Vernon & Alberty (1952). Statistics refer to means ± standard deviations.

Enzyme	No. of mice in each group	Enzyme activity		*P*
		0·9% NaCl	Iron–dextran	
Arylsulphatase	10, 10	0·126 ± 0·014	0·149 ± 0·022	0·01–0·02
Acid ribonuclease	10, 10*	0·837 ± 0·129	1·027 ± 0·082	<0·001
Acid deoxyribonuclease	10, 10*	0·258 ± 0·031	0·367 ± 0·060	<0·001
Adenosine triphosphatase	6, 6	2·98 ± 0·46	3·33 ± 0·42	0·01–0·02
Uricase	6, 9*	0·395 ± 0·050	0·402 ± 0·088	>0·3
Xanthine oxidase	10, 10	0·047 ± 0·027	0·032 ± 0·016	>0·1
DPNH–cytochrome *c* reductase	5, 5	5·44 ± 1·53	5·88 ± 1·01	>0·6
Glucose 6-phosphatase	6, 6‡	1·26 ± 0·23	0·89 ± 0·15	0·001–0·01
	0, 6†	—	0·76 ± 0·06	0·001–0·01
	10, 10*	1·51 ± 0·36	1·21 ± 0·49	>0·1
Catalase	10, 10	44·6 ± 11·6	27·8 ± 7·2	<0·001
Esterase	10, 10	16·1 ± 3·6	10·9 ± 2·5	0·001–0·01

Macrophage and related cell enzymes. On histochemical evidence Gedigk & Bontke (1957) have reported the presence of acid phosphatase, β-glucuronidase, phosphamidase, aminopeptidase and, less regularly, non-specific esterase; alkaline phosphatase and 5-nucleotidase were not present. Gropp & Hupe (1958) found that macrophages and epithelioid cells cultured *in vitro* gave positive histochemical tests for adenosine triphosphatase, non-specific esterase and acid but not alkaline phosphatase. It has been suggested by these and other workers (Weiss & Fawcett, 1953) that enzymic changes of this kind develop during the activation of resting histiocytic cells. Moreover, they appear to persist. The high enzyme activity of the foreign-body granuloma, for example, remains relatively constant over a long period and is only lost when fibrous scar tissue finally replaces the granuloma (Gedigk & Bontke, 1957).

The changes undergone by iron–dextran or saccharated iron oxide within splenic macrophages were studied by Richter (1959). He was able to

show by electron microscopy that within 6 hr. transformation to ferritin was taking place. Whereas saccharated iron oxide inhibits reticuloendothelial function for at least 48 hr., iron–dextran has a stimulatorv effect (L. Golberg, L. E. Martin, C. Harrison & C. M. Bates, unpublished observations). It is likely therefore that under these conditions 'activation' and proliferation of liver histiocytic and Kupffer cells is taking place, and that this process is responsible for at least a part of the rise in acid phosphatase. The situation is clearer in injected muscle and subcutaneous tissues, where macrophage accumulation of striking proportions has been shown to take place within 48 hr. of massive iron–dextran injection (Beresford, Golberg & Smith, 1957; L. Golberg & S. Baker, unpublished observations).

Polymeric pigments. The presence of hydrolytic enzymes in lipofuscin or ceroid has been demonstrated by Gedigk & Bontke (1956) and Gedigk & Fischer (1959). Acid phosphatase is invariably found and non-specific esterase less constantly. The suggestion made by these authors, that lipofuscin is deposited at cytoplasmic sites of high acid-phosphate activity, is in keeping with the observation of Novikoff (1959) referred to below.

Parenchymal iron is always present in the liver of the siderotic rat or mouse and in the course of time its peribiliary location becomes more and more manifest. Histochemically demonstrable lipofuscin pigments develop only after many weeks (Golberg & Smith, 1960). However, the haemosiderin matrix which begins to form almost at once at the sites of iron deposition has many histochemical properties in common with ceroid (Weber, 1954), although Gedigk & Fischer (1958) have drawn attention to differences between them. The formation of lipofuscin around bile canaliculi led Pfuhl (1941) to suggest that the polymeric pigment develops in the Golgi body, a cytoplasmic structure which appears to be closely associated with, if not actually identical with, the 'acid phosphatase-rich granule' of Deane (1947). The first appearance of lipofuscin coincides with the disappearance of these peribiliary acid-phosphatase granules (Novikoff, 1959).

Although none of the above-mentioned histochemical studies includes comment on this point, it is difficult to decide whether haemosiderin shows a positive acid-phosphatase reaction. The Gomori (lead phosphate) technique fails, since, as would be expected, lead is fixed by the haemosiderin 'carrier substance' or matrix. The azo method (Pearse, 1953) reveals a distinct increase in acid phosphatase but is too diffuse to permit precise localization of enzyme activity. Haemosiderin granules when isolated by the technique of Ludewig (1957) show no acid-phosphatase activity *in vitro*.

Cytochondrial enzymes. Intense acid-phosphatase activity is the common feature of biochemical entities, the lysosomes, and cytological units: the Golgi bodies, 'acid-phosphatase granules' and 'peribiliary dense bodies' (Novikoff, 1959). In order to assess whether the increase of acid phosphatase is an index of generally enhanced lysosomal activity, other enzymes of this category were studied in siderotic liver: alkaline phosphatase, β-glucuronidase, cathepsin, arylsulphatase and uricase. Other phosphatases measured were adenosine triphosphatase and glucose 6-phosphatase, which is exclusively microsomal (Hess, Berthet, Berthet & de Duve, 1951), as well as esterase, said to be truly microsomal (Underhay, Holt, Beaufay & de Duve, 1956). Attempts to measure cytochrome oxidase as a typical mitochondrial enzyme were unsuccessful, owing to interference by iron as mentioned above.

Of the other enzymes measured, xanthine oxidase has been reported to be intimately linked with ferritin and with the liberation of ionic iron from its storage form (Mazur, Green, Saha & Carleton, 1958). DPNH–cytochrome *c* reductase was of interest in view of the possibility that α-tocopherol functions as the coenzyme. Hence inactivation of tocopherol, or its maintenance in the oxidized form, by the overwhelming accumulation of stored iron, might be expected to reduce drastically the observed activity of the enzyme.

The outcome of these measurements was to show that, in addition to acid phosphatase, a number of other hepatic enzymes had increased activity: alkaline phosphatase, β-glucuronidase and especially cathepsin; arylsulphatase, acid ribonuclease and deoxyribonuclease; adenosine triphosphatase. All of these except the last are lysosomal enzymes. Uricase remained unchanged. From the most recent evidence of de Duve (1959) uricase does not appear to run parallel with acid phosphatase. It may be present with catalase in some cytoplasmic particle other than the lysosome (Thomson & Klipfel, 1957). The fact that adenosine triphosphatase is not regarded as a lysosomal enzyme does not prevent the conclusion being drawn that injection of massive doses of iron exercises an effect on lysosomes and that the enhanced level of acid phosphatase is not entirely, though it may be partly, due to stimulation of reticuloendothelial-cell activity.

de Duve (1957) has criticized the use by Tsuboi (1952) of phenyl phosphate as substrate for the study of mouse-liver acid phosphatase at pH 5·5. This substrate is also split by glucose 6-phosphatase (Beaufay & de Duve, 1954), which is abundant in liver and has a pH optimum of 6·0. Tsuboi's high absolute values for acid phosphatase and low overall recoveries on fractionation of liver homogenate

are thus attributed by de Duve to this cause. In the present experiments carried out at pH 4·9 the contribution of glucose 6-phosphatase is slight. Direct measurements with glucose 6-phosphate as substrate at pH 6·5 (Table 8) showed either no rise or a distinct decrease in siderotic livers, whereas in the same livers there was a very large increase in acid phosphatase. Other experiments, to be reported separately, have involved the use of other substrates and various inhibitors, establishing beyond question that the enzyme involved is acid phosphatase and not glucose 6-phosphatase.

Role of iron

de Duve (1959) has reviewed the frequent, though not invariable, association of iron granules of high electron density with those cytoplasmic particles in liver, kidney and spleen which possess high acid-phosphatase activity. In earlier experiments, de Duve & Beaufay (1957) had demonstrated a highly significant correlation between the iron content and the acid-phosphatase activity of the lysosome-rich fractions of liver homogenate. Up to 10% of the cell ferritin was estimated to be associated with lysosomes or related particles, most of the remainder being in the final supernatant. More recently the same group (Beaufay, Bendall, Baudhuin & de Duve, 1959) have once more drawn attention to this correlation, suggesting that lysosomes and peribiliary dense bodies may be identical.

Bessis & Breton-Gorius (1959) have described iron accumulation within the mitochondria of sideroblasts and siderocytes, which rupture with the release of so-called '*micelles ferrugineuses*', supposedly a form of iron ready for incorporation into haem. Similarly, Richter (1958) has described particles containing much iron, the 'siderosomes', possibly derived from mitochondria. All these scattered observations suggest a relationship between iron in its storage forms and acid phosphatase in certain specialized cytoplasmic structures. In the present state of knowledge the functional connexion is obscure.

SUMMARY

1. Very high doses of iron, as iron–dextran or saccharated iron oxide, injected into mice, bring about stunting of growth and hyperplastic enlargement of the liver. There is a disproportionately large, and apparently permanent, increase in the acid-phosphatase activity of liver. Enhanced activity is seen in the liver of the rat and the hamster, but not of the guinea pig.

2. Injection of iron in doses which were less massive, yet well above the clinical level, or the administration of sodium chloride solution or dextran, had no such result.

3. In the mouse and rat the injection sites in muscle and in subcutaneous tissue have a raised acid-phosphatase activity after the administration of heavy doses of iron–dextran. The spleen and kidney of the mouse also show a higher level of acid phosphatase.

4. Of various other enzyme activities measured in the siderotic liver, the following were significantly raised: cathepsin, β-glucuronidase, alkaline phosphatase, arylsulphatase, acid ribonuclease, acid deoxyribonuclease and adenosine triphosphatase.

5. The significance of these observations is considered in the light of the possible relationships between intracellular iron and acid phosphatase on the one hand, and cytoplasmic particles or structures on the other. It is suggested that the observed changes may be partly lysosomal in origin but that the activity of macrophages and related cells is likely to be a contributory factor.

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The Inhibitory Action of Relaxing-Factor Preparations on the Myofibrillar Adenosine Triphosphatase

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Marsh (1952) reported that whole-muscle homogenates contained a factor (known as the Marsh factor, Marsh–Bendall factor or relaxing factor) which prevented adenosine triphosphate from inducing the normal synaeresis which occurred on addition of the nucleotide to washed muscle-cell fragments. Under certain conditions in the presence of the factor an elongation of the fibre fragments took place and the process was considered to be analogous to relaxation. In whole-muscle homogenates Marsh also observed that the adenosine-triphosphatase activity of the myofibrils was suppressed and subsequent work by others (Bendall, 1953; Bozler & Prince, 1953) showed that relaxation in muscle-model systems was associated with a low rate of hydrolysis of adenosine triphosphate. A number of investigators have demonstrated that so-called relaxing-factor preparations can also inhibit the Mg^{2+}-ion-activated adenosine triphosphatase of isolated myofibril preparations (Hasselbach & Weber, 1953; Portzehl, 1957*a*; Bendall, 1958).

The nature of this relaxing factor has been a matter of some controversy and although various phosphorylating systems (Bendall, 1954; Goodall & Szent-Györgyi, 1953; Lorand, 1953) have been claimed to possess activity it is now clear that particulate material can be sedimented from homogenates of rabbit skeletal muscle which will induce the relaxation of fibre models in the presence of adenosine triphosphate (Ebashi, Takeda, Otsuka & Kumagai, 1956; Portzehl, 1957*a*) and simultaneously inhibit the Mg^{2+}-ion-activated myofibrillar adenosine triphosphatase. These prepara-