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Biochemical Changes in the Tissues of Animals Injected with Iron

4. THE NATURE OF ACID-PHOSPHATASE ACTIVITY*

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Golberg, Martin & Batchelor (1960) showed that treatment of mice and rats with heavy doses of iron-dextran complex increased the activity of acid phosphatase and other lysosomal enzymes, not only in liver but also in organs such as spleen and kidney which display low activity in the untreated animal. Some of the largest increases were found at the sites of repeated injections given into the thigh muscles or in the subcutaneous tissues of the abdominal wall, changes clearly reflecting the morphological and cytological alterations which are induced locally under these circumstances (Baker, Golberg, Martin & Smith, 1961; Muir & Golberg, 1961*a*). There is an influx of macrophages into the injection site (Beresford, Golberg & Smith, 1957; Baker *et al.* 1961), and since such cells are known to possess particularly high acid-phosphatase activity (Gomori, 1941), the early rise found in the local tissues (Golberg *et al.* 1960) can be readily accounted for. What is more difficult to understand is the persistence of activity of this kind long after the phagocytosis of iron-dextran and its transformation into ferritin are apparently complete (Muir & Golberg, 1961*b*). The answer is probably to be found in the complex and enduring effects exercised by the stored iron (Muir & Golberg, 1961*a*). Macrophages appear to be affected in three main ways: they die and are disrupted, when the scattered ferruginous masses are taken up by other macrophages; or they die and are engulfed in a compacted form, again by macrophages; or they appear to be replaced by ferritin-laden fibroblasts. Even after a year or more, continuing muscle necrosis provides evidence of local disturbance. In

the liver, which appears histologically more quiescent at this time, acid-phosphatase activity tends to revert to values found in untreated animals.

Consideration of this difference requires an insight into the origin of acid-phosphatase activity. In the liver cell this enzyme is localized in lysosomes (de Duve, 1959) which have been correlated morphologically with the peribiliary dense bodies (Daems & van Rijssel, 1961) and also with the large spherical bodies of Kupffer cells, with microbodies and with lipofuscin granules (Essner & Novikoff, 1961). In other reticulo-endothelial cells the same or similar organelles are associated with phagocytosis and storage (de Duve, 1959; Novikoff, 1960). Also a continuous transition has been established between the initial ingestion vacuole and the dense cytoplasmic structure in which the material taken up is stored (Parks, Peachey & Chiquoine, 1956; Felix & Dalton, 1956; Harford, Hamlin & Parker, 1957; Muir & Golberg, 1961*b*). Thus it is likely that a relationship exists between lysosomal acid phosphatase and the plasma membrane of the reticulo-endothelial cell, though it is impossible to say at present whether the ingestion vacuoles fuse with existing lysosomal structures or create new specialized bodies of this kind. In any event the appearance of acid phosphatase in these vacuoles raises the question whether the enzyme has been formed simultaneously with the vacuole or has been segregated from elsewhere in the cell. Holt & Hicks (1961) have suggested that 'readiness' for phagocytic activity varies in different cells, as exemplified by the state of their acid phosphatase and other hydrolytic enzymes.

A possible approach to the clarification of these problems would be the induction of an increase in

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acid phosphatase and a comparison of the characteristics of the enzyme then present with those of the enzyme found in untreated animals. The heterogeneity of acid phosphatase (Barka, 1961) might facilitate the detection of any changes brought about in this way. Methods available for achieving such an increase include partial hepatectomy, the injection of zymosan or *Escherichia coli* endotoxin, infection with *Mycobacterium tuberculosis* B.C.G. strain, or the implantation of sarcoma 180 (Thorbecke, Old, Benacerraf & Clarke, 1961), injection of trypan blue or typhoid vaccine (Barka, Schaffner & Popper, 1961), injection of clinical dextran (van Duijn, Willighagen & Meijer, 1959; de Man, Daems, Willighagen & van Rijssel, 1960), high- and low-molecular-weight polymers of vinylpyrrolidone, carbon particles (Meijer & Willighagen, 1961) and iron-dextran (Golberg, Martin, Bates & Batchelor, 1959; Golberg *et al.* 1960). Of all these, the last appears to provide by far the greatest rise in liver phosphatase—an effect which is not reversed on repeated injection as with trypan blue or typhoid vaccine (Barka *et al.* 1961). Use of this technique has the further advantage that the association of iron with lysosomes is an intimate one, and possibly of fundamental physiological and pathological significance (Beaufay, Bendall, Baudhuin & de Duve, 1959; Essner & Novikoff, 1960). Hence a comparison of the type suggested above might also provide information on this relationship.

METHODS AND MATERIALS

Iron dosage. For most of the experiments Tuck no. 1 strain albino mice were used; but this strain was recently discontinued by the breeder and some of the later work with liver mitochondrial fractions was carried out on Tuck T.T. strain mice. Mice weighing 18–20 g. were given eight daily intramuscular injections of 0.1 ml. of iron-dextran complex (Imferon) containing 50 mg. of ferric iron/ml. They were killed 72–96 hr. after the last injection. Similarly albino rats weighing 150 g. were killed 96 hr. after the last of five daily injections of 1 ml. of iron-dextran.

Preparation of liver, muscle and skin homogenates. Non-starved mice or rats were decapitated and excess of blood was drained out. The liver was rapidly excised, weighed and transferred to a Potter-Elvehjem smooth-glass mortar packed in ice and containing sufficient water at 2° to give a 10% (w/v) homogenate. The tissue was ground for 2 min. with a Teflon pestle, running at 1000 rev./min., and the homogenate was transferred to ice-cold McCartney bottles. A sample of the homogenate was diluted with water to give a final concentration in the range 0.5–3 mg. of protein/ml., the higher dilution being made necessary by the greater acid-phosphatase activity of siderotic liver. The skin was reflected back and the most deeply stained muscle tissue removed, cut up into small pieces and homogenized under the same conditions as for liver. The heavily stained area of skin was also treated in a similar manner.

Preparation of liver-mitochondrial and other fractions.

For this purpose four mouse livers were homogenized together, by the same procedure but with ice-cold 0.25M-sucrose in place of water. The homogenate was centrifuged at 600g for 10 min. to remove unbroken cells, nuclei and debris. The supernatant was transferred to chilled cellulose acetate tubes and centrifuged again at 8500g for 10 min. The layer of fat at the surface was removed with a throat swab and the supernatant layer carefully decanted and discarded. Ice-cold 0.25M-sucrose was then added (1 ml./2 g. of fresh liver taken). The mitochondrial pellet was broken up with a glass rod and a uniform suspension produced by repeated aspiration with a pipette. The mitochondrial fraction was separated at 8500g for 10 min., suspended again in 0.25M-sucrose, separated again as before and finally suspended in water, to be homogenized in a MSE Nelco blender for 3 min. to liberate all the acid-phosphatase activity. The final protein concentration of the diluted mitochondrial suspension was adjusted to 1–4 mg. of protein/ml., depending on the enzyme activity it was intended to measure. In preliminary experiments it was found that total acid-phosphatase activity was the same whether the mitochondria were lysed with Triton X-100 or disrupted mechanically (Wattiaux & de Duve, 1956). Since Triton X-100 interfered with the estimation of phosphate by the method of Fiske & Subbarow (1925) the Nelco blender was used instead. Protein was determined by the biuret reaction (Golberg *et al.* 1960). The morphology of washed mitochondrial fractions, before lysis with water, was studied by light- and electron-microscopy. For the latter, the mitochondrial pellet was fixed in 1% (w/v) osmium tetroxide in sodium acetate-sodium barbitone buffer, pH 7.4 (Palade, 1952), at 0° for 1 hr. The mitochondria were washed with buffer, separated by centrifuging and covered with a layer of 1% (w/v) agar. When the agar had solidified, a layer of 10% (v/v) ethanol was poured over it. Sections, 20–40 m μ thick, were cut by Dr A. R. Muir on a Porter-Blum microtome with a glass knife and mounted on copper grids (Athene 483) covered with a carbon-collodion membrane. The electron microscope was an A.E.I. E.M. 6, operated at 40 kv with a 50 μ objective aperture.

The small-particle fraction was obtained by centrifuging the supernatant from the mitochondrial fraction at 20 000g for 90 min. The particles were suspended in 0.25M-sucrose, homogenized in the Nelco blender for 3 min. and made up to volume with 0.25M-sucrose. The supernatant from the small-particle fraction was diluted and blended in the same manner.

Buffers. For the most part the buffers used were 0.1M-sodium acetate-acetic acid, pH 4.9 or 5.8, and 0.1M-sodium citrate-HCl, pH 4.9 or 5.8; for 0.1M-citrate-acetate buffer, equal volumes of the above-mentioned buffers, made up in 0.2M-concentration, were mixed before addition of the substrate. 0.1M-Malonate-acetate buffer was prepared by mixing equal volumes of 0.2M-disodium malonate and 0.2M-sodium acetate; the pH was adjusted to 5.8. In all cases the pH of the buffer was re-adjusted after addition of the substrate.

Phenyl phosphatase and related activities. Disodium phenyl phosphate was purified by the method of Tsuboi (1952). Samples (0.2 ml.) of diluted liver homogenate or lysed mitochondria were added to 4 ml. of buffered disodium phenyl phosphate which had been heated at 37° for 5 min. The various buffers and substrate concentrations

Table 1. *Phosphomonoesterase activities of mouse-liver homogenates*

Enzyme activity is expressed as μg . of P/100 mg. of fresh tissue/min., except with 2-carboxyphenyl phosphatase, where it is the change in extinction at 310 m μ /100 mg. of fresh tissue/5 min. Values are means $\pm$ s.d. for groups of ten mice (in experiments at pH 4.9) or six mice (in all other experiments).

Substrate	Concn. of substrate (mm)	Buffer			Enzyme activity		Ratio, iron-treated:untreated
		Molarity	pH	Composition	Untreated	Iron-treated	
Phenyl phosphate	5	0.1	4.9	Acetate	13.4 $\pm$ 2.47	23.1 $\pm$ 4.27	1.7
	5	0.1	4.9	Citrate	12.2 $\pm$ 2.21	72.1 $\pm$ 10.12	5.9
	5	0.0125	4.9	Veronal-acetate	16.5 $\pm$ 1.32	27.5 $\pm$ 8.07	1.7
	5	0.1	5.8	Acetate	26.5 $\pm$ 4.47	55.0 $\pm$ 7.49	2.1
	5	0.1	5.8	Citrate	20.4 $\pm$ 5.92	81.1 $\pm$ 17.00	4.0
β -Glycerophosphate	100	0.1	5.8	Acetate	59.8 $\pm$ 4.60	101.2 $\pm$ 11.3	1.7
	100	0.1	5.8	Citrate	269.0 $\pm$ 77.0	269.0 $\pm$ 77.0	4.1
	100	0.1	5.8	Malonate	58.6 $\pm$ 3.00	186.6 $\pm$ 36.2	3.1
	16	0.1	5.8	Acetate	5.32 $\pm$ 0.32	8.75 $\pm$ 0.56	1.6
4-Nitrophenyl phosphate	7.6	0.1	5.8	Citrate	2.53 $\pm$ 0.19	6.03 $\pm$ 0.34	2.4
	7.6	0.1	5.8	Acetate	16.6 $\pm$ 2.02	18.6 $\pm$ 4.97	1.1
2-Carboxyphenyl phosphate	6	0.1	5.8	Citrate	19.3 $\pm$ 1.06	41.3 $\pm$ 1.50	2.1
	6	{0.1} {0.1}	6.0	Acetate {Citrate-acetate}	1.7 $\pm$ 0.64	2.4 $\pm$ 0.87	1.4
Phenolphthalein phosphate	1	0.1	6.0	acetate	2.4 $\pm$ 0.47	7.5 $\pm$ 2.40	3.2
	1	0.1	4.9	Acetate	22.3 $\pm$ 2.04	36.6 $\pm$ 4.13	1.6
	1	0.1	4.9	Citrate	57.5 $\pm$ 8.84	111.9 $\pm$ 15.4	1.9

used are indicated in the text. The solution was heated for a further period of 15 min., and the liberated phenol was estimated with the Folin & Ciocalteu (1927) reagent according to the method of King & Wootton (1956). Where comparative studies were undertaken with β -glycerophosphate and other non-aromatic phosphate substrates the inorganic phosphate liberated was determined by the method of Ernster & Lindberg (1956). The other aromatic phosphate substrates, employed in a manner similar to phenyl phosphate, were: 7.6 mm-disodium 4-nitrophenyl phosphate with 0.2 ml. of liver homogenate (Sommer, 1952); 6 mm-trisodium 2-carboxyphenyl phosphate (L. Light and Co.) with 0.6 ml. of liver homogenate (Singer & Fruton, 1957); 1 mm-disodium phenolphthalein phosphate with 0.5 ml. of liver homogenate (Huggins & Talalay, 1945).

β -Glycerophosphatase activity. Samples (0.6 ml.) of homogenate of lysed mitochondrial suspension were added to 1.2 ml. of 16 mm- β -glycerophosphate in the appropriate buffer and the liberated phosphate was determined according to the method of Ernster & Lindberg (1956) because citrate and ascorbic acid interfered with the method of Fiske & Subbarow (1925). In selected cases, when citrate buffer was used, the liberated glycerol was determined by periodate oxidation to formaldehyde (Nigam, Davidson & Fishman, 1959), which was distilled into chromotropic acid reagent (MacFadyen, 1945).

Other enzyme activities. Phosphoprotein phosphatase was determined by the method of Revel & Racker (1960), with 4.1% (w/v) casein as substrate with 0.2 ml. of homogenate or lysed-mitochondrial suspension. Cysteine, ascorbic acid or sodium thioglycollate was used as activator.

For the estimation of phosphamidase activity 0.2 ml. of homogenate was added to 0.8 ml. of 75 mm-sodium phosphamidate (Albright and Wilson Ltd.) in acetate buffer, pH 6.0 (Singer & Fruton, 1957).

Adenosine-triphosphatase activity was measured in 0.2 ml. samples of homogenate or lysed-mitochondrial suspension added to 1.8 ml. of buffer, pH 5.8, containing 5 mm-ATP, at 37°. After 15 min. at 37° the mixture was treated with 2 ml. of ice-cold 12% (w/v) trichloroacetic acid, centrifuged at 1500g and inorganic phosphate estimated in the supernatant by the method of Ernster & Lindberg (1956). In control tubes homogenate was added after the trichloroacetic acid. The standard curve was prepared with KH_2PO_4 solution in place of homogenate.

Tissue iron. Total tissue iron was determined as described by Golberg *et al.* (1960). The concentration of free ionic iron was determined in 2 ml. of liver homogenate by adding 1 ml. of 6N-HCl, followed immediately by 2 ml. of 20% (w/v) trichloroacetic acid. The precipitated protein was removed by centrifuging and the iron content of the supernatant determined by the method of Ventura & Klopfer (1951).

Materials. Except where otherwise stated, all reagents were AnalaR grade (British Drug Houses Ltd.); or, if this grade was not available, laboratory-reagent grade was used.

RESULTS

Phosphomonoesterase activities

Comparisons were made initially between liver homogenates from untreated and iron-treated mice. The effects of various substrates and buffers on

phosphomonoesterase activities are shown in Table 1. Phenyl phosphatase was more active at higher pH values; from pH-activity plots, the optimum pH was found to be 5.8 in both types of liver homogenate. Over the range of substrate concentration 0.005–0.2M, the kinetics of phenyl phosphatase proved to be zero order above 0.05M. With 0.1M-acetate buffer, pH 5.8, 0.1M-phenyl phosphate and an incubation period of 15 min., there was a linear relationship between the amount of phosphate liberated and the weight of liver homogenate taken, whether normal or siderotic, over the range studied (0.2–5 mg. of tissue per tube). When citrate or malonate buffers were substituted for acetate there was a striking increase in phenyl-phosphatase activity of siderotic liver, without any comparable change in the normal liver. The ratio of activities in the two types of liver was more than doubled by the use of citrate. A similar effect was observed with related substrates, 4-nitrophenyl phosphate and 2-carboxy-phenyl phosphate. With phenolphthalein phosphate, citrate brought about a parallel increase in enzyme activity of both normal and siderotic liver.

With a non-aromatic substrate, β -glycerophosphate, there was still a distinct stimulatory effect of iron; but, in both types of liver, citrate brought about enzyme inhibition, as measured by the phosphate and glycerol liberated. Citrate inhibition extended also to mitochondrial preparations from the liver homogenates.

In rat-liver homogenates the effect of treating the animal with iron was less striking than in mice but nevertheless distinct; replacement of acetate buffer by citrate brought about little change in phenyl-phosphatase activity (Table 2).

Other phosphatases

Phosphoprotein-phosphatase activity was also higher in liver homogenates from iron-treated, as compared with untreated, mice (ratio 1.3). The results of more detailed studies with mitochondrial preparations are reported below. Phosphoamidase activity, on the other hand, was not significantly affected by the iron treatment. With 0.1M-acetate buffer, pH 6.0, the results, in μ moles of ammonia/

100 mg. of fresh tissue/min., were 1.5 ± 0.23 for untreated mice and 1.4 ± 0.20 for treated mice. It thus appears that the additional phosphatase activity of siderotic liver cleaves P–O bonds but not P–N bonds.

We have previously reported that the activity of adenosine triphosphatase is enhanced in liver of iron-treated mice (Golberg *et al.* 1960). This was the magnesium-activated enzyme which hydrolyses the terminal phosphate group of ATP (Kielley, 1955). Revel & Racker (1960) showed that a parallel existed between the activities of ox-spleen phosphoprotein phosphatase and of an adenosine triphosphatase which was not activated by magnesium, functioning as a pyrophosphatase by splitting off the two terminal phosphate groups of the substrate. We checked whether the findings of Revel & Racker (1960) were applicable to mouse liver by using 5 mM-ATP and 0.1M-acetate buffer, pH 5.8, when the results for liver homogenates from untreated and iron-treated mice were 0.76 and 2.68 μ g. of P/mg. of protein/min. The corresponding figures on the addition of 0.1M-citrate were 0.99 and 7.74; with 0.1M-malonate in place of citrate they were 1.00 and 6.14 respectively. Thus there exists ample evidence of increased activity of this enzyme in siderotic liver.

Citrate effect

The mechanism of action of citric acid with prostatic phosphatase was studied by Anagnostopoulos (1953), who also reviewed previous observations on the activation of acid phosphatases by citrate. By varying the concentration of citrate present in citrate buffer or added to 0.1M-acetate buffer (both buffers pH 5.8), and by using 0.5 mM-phenyl phosphate, the citrate effect was investigated with liver homogenates from untreated and iron-treated mice. The results in Fig. 1 reveal an effect only in siderotic liver, where phenyl phosphatase attained maximum activity at concentrations of citrate equal to or greater than 75 mM. (This was not the consequence of an increase in total ionic strength of the buffer, since the addition of 0.1M-acetate to a 0.1M-citrate buffer did not affect activity.)

Table 2. *Phenyl-phosphatase activity of rat-liver homogenates*

Enzyme activity is expressed as μ g. of P/100 mg. of fresh tissue/min. Values are mean $\pm$ S.D. for groups of four rats (experiments with 0.1M-buffers) or three rats (in other experiments).

Concn. of substrate (mM)	Buffer pH 5.8	Enzyme activity		Ratio, iron-treated:untreated
		Untreated	Iron-treated	
5	Acetate	30.5	50.0	1.6
5	Citrate	23.5	67.8	2.9
100	Acetate	54.0 ± 4.5	106.1 ± 10.0	2.0
100	Citrate	50.7 ± 7.3	117.6 ± 18.9	2.3

It was of interest to ascertain whether the activating effect of citrate on phenyl phosphatase was displayed when other tissues of iron-treated mice were used, such as muscle and skin taken from the sites of injection of iron-dextran. In Fig. 2 are shown pH-activity curves, expressed as ratios of

Fig. 1. Comparison of the effects on phenyl-phosphatase activity of sodium citrate-HCl buffer, pH 5.8, alone or mixed with 0.1M-sodium acetate-HCl buffer, pH 5.8. Untreated liver homogenate in acetate-citrate (●) and citrate (■) buffers; liver homogenate from iron-treated mice in acetate-citrate (○) and citrate (□) buffers; 5 mm-phenyl phosphate was used. For other conditions of enzyme assay see text.

Fig. 2. pH-activity curves of phenyl phosphatase in liver, muscle and skin homogenates from untreated and iron-treated mice. The results are expressed in two ways: first, as ratios of activity in 0.1M-citrate buffer, pH 5.8, to activity in 0.1M-acetate buffer, pH 5.8, for homogenates from untreated (●) and iron-treated (○) mice; and, secondly, as ratios of activity of homogenates from iron-treated mice to activity of homogenates from untreated mice when acetate (■) and citrate (□) buffers were used. The conditions of enzyme assay are described in the text; the concentration of phenyl phosphate was 5 mm.

(a) the enzyme activities observed by using 0.1M-citrate and 0.1M-acetate buffers, and (b) the enzyme activities in liver, muscle and skin of untreated mice as compared with the activities in the corresponding tissues of iron-treated animals, measured under identical conditions. In many respects the behaviour of phenyl phosphatase in liver was quite distinct from that of the enzyme present in muscle and skin, particularly after iron treatment. In the untreated mouse, citrate had only a slight activating effect on liver phosphatase above pH 4.8 but inhibited the enzyme in muscle and skin. In siderotic liver there was, as before, a striking activation over the whole pH range studied, more especially at pH 4.8-6.0. The effect of citrate on the muscle enzyme was slight, whereas skin showed a well-defined peak of citrate stimulation at pH 5.8. Comparing the effects of iron treatment on enzyme activity in the various tissues, in liver the effect was slight with acetate buffer, whereas in siderotic muscle and skin the effect was very much more distinct. In citrate buffer all siderotic tissues had higher phosphatase activities, but again the results with muscle and skin were very similar and the ratios were considerably higher than in liver. The absolute enzyme activity of liver was, of course, of an altogether higher order than that of the other tissues.

It was clearly important to decide whether, and to what extent, citrate owed its stimulatory effect to the removal of an inhibitor. The hydrolysis of phenyl phosphate was followed at various substrate concentrations and the results were plotted according to Lineweaver & Burk (1934). The outcome of one such experiment is shown in Fig. 3. In untreated mouse liver the addition of citrate brought about a slight activation but the V_{max} value was unchanged. Iron treatment led to strikingly different lines for acetate and acetate-citrate buffers, with a greatly increased value for V_{max} in the presence of citrate (333 as against 133 μ g. of P/100 mg. of fresh tissue/min.). The differences were less distinct in rat livers (143 as against 105 μ g. of P/100 mg. of fresh tissue/min.). Thus though the change in K_m with untreated mouse liver indicates that citrate may exercise its effect by protecting the enzyme or removing an inhibitor, neither mechanism can adequately account for the results with siderotic liver. Indeed, with this type of liver homogenate, the use of citrate-acetate buffer gave rise to low K_m values, about 2-3 mm, similar to those found by Weber, Weber & Niehus (1961) on adding EDTA to acid phosphatases from wheat germ and *Xenopus* liver. It is possible that citrate activated phenyl phosphatase at low substrate concentrations, perhaps by stabilizing the enzyme or by increasing the available active sites; as the substrate concentration was raised, the

effect became less striking and the maximum velocities in acetate and citrate-acetate buffers approached one another.

Under the circumstances of citrate stimulation in siderotic liver, phosphate acted as an inhibitor. Macdonald (1961) demonstrated competitive inhibition by phosphate in normal mouse liver with malonate as buffer. There was a mixed type of inhibition by phosphate in siderotic mouse liver with citrate-acetate as buffer (Fig. 4). Thus siderotic liver represents a considerably more complex system with regard to acid-phosphatase activity than the homogenate from untreated mice.

Action of iron

The evident connexion between the magnitude of the 'citrate effect' and the presence of iron led us to check the action of this metal in ionic or in complex form *in vitro* with homogenates or mitochondrial preparations from livers of untreated mice. In acetate buffer, Fe^{3+} ions strongly inhibited phenyl phosphatase and phosphoprotein phosphatase, but apparently did not affect glycerophosphatase (Table 3). In a further experiment, in which ferric chloride was added to give a final con-

centration of 0.14 mM, no effect was seen in any of the three enzyme activities measured. Iron-dextran added *in vitro* over the range 8.3–16.7 $\mu\text{g.}$ of Fe/ml. did not affect phosphomonoesterase activity.

In the phenyl-phosphatase assay system containing siderotic-liver homogenate and acetate buffer, the content of free ionic iron was 14 $\mu\text{g.}$; when citrate was added to give a concentration of 0.1M, ionic iron decreased to 7 $\mu\text{g.}$ At pH 4.9 EDTA (sodium salt) and its hydroxyethyl derivative chelated all the ionic iron present in both citrate and acetate test systems. Neither of these chelating agents stimulated phenyl-phosphatase activity [cf. Weber *et al.* (1961), cited above].

The possibility had also to be considered that the citrate stimulation of phenyl-phosphatase activity might be due to some factor in the siderotic liver unconnected with iron as such. All measurable phenyl phosphatase in an homogenate of siderotic liver was destroyed by heat and different proportions were added to liver homogenate from untreated mice. The first experiment pointed to a slight but distinct activation but this result could not be reproduced.

Effects of inhibitors of phenyl phosphatase

Several known inhibitors of acid phosphatase were compared in homogenates of untreated liver and liver, muscle and skin from siderotic mice;

Fig. 3. Double-reciprocal plots (Lineweaver & Burk, 1934) of the hydrolysis of phenyl phosphate by liver homogenates from untreated mice, carried out in 0.1M-acetate buffer, pH 5.8 (Δ), and 0.1M-citrate in 0.1M-acetate buffer, pH 5.8 ($\bullet$). Similar plots are presented for liver homogenates from iron-treated mice, activity being measured in acetate ($\triangle$) and citrate-acetate ($\circ$) buffers as before. The reaction velocity is expressed in $\mu\text{g.}$ of P/100 mg. of fresh tissue/min. The conditions of enzyme assay are described in the Methods and Materials section.

Fig. 4. Double-reciprocal plots (Lineweaver & Burk, 1934) of the hydrolysis of phenyl phosphate by liver homogenates from iron-treated mice, carried out in 0.1M-acetate buffer ($\triangle$), 0.1M-citrate in 0.1M-acetate buffer ($\circ$) and citrate-acetate buffer containing 0.01M-sodium phosphate ($\square$). The reaction velocity is expressed in $\mu\text{g.}$ of P/100 mg. of fresh tissue/min. The pH of all buffers was 5.8 and the conditions of enzyme assay are described in the Methods and Materials section.

both acetate and citrate buffers, pH 4.9 and 5.8, were used (Table 4). With fluoride, DL-tartrate, L(+)-tartrate or Cu^{2+} ions, no important difference emerged between the effects on phenyl phosphatase in the four tissues studied. Citrate was capable of partially reversing the inhibition due to 0.7 mM-DL-tartrate at pH 4.9, 0.02 M-L(+)-tartrate at pH 5.8, and copper at pH 5.8.

Mitochondrial fraction of liver

Stored iron is present in the livers of iron-treated animals in various states of aggregation, ranging from individual ferritin molecules dispersed in the cytoplasm to dense haemosiderin masses of complex structure (Richter, 1959; Muir, 1960). The characteristics of homogenates of siderotic liver

Table 3. *Action of ferric ions on mouse-liver phosphatases*

The substrate concentrations were: 10 mM-phenyl phosphate, 16 mM- β -glycerophosphate, 5% (w/v) casein. The buffer was 0.1 M-acetate, pH 5.8. Ferric chloride was added to give a concentration of 1 mM. Inhibition is expressed as a percentage of the enzyme activity, measured before the addition of iron.

Mouse-liver homogenates	Mitochondrial preparations	Enzyme inhibition (%)		
		Phenyl phosphatase	Glycero-phosphatase	Phosphoprotein phosphatase
Untreated	—	61	3*	67
Iron-treated	—	79	9*	59
—	Untreated	62	19*	74
—	Iron-treated	57	6	83

* Activation.

Table 4. *Inhibitor effects on phenyl-phosphatase activity*

Phenyl phosphate and buffer concentrations were 5 mM and 0.1 M respectively. Inhibition is expressed as a percentage of the enzyme activity measured in the absence of added inhibitor. Each result is the mean for two homogenates, except for DL-tartrate, where a single homogenate of each tissue was used.

Inhibitor	Concn. of inhibitor (mM)	Buffer pH	Inhibition of phenyl phosphatase (%)							
			Untreated liver		Iron-treated					
			Acetate	Citrate	Liver		Muscle		Skin	
Fluoride	10	4.9	37	57	73	74	62	46	75	50
	10	5.8	40	37	43	48	41	28	53	34
DL-Tartrate	0.7	4.9	46	4	48	10	—	—	—	—
	20	4.9	51	54	43	42	—	—	—	—
L(+)-Tartrate	20	4.9	42	37	33	29	—	—	—	—
	20	5.8	45	7	15	6	27	6	17	7
Cu^{2+} ions	0.2	5.8	94	30	97	31	83	36	77	30

Table 5. *Effects of various acids on phenyl-phosphatase activity*

The acids were used in a concentration of 0.1 M in 0.1 M-acetate buffer, pH 5.8. The concentration of substrate was 0.1 M. Enzyme activity is expressed in relation to the activity of untreated mouse-liver homogenate, taken as unity (actual value 7.00 μg . of P/mg. of protein/min.).

Acid added	Enzyme activity			
	Untreated		Iron-treated	
	Liver homogenate	Mitochondrial fraction	Liver homogenate	Mitochondrial fraction
None	1.00	0.78	1.85	2.34
Citrate	0.95	1.92	3.91	8.03
Isocitrate	1.11	1.93	3.60	7.01
Oxaloacetate	1.10	3.10	4.17	8.52
α -Oxoglutarate	1.06	2.19	3.29	6.19
Malonate	1.05	1.29	3.54	5.83
Aspartate	0.70	1.35	3.61	6.01

were adversely affected by the abrasive action of haemosiderin aggregates, which tended to disrupt nuclei and cytoplasmic organelles. On centrifuging the siderotic homogenate, haemosiderin separated to some extent with the nuclear fraction whereas dissolved ferritin remained in the supernatant. The principal iron-containing particles, corresponding to the 'siderosomes' described by Richter (1957), have the approximate dimensions of mitochondria or are somewhat smaller. In view of their increased density, they might be expected to sediment in the mitochondrial fraction of liver homogenate. Lysosomes containing a great deal of iron would separate in the mitochondrial fraction whereas those containing little iron would under our conditions pass partly into the small-particle fraction.

These considerations are borne out by the results of morphological and analytical studies carried out on the mitochondrial fractions. Electron-microscopy of these fractions by Dr A. R. Muir revealed a heterogeneous collection of iron-containing particles, including lysosomes and free ferritin, among the mitochondria from iron-treated liver. Some analyses were carried out on fractions of the liver homogenates from untreated and iron-treated mice. Though the distribution of protein nitrogen was similar in the two series of fractions, that of iron was very different, for reasons which were explained above. The relative specific acid-phosphatase activity was highest in mitochondrial and small-particle fractions of untreated liver but with siderotic-liver homogenate the nuclear fraction as well as the other two fractions already mentioned showed high activity.

The phenyl-phosphatase activities of liver homogenate and its mitochondrial fraction were compared, with citric acid, malonic acid and four related acids (Table 5). Whereas only slight activation, if any, was brought about in liver homogenate from the untreated mouse, in the mitochondrial fraction there was powerful activation by all the acids tested. The stimulatory effect was even greater with the siderotic liver. The results with malonate and aspartate were similar; both were less effective as activators than citrate.

Heat-inactivation of acid phosphatase was studied with mitochondrial fractions from livers of untreated and iron-treated mice. Heating at 37° for 30 min. partially inactivated phenyl phosphatase in both types of mitochondrial fraction and the presence of citrate did not prevent the loss of activity (Table 6). Phosphoprotein phosphatase was also partially inactivated by heat and citrate exercised a weak inhibitory effect. On the other hand, β -glycerophosphatase was stable to heat and strongly inhibited by citrate. The enzymes were affected in the same way in both types of mitochondrial preparation.

Table 6. *Relative enzyme activities of liver mitochondrial fractions before and after heating, and the effect of some added compounds on these activities*

Compound added	Phenyl-phosphatase activity						Phosphoprotein-phosphatase activity						β -Glycerophosphatase activity					
	Untreated			Iron-treated			Untreated			Iron-treated			Untreated			Iron-treated		
	Before heating	After heating		Before heating	After heating		Before heating	After heating		Before heating	After heating		Before heating	After heating		Before heating	After heating	
	1.00	0.54	1.13	0.73	0.85	2.10	1.00	0.41	4.55	1.12	0.97	1.06	1.00	0.51	0.93	1.80	0.81	1.83
None	1.49	0.57	5.85	2.10	8.03	4.15	0.83	0.32	3.08	0.97	6.62	0.52	0.51	0.93	2.02	—	—	—
Citrate	1.69	1.15	8.03	4.15	11.38	10.48	2.20	1.26	9.99	6.62	13.16	0.94	0.94	—	—	—	—	—
Cysteine	—	—	—	—	—	—	3.06	2.70	15.33	13.16	—	—	—	—	—	—	—	—
Thioglycollate	2.20	1.76	10.28	8.42	—	—	1.36	1.19	6.79	5.82	—	0.82	0.75	0.82	1.43	—	—	—
Ascorbate																		

Mitochondrial fractions of mouse liver were suspended in 0.1 M-acetate buffer, pH 5.8, and kept at 37° for 30 min. The following were the concentrations of the compounds added before heating: citrate, 0.1 M; cysteine and thioglycollate, 10 mM; ascorbate, 5 mM. The substrate concentrations were: phenyl phosphate, 0.1 M; β -glycerophosphate, 16 mM; casein, 4.1 % (w/v). Each result is the mean of three measurements; it is expressed as a ratio, taking as unity each enzyme activity of the mitochondrial fraction from untreated liver, before heating. The actual values for the three enzymes, in μ g. of P/mg. of protein/min., were: phenyl phosphatase, 8.6; phosphoprotein phosphatase, 0.67; β -glycerophosphatase, 0.98.

Table 7. *Relative adenosine-triphosphatase activities of liver mitochondrial fractions, and the effect of some added compounds on these activities*

The substrate concentration was 5 mM. All other experimental conditions were as stated in Table 6, save that the mitochondrial fractions were not heated before assay of enzyme activity, which is expressed in relation to the activity of untreated mouse-liver mitochondrial fraction, taken as unity (actual value 4.6 μ g. of P/mg. of protein/min.).

Compound added	Relative enzyme activity	
	Untreated	Iron-treated
None	1.00	2.51
Citrate	1.57	7.61
Cysteine	1.28	5.07
Thioglycollate	1.22	2.88
Ascorbate	1.77	5.05

Table 6 also shows the effects of the addition of cysteine, thioglycollate or ascorbate. In both siderotic and non-siderotic preparations phenyl phosphatase and phosphoprotein phosphatase were strongly activated and the loss of activity on heating was greatly reduced. β -Glycerophosphatase was not activated by cysteine whereas ascorbate exercised a weak inhibition in both types of mitochondrial fraction.

With 5 mM-ATP as substrate, citrate, malonate, cysteine and ascorbate were all stimulatory, particularly in siderotic preparations (Table 7).

DISCUSSION

Characteristics of phenyl phosphatase in liver homogenates

Macdonald (1961) analysed in detail the hydrolysis of phenyl phosphate by mouse liver. Tsuboi (1952) worked with the same tissue, but Macdonald found a higher optimum pH, 5.9–6.1, and higher enzyme values which he attributed to complete liberation of active enzyme as well as avoidance of inactivation during the preparation of homogenates and enzyme assay. We obtained equally high values at an optimum pH of 5.8. Though age (Golberg *et al.* 1960) and strain differences may have been partly responsible for the lower results reported by Tsuboi, a contributory factor was the suboptimum substrate concentration (especially since the concentration in Fig. 2 of his paper seems to represent μ moles/3 ml. volume of assay mixture). Like Macdonald, we found zero-order kinetics with 0.1 M-substrate; and the other provisions laid down by Macdonald were observed under our experimental conditions.

Citrate activation of prostatic acid phosphatase was studied by Anagnostopoulos (1953), using a

purified enzyme. The effect of added citrate was less distinct in cruder preparations (Abul-Fadl & King, 1949), possibly because small amounts of citrate were already present as part of the prostatic secretions. Goodlad & Mills (1957) showed that citrate caused a marked inhibition of rat-liver acid phosphatase below pH 4.5 but had no effect under less acid conditions. Lundquist (1947) reported citrate stimulation of β -glycerophosphate hydrolysis. In liver homogenates the striking enhancement of phenyl-phosphatase activity by malonate and citrate was attributed by Macdonald (1961) to the prevention of enzyme inactivation; also, citrate was somewhat inhibitory whereas malonate was non-inhibitory. Our studies have demonstrated a striking activation by citrate, especially at the optimum pH 5.8, of phenyl phosphatase in homogenates of liver, skeletal muscle and skin from siderotic animals. By using liver mitochondrial preparations, the stimulatory effect was observed with several di- and tri-carboxylic acids in untreated as well as iron-treated liver. This result is hard to reconcile with the suggestion by Macdonald (1961) that citrate and malonate owe their effect to the conversion of inhibitory Fe^{3+} ions into non-inhibitory metal complexes.

The fact that phosphate acts as a competitive inhibitor of (untreated) mouse-liver phenyl phosphatase was established by Schönheyder (1952) and confirmed by Macdonald (1961). It is possible that inorganic phosphate, present initially, inhibits the enzyme, and that this effect is overcome when phosphate is displaced by citrate or a similar acid. Inhibition of bitter-almond β -glycerophosphatase by molybdate was eliminated by citrate or phosphate, both of which form complexes with molybdate (Courtois & Bossard, 1944). Of the acids tested by us on phenyl phosphatase in liver mitochondrial fractions, malonate and aspartate had the least activating effect (Table 5). Perhaps tricarboxylic or oxodicarboxylic acids displace phosphate more readily than dicarboxylic acids are able to do. In siderotic liver, and particularly the mitochondrial fraction, the effects of acids listed in Table 5 were even more distinct. Yet analysis of the actions of citrate and phosphate (Fig. 4) revealed relationships too complex to be explained on the basis of a simple displacement of phosphate by citrate.

Ku (1958) found that heparin inhibited liver and other phenyl phosphatases, an effect attributed to a possible direct combination through a salt linkage of heparin sulphonic acid groups with amino groups of the enzyme. Coddington & Perkins (1960) showed that citrate makes possible a union of Fe^{3+} ions with serum albumin, in line with previous observations quoted by these authors which demonstrated that the formation of an iron-citrate complex possessing a net negative charge

permits binding of iron to positively-charged sites on proteins. The close proximity of stored iron and acid phosphatase in siderotic-liver lysosomes and phagosomes (de Duve & Beaufay, 1957; Beaufay *et al.* 1959; Novikoff, 1960; Essner & Novikoff, 1960, 1961) raised the possibility that under these circumstances citrate forms a complex with Fe^{3+} ions bound to apoferritin; and that this iron-citrate complex then becomes attached to the enzyme, perhaps bringing about activation by displacing phosphate or one of a number of other possible inhibitors (see below). Reducing agents such as cysteine or ascorbic acid are known to mobilize Fe^{2+} ions from ferritin (Mazur, Baez & Shorr, 1955), provided that an avid iron-binding agent is present to combine with them. The effect of reducing agents on phenyl phosphatase (Table 6) was distinctly one of activation in mitochondrial fractions from untreated liver, with partial protection against loss of activity on heating. In the siderotic mitochondrial fractions the degree of activation was far more striking even than that brought about by citrate, and this finding raises the question whether a common mechanism related to iron is involved.

Our finding of inhibition by Cu^{2+} and Fe^{3+} ions, fluoride and $\text{L}(+)\text{-tartrate}$ agrees with similar observations made by Macdonald (1961) and by previous workers. In their response to these inhibitors, the phenyl phosphatases of untreated and iron-treated liver were indistinguishable. The use of citrate often prevented, partially or almost completely, the inhibition caused by these agents. Lundquist (1947) observed a similar effect with prostatic phenyl phosphatase. It is not inconceivable that a naturally occurring inhibitor of the liver enzyme may be affected in the same manner.

β -Glycerophosphatase and phosphoprotein phosphatase

Without attempting to decide whether mouse-liver β -glycerophosphatase is a distinct and separate enzyme from phenyl phosphatase, it is clear from our results that the two possess very different properties. Thus there is the strongly inhibitory effect of citrate on phosphate release from β -glycerophosphate by homogenates or mitochondrial fractions from untreated or iron-treated mouse liver; the absence of an inhibitory effect by Fe^{3+} ions; the complete heat-stability in mitochondrial fractions; and the failure of cysteine or ascorbate to bring about activation.

Phosphoprotein phosphatase, on the other hand, follows phenyl phosphatase in all these respects; in fact, the effect of siderosis is even more distinct than with phenyl phosphatase. The work of other authors who have investigated these enzymes in mouse and rat liver is in agreement with our

findings as regards the optimum pH and powerful inhibition by phosphate (Norberg, 1950), inactivation on heating, and activation by ascorbic acid and thiol compounds (Paigen, 1958; Feinstein & Volk, 1949). Published observations on purified phosphoprotein phosphatase from ox spleen show it to be closely related to the enzyme in liver. Hofman (1958) separated two enzymes from ox spleen, one acting on β -glycerophosphate and the other attacking casein, phenyl phosphate and ATP but not β -glycerophosphate. Revel & Racker (1960), working with a highly purified preparation of ox-spleen phosphoprotein phosphatase, found that enzyme activity was greatly increased by reducing agents, especially ascorbic acid and Fe^{2+} ions; no other metal tested had this stimulating effect [Sabel, Glomset & Porath (1961) found that Ni^{2+} and Co^{2+} ions had some effect]. Short exposure to reducing agents in the absence of substrate produced inactivation which was prevented by substrate or Fe^{2+} ions or both. Revel & Racker (1960) suggested that the enzyme as isolated from spleen is a ferric iron-enzyme complex activated by reduction to a ferrous iron-enzyme complex which is stable only in the presence of substrate or of high concentrations of Fe^{2+} ions. Paigen (1958) put forward the suggestion that activation of mouse-liver phosphoprotein phosphatase by Fe^{2+} ions may be effective not so much by a mechanism involving substrate binding as by the fact that the iron serves to hold the enzyme in an active configuration. The proposed configurational change involving rotation of a peptide chain carrying a terminal sulphhydryl group cannot be accepted since Sabel *et al.* (1961) failed to find any free sulphhydryl groups in the unactivated or activated ox-spleen enzyme.

However, it is clearly possible that, under the conditions of our experiments, the activating Fe^{2+} ions may be derived from ferritin, and that citrate or similar acids help to bring about the transfer and binding of the iron.

SUMMARY

1. The characteristics of acid phosphatase in the livers of untreated and iron-treated mice have been compared. Some experiments were also carried out on muscle and skin.

2. Of various substrates used to show increased acid-phosphatase activity in siderotic liver, attention has been centred principally on phenyl phosphate, β -glycerophosphate, casein and ATP.

3. In siderotic liver, phenyl phosphatase was strongly activated by citrate or malonate; by comparison, the effect in untreated liver was slight, unless mitochondrial fractions were used, when distinct activation was seen with a number of acids akin to citric acid and an even greater stimulation

of enzyme activity in siderotic mitochondrial fractions. β -Glycerophosphatase was strongly inhibited by citrate under all circumstances.

4. Kinetic analysis of the effect of added citrate on phenyl phosphatase showed that the value for $V_{\max}$ was unchanged in untreated liver but greatly increased in siderotic liver. Whereas phosphate acts as a competitive inhibitor in untreated mouse liver, there was mixed inhibition in siderotic liver.

5. Added Fe^{3+} ions caused enzyme inhibition with phenyl phosphate and casein as substrates but not with β -glycerophosphate. Other inhibitors such as Cu^{2+} ions, fluoride and $\text{r}(+)$ -tartrate had their effects partially reversed by citrate.

6. Phenyl phosphatase and phosphoprotein phosphatase were both inactivated by heat to a similar extent in siderotic and untreated livers; β -glycerophosphatase was unaffected.

7. Cysteine, thioglycollate and ascorbate strongly activated both phenyl phosphatase and phosphoprotein phosphatase. They also greatly reduced the loss of activity on heating.

8. A possible explanation is suggested for the action of citrate on acid phosphatase in siderotic liver.

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The Breakdown of Cellulose and its Derivatives by Enzymes from *Myrothecium verrucaria*

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Halliwel (1961) examined some of the circumstances controlling the slow solubilization of native cellulose and the complete solubilization of swollen cellulose powder and swollen fibres by filtrates from *Myrothecium verrucaria*. The organism itself experiences no difficulty in rapidly decomposing undegraded cellulose of the type associated with cotton fibres. However, the purified 'cellulases' described so far show marked activity on carboxymethylcellulose, but are less effective than culture filtrates against undegraded cellulose (cf. Youatt & Jermyn, 1959).

Cell-free filtrates were used in the present work, which extends my previous findings (Halliwel, 1961) and presents further properties of the cellulolytic enzymes together with an analysis of the requirements for the quantitative determination of carboxymethylcellulase.

MATERIALS AND METHODS

Source of enzymes

Preparations of cell-free culture filtrates from *M. verrucaria* and from rumen bacteria were used as sources of cellulolytic enzymes and were as described by Halliwel (1961, 1957b). In the present work, rumen fluid was strained through surgical gauze and centrifuged at 300g for 1 min. to remove most of the protozoa. The supernatant liquor was subsequently centrifuged at 20 000g for 30 min. at 1° (Spinco model L centrifuge, rotor 30). The precipitate was resuspended in the minimal volume of supernatant, decanted to a conical flask at 1°, and 0.4 vol. of butan-1-ol (AnalaR) was added slowly with stirring. After the addition of butanol had been completed, stirring was continued for a further 15 min. The mixture was centrifuged, at 20 000g as above, and the aqueous phase was carefully separated from over- and under-lying layers and recentrifuged to give a clear orange phase. An occasional opacity

in some preparations was removed by the dropwise addition of water and mixing. Crystalline pepsin (porcine mucosa) and crystalline trypsin (bovine pancreas) were commercial preparations (Armour Pharmaceutical Co. Ltd.).

Substrates

Dewaxed cotton fibres, cellulose powder (Whatman), swollen cellulose powder and hydrocellulose were as described by Halliwel (1961, 1957a).

Carboxymethylcellulose (Cellofas B, ref. no. MH.Y.2, degree of substitution about 0.5) solutions (1%, w/v) were prepared by mixing 0.3 g. in 30 ml. of boiling water and heating at 100° for 30 min. with occasional stirring to dissolve particulate matter. After cooling and making up to 30 ml. with water the 'solution' was centrifuged at 1800g for 15 min. to remove fibrillar matter. The viscous but clear supernatant phase was removed with a pipette and served as the stock solution for carboxymethylcellulase assays. It could be stored at room temperature (20°) or 3° for about 3 days (see the Results section).

Analytical procedure

Determination of the activities of cellulolytic enzymes. The methods of assay with the soluble substrate, carboxymethylcellulose, and with insoluble forms of cellulose were as described by Halliwel (1961). In the carboxymethylcellulase assay a point of particular importance was the need for care in pipetting small volumes of reagents into a compact reaction volume; in this respect conical Quickfit tubes of the type described by Levvy & McAllan (1959) were useful. With cellulose, hydrolysis was measured by two procedures, namely the indirect procedure, based on the amount of soluble carbohydrates transferred to the aqueous phase, and the direct procedure (Halliwel, 1958). The latter was only used with fibres. It was modified to include a preliminary alkaline digestion of the fibres and the volumes of reagents were increased 4.5-fold to deal with the greater weight of fibres present (5 mg.). In addition the heating period at 100° was extended to 1½ hr. After