lighter fraction, mainly free from pigmented cells, is washed with 50% (v/v) aqueous acetone at 0° until free from phenols, and then with 80%, and finally anhydrous, acetone to dry it. The acetone-dried white powder (25 g.) is extracted with 0·2M-Na barbital buffer (500 ml.), pH 8·0, in a blender, after first passing it as a paste through a triple-roll mill. After centrifuging, the extract is adjusted to pH 4·0 with 0·2N-HCl, filtered, and 2 vol. of ethanol added to the acid filtrate at 0°. The resultant precipitate is centrifuged off and dissolved in 50 ml. of McIlvaine buffer, pH 4. It is stable at 0° for at least 4 weeks. The activity of such a preparation towards the anthocyanins is shown in Fig. 1. It is necessary to use anaerobic conditions to preclude polyphenol oxidase activity. The hydrolysis is most readily followed by colorimetry. A Spekker photoelectric absorptiometer was used with a 0·5 cm. cell and a yellow-green filter (no. 7 of Kodak set H 769; peak transmission 540 mμ.). Under the conditions used, the resultant cyanidin is mainly, but not entirely, in the colourless pseudo-base form. This accounts for the residual adsorption (Fig. 1), although the sugars are liberated stoicheiometrically as confirmed by direct quantitative chromatography.

It would appear that β-D-galactopyranosyl activity is the major glycosidase function present and that the isolated pigments are indeed 3-β-D-galactosidyl cyanidin chloride and the corresponding 3-α-L-arabinosidyl cyanidin chloride of closely similar structure.

Huang (1956) has used a similar technique with a fungal β-glucosidase to confirm that the anthocyanin pigment of the blackberry is a β-glucoside.

Confirmation by infrared spectra. The infrared spectra of all the 3-monoglycosides were determined with the KBr pellet technique (Li & Wagenknecht, 1956). The results of this analysis confirmed that the slower-moving cacao anthocyanin was indeed the 3-β-D-galactosidyl cyanidin chloride and the faster one the 3-α-L-arabinosidyl cyanidin chloride.

SUMMARY

The anthocyanin pigments of the cacao bean are 3-β-D-galactosidyl and 3-α-L-arabinosidyl cyanidin salts.

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REFERENCES

Bate-Smith, E. C. (1949). *Symp. biochem. Soc.* **3**, 62.
Bate-Smith, E. C. (1954). *Biochem. J.* **58**, 122.
Bate-Smith, E. C. & Westall, R. G. (1950). *Biochim. biophys. Acta*, **4**, 427.
Brown, H. B. (1954). *Nature, Lond.*, **173**, 492.
Flood, A. E., Hirst, E. L. & Jones, J. K. N. (1947). *Nature, Lond.*, **160**, 86.
Forsyth, W. G. C. (1952). *Biochem. J.* **51**, 511.
Forsyth, W. G. C. (1955). *Biochem. J.* **60**, 108.
Huang, H. T. (1956). *Nature, Lond.*, **177**, 39.
Li, K. C. & Wagenknecht, A. C. (1956). *J. Amer. chem. Soc.* **78**, 979.
MacDowell, R. H., Robinson, R. & Todd, A. R. (1934). *J. chem. Soc.* p. 806.
Robertson, A. & Robinson, R. (1929). *Biochem. J.* **23**, 35.
Robinson, G. M. & Robinson, R. (1931). *Biochem. J.* **25**, 1687.
Robinson, R. & Smith, H. (1955). *Nature, Lond.*, **175**, 634.
Scott-Moncrieff, R. (1930). *Biochem. J.* **24**, 763.
Shu, P. (1950). *Canad. J. Res.* B, **28**, 527.

Manganese Oxidation in the Pea Plant (*Pisum sativum* L.) Grown Under Conditions of Manganese Toxicity

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Manganese is known to be an essential micronutrient of plants. It has frequently been suggested that its physiological effects may, at least in part, be due to its capacity for valency change. Kenten & Mann (1949, 1950) showed that Mn^{2+} ions at physiological concentrations are oxidized by peroxidase systems and suggested that *in vivo* manganese is involved in a cycle of oxidation and reduction which may be partly responsible for the stimulating effects of manganese on plant respiration shown by Lundegårdh (1939). But there is as yet no direct evidence that manganese is oxidized in plants grown under normal conditions of manganese supply. If the oxidation of manganese does in fact occur *in vivo* under such conditions, then, as suggested by Kenten & Mann (1952*a*), it might be expected that the oxidation product would be reduced by plant metabolites as rapidly as it was

formed. Under conditions of manganese toxicity, however, the rate of oxidation of Mn^{2+} ions might be more rapid than the rate of reduction of the oxidation product, thus leading to accumulation of the latter. Kelley (1914) obtained evidence of the presence of 'manganese dioxide' in the tissues of plants showing manganese toxicity. The present work was undertaken to investigate whether it was possible to demonstrate the presence of compounds containing manganese of valency greater than two ('higher-valency forms of manganese') in plants grown in water culture at high levels of Mn^{2+} ions.

MATERIALS AND METHODS

Plant material. The seeds were germinated in acid-washed sand and the seedlings obtained were then transferred to the culture medium in the greenhouse. The medium was contained in 300 ml. bottles and four seedlings were grown in each bottle. The medium was changed weekly.

Composition of culture medium. The nutrient solution, in glass-distilled water, contained as macronutrients mM-KH_2PO_4, 4 mM-$Ca(NO_3)_2,4H_2O$, 2 mM-$MgSO_4,7H_2O$ and 6 mM-KNO_3. In addition, H_3BO_3, $ZnSO_4,7H_2O$, $CuSO_4,5H_2O$ and ammonium molybdate were added to give these concentrations: boron, 1 p.p.m.; zinc, 0·1 p.p.m.; copper, 0·04 p.p.m.; molybdenum, 0·02 p.p.m. Iron was added as a solution of 0·5% (w/v) $FeSO_4,7H_2O$ in 0·4% (w/v) tartaric acid. A volume (0·6 ml.) of this solution was added per litre of culture solution once weekly. Sufficient Mn^{2+} ions as $MnSO_4,4H_2O$ were added to give concentrations from 1 to 220 p.p.m.

Estimation of manganipyrophosphate. The method used was based on that of Kenten & Mann (1955) which depends on the fact that solutions of manganipyrophosphate have an absorption maximum at 258 mμ.

Estimation of total manganese. After wet digestion of the plant material the manganese was oxidized with KIO_4 to permanganate, which was estimated colorimetrically.

Spectrophotometry. A Unicam SP. 500 quartz spectrophotometer was used with 1 cm. quartz cells.

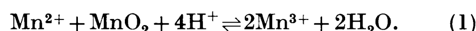
Buffers. Phosphate buffers (0·1 M) were prepared from solutions of KH_2PO_4 and KOH. Pyrophosphate buffers (0·1 M) were prepared from solutions of $Na_2H_2P_2O_7$ and $K_4P_2O_7$.

Hydrated manganese dioxide. This was prepared by the method of Heintze & Mann (1949).

EXPERIMENTAL AND RESULTS

Colorimetric evidence of manganese oxidation

With pea plants at high levels of Mn^{2+} ions in the culture medium (220 p.p.m.) a brown colour developed in the stems, more particularly at the nodes, after 1–2 weeks. This generally increased in intensity with time. Similar though less-distinct symptoms were observed at levels of 110, 55 and 22 p.p.m., but at the latter level the symptom was one of brown speckles on the stem. Sections cut from the brown parts of the stem and immersed in benzidine solution (Kenten & Mann, 1952*b*) generally turned blue, which suggested that 'higher-valency forms of manganese' might be present. When the brown tissue was extracted by grinding in a mortar with 0·1 M pyrophosphate, part of the brown material usually went into solution, and on centrifuging the supernatant solution gave a blue colour with benzidine. Manganic manganese dissolves in pyrophosphate at pH 7 and forms a stable manganipyrophosphate. Although MnO_2 is insoluble in neutral pyrophosphate it dissolves when Mn^{2+} ions are also present, to give manganipyrophosphate.



$$Mn^{2+} + MnO_2 + 4H^+ \rightleftharpoons 2Mn^{3+} + 2H_2O. \qquad (1)$$

After extraction the tissue generally still remained brown, but when tested with benzidine directly it gave negative or only faintly positive tests. The residual pigment was not dissolved either by pyrophosphate alone or by pyrophosphate containing $MnSO_4$.

Spectrophotometric evidence of manganese oxidation

Kenten & Mann (1955) have shown that solutions of manganipyrophosphate have an absorption at 258 mμ. and have based a method of estimation of manganipyrophosphate on the intensity of this absorption. It was thought therefore that by extracting the plant material with 0·1 M pyrophosphate, pH 7, centrifuging, and measuring the absorption at 258 mμ. the difference between the absorption of extracts of the experimental and control plants (Mn^{2+} ions, 1 p.p.m.) might give a measure of manganese oxidized. Fig. 1 shows the absorption curves of pyrophosphate extracts from plants grown for 4 weeks in media containing Mn^{2+} ions in the concentrations 1, 22, 55 and 220 p.p.m. The curves obtained with the extracts of the experimental plants resembled the absorption curve of manganipyrophosphate (Fig. 2). However, on treating the extracts with hydrazine sulphate (0·1 ml. of 0·1 M/3·5 ml. of extract) only a comparatively small decrease in the absorption at 258 mμ. was observed, although Kenten & Mann (1955) have shown that such treatment with hydrazine completely destroys the absorption at 258 mμ. of manganipyrophosphate solutions. Much of the increased absorption observed with the experimental plants was therefore due to the presence of compounds other than manganipyrophosphate. Nucleoproteins and nucleic acids and their degradation products give similar absorption curves. Tests showed that the absorption curves of yeast nucleic acid, prepared by the method of Holden & Pirie (1955*a*), before and after partial digestion with leaf ribonuclease (a preparation corresponding to fraction *J* of Holden & Pirie, 1955*b*), were unaffected by hydrazine.

Further experiments showed that it was possible to remove much of the absorbing material not affected by the addition of hydrazine by preliminary extraction of the plant material with 0·1 M phosphate buffer, pH 7. The absorption at 258 mμ. of the subsequent pyrophosphate extracts from the plants grown at high levels of Mn^{2+} ions was then often considerably diminished by hydrazine. The leaves and side shoots were removed from the stems and the latter were then weighed and sliced into segments 1–2 mm. long. Samples of the slices (e.g. 0·25 g.) were ground in a mortar with 5 ml. of 0·1 M phosphate, pH 7. The mixture was centrifuged and the residue was again washed on the centrifuge with a further 5 ml. of phosphate. The residue was suspended in 5 ml. of 0·1 M pyrophosphate, pH 7, and extracted at 0° in the dark for about 30 min. It was then centrifuged, and the absorption spectrum of the extracts, after suitable dilution with phosphate, was measured. An extract obtained by the same procedure from plants grown with normal manganese supply (1 p.p.m.) was used in the blank cell. Hydrazine sulphate (0·1 ml. of 0·1 M soln./3·5 ml.) was added to both cells and the absorption at 258 mμ. again measured. Fig. 2 shows that the phosphate extracts had an absorption spectrum similar to that of manganipyrophosphate but with an absorption maximum at about 265 mμ. The absorption of these extracts was not altered by hydrazine. The subsequent pyrophosphate extracts had absorption spectra almost identical with that of manganipyrophosphate with an absorption maximum at 258 mμ. The absorption of the pyrophosphate extracts was considerably diminished by adding hydrazine. The decrease in absorption when hydrazine was added to the pyrophosphate extract was taken as a measure of the manganipyrophosphate present. In the experiment of Fig. 2 this gave a value of 225 μg. of Mn^{3+} ions/g. of tissue. The amount of Mn^{3+} ions found in this and other similar experiments was not increased by adding Mn^{2+} ions to the pyrophosphate used for extraction. This suggests that if the 'higher-valency form of manganese' present in the plant is MnO_2, sufficient Mn^{2+} ions are also present to satisfy the requirements of equation (1).

Attempts were made to eliminate the possibility that the oxidation of manganese by enzyme systems took place during the extraction procedure. Heat treatment of the tissue (10 min. at 100°) before extraction was tried with the object of inactivating

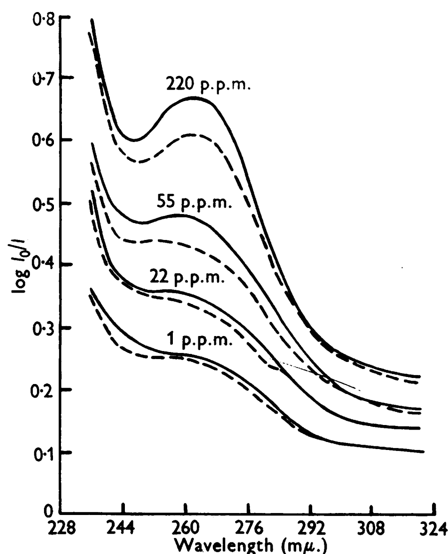


Fig. 1. Absorption curves of the pyrophosphate extracts, before and after hydrazine treatment, of the stems of pea plants grown at varying concentrations of manganese sulphate. Stems were extracted with 10 times their weight of 0·1 M pyrophosphate, pH 7, centrifuged, and the supernatant diluted with 10 times the volume of 0·1 M pyrophosphate, pH 7. Hydrazine sulphate, where added, was 0·1 ml. of 0·1 M soln./3·5 ml. of diluted extract. 0·1 M Pyrophosphate (±hydrazine) was present in the blank cell. —, Without hydrazine; - - -, with hydrazine.

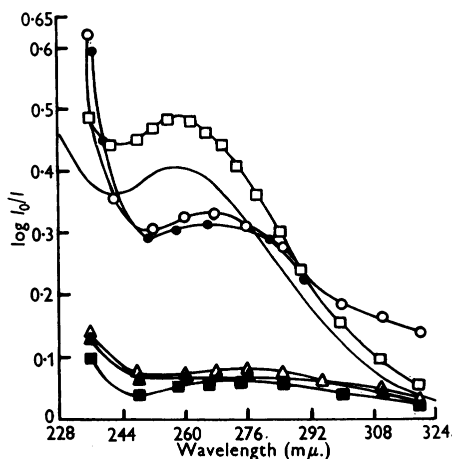


Fig. 2. Absorption curves of consecutive phosphate and pyrophosphate extracts, before and after hydrazine treatment, of the stems of pea plants grown at a concentration of Mn^{2+} ions of 110 p.p.m. for 4 weeks. Similar extracts of control plants (Mn^{2+} ions 1 p.p.m.) with and without hydrazine were used in the blank cell. First phosphate extract diluted with 2 vol. of phosphate: ○, without hydrazine; ●, with hydrazine. Second phosphate extract, undiluted: △, without hydrazine; ▲, with hydrazine. Pyrophosphate extract diluted with 2 vol. of pyrophosphate: □, without hydrazine; ■, with hydrazine. —, Manganipyrophosphate (3·5 μg. of Mn^{3+} ions/ml.) in 0·1 M pyrophosphate, pH 7. Only a few of the experimental points are plotted, for the sake of clarity.

the oxidizing system, and also the effect of adding Mn^{2+} ions to control tissue during the extraction procedure was tried. The results (Table 1) show that when the stems are immersed in boiling water for 10 min. before extraction much less Mn^{3+} ions were found in the pyrophosphate extract. This was probably due to reduction during the heat treatment. When the stems from control plants were ground in a mortar with MnO_2 and phosphate the amount of Mn^{3+} ions obtained by extracting the residue after centrifuging was little less than that obtained when the MnO_2 was similarly treated in absence of tissue, suggesting that only a small fraction of the MnO_2 was reduced to manganous manganese under these conditions. If, however, the macerate of stem, MnO_2 and phosphate was heat-treated before extraction with pyrophosphate the amount of Mn^{3+} ions obtained was considerably less. This suggests that the comparatively low values obtained after heat treatment with plants grown at high levels of Mn^{2+} ions were due to reduction of the 'higher-valency forms of manganese' present. The results (Table 1) provide evidence that the higher oxides found were not formed by oxidation of Mn^{2+} ions during the extraction procedure. Thus when Mn^{2+} ions were added to the stem tissue of control plants, either immediately before the phosphate extraction or to the pyrophosphate used for the final extraction, little or no Mn^{3+} ions were found.

Manometric evidence of manganese oxidation

In plants grown at high levels of Mn^{2+} ions (220 p.p.m.) it was possible to confirm the fact that 'higher-valency forms of manganese' accumulated in the plants by the manometric method of estimation with hydrazine described by Kenten & Mann (1949). In such experiments several stems were taken from the plants grown with toxic concentrations of Mn^{2+} ions and from those grown in the normal media. The two sets of stems were sliced and samples were taken for the manometric and spectrophotometric estimations. In the manometric experiments about 0·5 g. of the slices, suspended in $6 \cdot 7 \times 10^{-2}$ M pyrophosphate, pH 7, were present in each vessel. The vessels were filled with nitrogen. After equilibration 0·2 ml. of a saturated solution of hydrazine sulphate was added from the side arms. In the vessels containing the experimental plant tissue (Mn^{2+} ions, 220 p.p.m.) a gas output was observed. Little or no gas output was observed with the control tissue (Mn^{2+} ions, 1 p.p.m.), either alone or when $MnSO_4$ was added to the vessel contents to give concentrations of Mn^{2+}

Table 1. *Effect of heating the stem tissue in the presence and the absence of added* MnO_2 *on the amount of* Mn^{3+} *ions found in the pyrophosphate extract. Effect of* Mn^{2+} *ions added to the stem tissue immediately before or during the extraction procedure on the amount of* Mn^{3+} *ions found*

Experimental plants were grown at concentrations of Mn^{2+} ions of 1, 55 or 110 p.p.m. In the experiments with added Mn^{2+} ions stem sections of plants grown in culture solution containing 1 p.p.m. were extracted in the usual way, with phosphate followed by pyrophosphate, except that Mn^{2+} ions were added either before the phosphate extraction or during the pyrophosphate extraction. In the heat treatment the stems were cut into sections 2 cm. long and samples (0·3 g.) were immersed in boiling water for 10 min. The usual extraction procedure was then used. In experiments with added MnO_2 (equivalent to 420 μg. of Mn^{3+} ions) the stem samples (0·3 g.) from plants grown at a Mn^{2+} ion concentration of 1 p.p.m. were ground in a mortar with hydrated MnO_2 and 20 times the weight of phosphate, pH 7. The macerates were heated for 10 min. in a boiling-water bath, centrifuged, and the residues washed with phosphate and extracted with pyrophosphate containing 200 μg. of Mn^{2+} ions/ml.

Concentration of Mn^{2+} ions in culture solution (p.p.m.)	Treatment of tissue	Mn^{3+} ions found (μg./g. of tissue)
1	No Mn^{2+} ions added	0
1	140 μg. of Mn^{2+} ions added with phosphate	0
1	550 μg. of Mn^{2+} ions added with phosphate	10
1	140 μg. of Mn^{2+} ions added with pyrophosphate	10
1	550 μg. of Mn^{2+} ions added with pyrophosphate	15
55	Boiled	20
55	Not boiled	85
110	Boiled	20
110	Not boiled	105

Control experiments	Mn^{3+} ions found (μg.)
MnO_2 with tissue (heated)	145
MnO_2 with tissue (not heated)	315
MnO_2 in the absence of tissue (heated)	365
MnO_2 in the absence of tissue (not heated)	370

ions of up to 370 µg./ml. The values for Mn^{3+} ions were always higher by the manometric method than by the spectrophotometric method (Table 2). This was probably due to reduction of the 'higher-valency forms of manganese' during the extraction procedure in the spectrophotometric method of estimation. The small amounts of Mn^{3+} ions found by the manometric method for the plants in Expt. 2 (Mn^{2+} ions, 1 p.p.m.) were almost certainly due to experimental error. No evidence was found either by the spectrophotometric method or by the benzidine test of the presence of 'higher-valency forms of manganese' in such plants.

Table 2. *Spectrophotometric and manometric estimation of 'higher-valency forms of manganese' in the stems of pea plants grown in culture solutions containing* Mn^{2+} *ions* (220 *p.p.m.*)

Expt. no.	Mn^{2+} ions in culture solution (p.p.m.)	Total Mn^{3+} ions found (µg./g. of tissue) Spectrophotometric method	Manometric method
1	220	190	325
	1	0	0
	220	125	225
	1	0	0
2	220	310	315
	1	0	15
	220	315	385
	1	0	25
3	220	250	280
	1	—	0
	220	260	375
	1	—	0

Table 3. *Effect of time of growth and concentration of* Mn^{2+} *ions in the culture solution on the accumulation of 'higher-valency forms of manganese' in the stems of pea plants*

Higher-valency forms of manganese present were estimated by the spectrophotometric method.

Concn. of Mn^{2+} ions in the culture solution (p.p.m.)	Time of growth (weeks)	Mn^{3+} ions found (µg./g. of tissue) Expt. 1	Expt. 2	Expt. 3
11	6	0	—	—
	7	0	—	—
22	2	—	0	—
	3	0	0	—
	4	0	20	—
	5	—	—	—
	6	25	—	—
	7	25	—	—
55	2	0	0	—
	3	0	40	—
	4	0	55	55
	5	30	—	45
	6	20	—	75
	7	55	—	—
110	2	0	10	—
	3	15	55	—
	4	45	250	45
	5	25	—	80
	6	125	—	95
220	2	—	—	—
	3	—	—	—
	4	—	—	275
	5	—	—	160
	6	—	—	—

Effect of time of growth and concentration of Mn^{2+} *ions*

A number of experiments were done in which plants grown at various concentrations of Mn^{2+} ions were tested at weekly intervals for the presence of 'higher-valency forms of manganese'. Some of the results obtained are set out in Table 3. There was a large variation in the content of 'higher-valency forms of manganese' of plants grown under apparently identical conditions. Despite these variations the following conclusions can be drawn. No accumulation of 'higher-valency forms of manganese' was found when the concentration of Mn^{2+} ions in the culture solution was less than 22 p.p.m. At concentrations of 22 p.p.m. upwards the time of appearance of the higher-valency forms and the final concentration reached was, in general, directly related to the concentration of Mn^{2+} ions in the culture solution. Concentrations of Mn^{2+} ions of 55 p.p.m. upwards caused a rapid appearance of chlorosis and stunting of growth. At a concentration of Mn^{2+} ions of 220 p.p.m. growth practically ceased after 2–3 weeks.

Relation between the amounts of total manganese and 'higher-valency forms of manganese' accumulating in the stems

In two experiments both the total manganese and its 'higher-valency forms' present in the stems were estimated. The results are shown in Table 4. In these experiments no 'higher-valency forms of manganese' were found in the stems of the plants grown at a concentration of Mn^{2+} ions of 22 p.p.m., though the total manganese was high in comparison with that of the control stems (Mn^{2+} ions, 1 p.p.m.). With higher levels of Mn^{2+} ions in the culture solution the Mn^{3+} ions found formed a considerable fraction of the total manganese in the stem and in one case more than one-half of the total manganese was accounted for as Mn^{3+} ions.

Injection experiments

Since it is known that certain micro-organisms can bring about the oxidation of Mn^{2+} ions the results so far obtained did not exclude the possibility that the 'manganese higher-valency forms' found were formed by such oxidation in the culture

solutions and then taken up in the oxidized form by the plant. The roots of the plants grown in culture solutions containing higher concentrations of Mn^{2+} ions were dark brown, and sometimes gave a strongly positive benzidine reaction due presumably to 'higher valency-forms of manganese'. As it was not easy to decide how far these were present as a deposit on the root surface rather than inside the roots, the stem tissue was used in the present work. Even when high concentrations of 'higher-valency forms of manganese' were present in the stems the leaves gave negative or only weakly positive benzidine reactions. To exclude the possibility that the 'higher-valency forms of manganese' found in the plants were not derived from the culture solution the oxidation of Mn^{2+} ions was also demonstrated, in experiments in which the Mn^{2+} ions were injected into the plants via the leaf petioles according to the technique of Roach (1945). Although it was not possible with this technique to demonstrate manganese oxidation in the plants at low concentrations of Mn^{2+} ions, 'higher-valency forms of manganese' accumulated in the stems within a few hours when the strength of the Mn^{2+} ions solution injected was 0·05–0·1 M (Table 5). Accumulations of the same order were also found when Mn^{2+} ions were injected into plants kept in the dark.

Table 4. *Relation between the total manganese contents of the stems of pea plants grown for four weeks in water culture at various concentrations of* Mn^{2+} *ions and the amount of 'higher-valency forms of manganese' that accumulated*

Expt. no.	Mn^{2+} ions in culture solution (p.p.m.)	Total Mn (μg./g. of tissue)	Mn^{3+} ions found (μg./g. of tissue)
1	1	5	0
	22	55	0
	55	155	45
	110	540	325
2	1	5	0
	22	40	0
	55	205	50
	110	550	155

Table 5. *Effect of varying the concentration of* Mn^{2+} *ions and the time of injection on the accumulation of 'higher-valency forms of manganese' in the stems of pea plants*

Pea seedlings were grown in soil until they reached a height of about 15 cm., and $MnSO_4$ solutions were then injected through the petioles.

Time of injection (hr.)	Concn. of $MnSO_4$ soln. (M) 0·02	0·05	0·1
	Mn^{3+} found (μg./g. of tissue)		
6	—	20	20
24	—	120	135
48	145	410	630
72	180	300	860
96	210	—	—

Results with other plants

A number of other plants [tomatoes (*Lycopersicum esculentum* Mill.), sprouts (*Brassica oleracea* var. *gemmifera* Zenk.) and barley (*Hordeum vulgare* L.)] were grown in water culture with added Mn^{2+} ions under conditions similar to those used with pea plants. The growth of tomatoes and sprouts was much more strongly inhibited by high concentrations of Mn^{2+} ions than that of pea plants, whereas barley survived at high concentrations of Mn^{2+} ions, as is already known from the work of Brenchley (1927). With none of these plants was definite evidence obtained of accumulation of 'higher-valency forms of manganese' in the tissues, although in some cases under toxic concentrations of Mn^{2+} ions browning of the tissues was observed.

DISCUSSION

The present work shows that 'higher-valency forms of manganese' accumulate in pea plants grown under conditions of manganese toxicity. The valency form of the manganese and the precise nature of these accumulations have not yet been established. The accumulations appear to be associated with a browning of the tissues, which may represent a deposition of a higher oxide of manganese with necrosis of the neighbouring tissues. But occasionally with the pea plant and invariably with the other plants, where the browning of the tissues occurred, it was not accompanied by accumulation of demonstrable amounts of 'higher-valency forms of manganese'. These brown areas may be sites where the rate of oxidation of manganese is high enough to bring about a failure of the normal metabolism and to cause necrosis but not sufficiently high to lead to the accumulation of the oxidation product of manganese. The evidence at present available suggests that such accumulation is due to the oxidation of Mn^{2+} ions by peroxidase systems (Kenten & Mann, 1950, 1952*a*), which at high concentrations of Mn^{2+} ions becomes more rapid than the reduction of the oxidation product by plant metabolites. The possibility that accumulation follows a photo-induced oxidation either by the chloroplasts, as suggested by the results of Kenten & Mann (1955), or by a system of the type described by Andreae (1955), would appear to be excluded by the results of the injection experiments in light and darkness. Negative results obtained with other plants investigated may be due to the rate of reduction of the higher-valency forms being as great as the rate of oxidation of Mn^{2+} ions. But these

negative results do suggest that manganese toxicity is not due in all cases to accumulation of 'higher-valency forms of manganese' in the plant tissues. It is of interest that accumulation of ferric iron in the vascular-plate tissues of the nodes of *Zea mays* L., particularly under conditions of potassium deficiency, was observed by Hoffer & Trost (1923) and Hoffer (1930).

The present results show that a valency change of manganese does occur in plants *in vivo* and support the hypothesis that the function of manganese as an essential element in plant nutrition may depend, at least to some extent, on such valency change. Kenten & Mann (1952*a*) have shown that *in vitro* the oxidation of Mn^{2+} ions can be brought about by enzyme systems producing hydrogen peroxide, such as α-hydroxy-acid oxidase, xanthine oxidase and plant amine oxidase systems, coupled with peroxidase systems. Peroxidase is known to be widely distributed among the higher plants, and Siegel & Galston (1955) and Pilet & Galston (1955) have recently put forward evidence of peroxide genesis in plant tissues. Although the components of the manganese-oxidizing system appear to be widely distributed in the tissues of higher plants there is as yet no evidence that any large part of plant respiration is dependent on this system. Lundegårdh (1954) finds that only a small fraction of the normal aerobic respiration of wheat and rye roots depends on a peroxidase system. It is possible that this fraction of the respiration depends on manganese oxidation. Since peroxidase catalyses the oxidation of Mn^{2+} ions the range of compounds which peroxidase systems can oxidize will be extended by addition of Mn^{2+} ions to include those compounds capable of oxidation by the oxidation product of manganese. Thus Kenten & Mann (1953) have shown that the oxidation of certain dicarboxylic acids is catalysed by peroxidase systems in the presence of Mn^{2+} ions. Although the fraction of the total respiration passing through the peroxidase–manganese system may be small it may be qualitatively important.

SUMMARY

1. Pea plants (*Pisum sativum* L.) grown in water culture containing toxic concentrations of manganese sulphate accumulate compounds containing manganese of valency greater than two in the stems.

2. The presence of these higher-valency forms was demonstrated colorimetrically by the benzidine test, and the amount present estimated spectrophotometrically as manganipyrophosphate and manometrically by the oxidation of hydrazine.

3. It is suggested that the oxidation is due to peroxidase systems. Under toxic conditions of manganese supply the rate of oxidation of Mn^{2+} ions may exceed the rate of reduction of 'higher-valency forms of manganese' by plant metabolites, thus leading to the observed accumulation of manganese higher oxides.

4. In other plants examined under similar conditions the accumulation of 'higher-valency forms of manganese' could not be demonstrated, possibly owing to the fact that in these plants they are reduced as fast as they are formed.

5. It is suggested that the function of manganese as an essential element in plant nutrition depends, to some extent, on such valency change.

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REFERENCES

Andreae, W. A. (1955). *Arch. Biochem. Biophys.* **55**, 584.
Brenchley, W. E. (1927). *Inorganic Plant Poisons and Stimulants*, 2nd ed., p. 89. Cambridge University Press.
Heintze, S. G. & Mann, P. J. G. (1949). *J. agric. Sci.* **39**, 80.
Hoffer, G. N. (1930). *Indiana Purdue Univ. Exp. Sta. Bull.* no. 298 (revised).
Hoffer, G. N. & Trost, J. F. (1923). *J. Amer. Soc. Agron.* **15**, 323.
Holden, M. & Pirie, N. W. (1955*a*). *Biochem. J.* **60**, 46.
Holden, M. & Pirie, N. W. (1955*b*). *Biochem. J.* **60**, 39.
Kelley, W. P. (1914). *Bot. Gaz.* **57**, 213.
Kenten, R. H. & Mann, P. J. G. (1949). *Biochem. J.* **45**, 255.
Kenten, R. H. & Mann, P. J. G. (1950). *Biochem. J.* **46**, 67.
Kenten, R. H. & Mann, P. J. G. (1952*a*). *Biochem. J.* **52**, 125.
Kenten, R. H. & Mann, P. J. G. (1952*b*). *Biochem. J.* **50**, 360.
Kenten, R. H. & Mann, P. J. G. (1953). *Biochem. J.* **53**, 498.
Kenten, R. H. & Mann, P. J. G. (1955). *Biochem. J.* **61**, 279.
Lundegårdh, H. (1939). *Planta*, **29**, 419.
Lundegårdh, H. (1954). *Ark. Kemi*, **7**, 451.
Pilet, P. E. & Galston, A. W. (1955). *Physiol. Plant.* **8**, 888.
Roach, W. A. (1945). *Tech. Commun. Bur. Hort., E. Malling*, no. 10.
Siegel, S. M. & Galston, A. W. (1955). *Arch. Biochem. Biophys.* **54**, 102.