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Determination of Manganese in Tissue extracts by means of Formaldoxime

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Sideris (1937) described a method for the estimation of Mn^{2+} ions in ashed tissue which was based on the spot-test reaction of manganese with formaldoxime ($CH_2=N\cdot OH$). Gum ghatti was used to stabilize the colour. The present modification of this method was developed for the study of the uptake of Mn^{2+} ions by tissues from dilute solutions (10^{-3} to $10^{-5}M$). The estimation is made directly on trichloroacetic acid or perchloric acid extracts of the tissue without prior ashing. The technique is simple, involving the addition of a single solution, and the colour developed is extremely stable without addition of any protective colloid. Ferric iron which also reacts with formaldoxime may be removed by extraction as ferric thiocyanate. Between 0.01 and 0.25 μ mole (0.55–13.7 μ g.) of manganese can be estimated in this way. Yellow tissue constituents absorb at the same wavelength as the formaldoxime-manganese complex and this may reduce the accuracy of the determination at low levels of Mn^{2+} ions. The method is therefore not suitable for the determination of endogenous tissue manganese without prior ashing, but it is very convenient for the determination of manganese at the levels often used in enzyme experiments.

METHOD

Solutions. Manganous chloride (A.R., 0.1M) in glass-distilled water was diluted to give a working standard of 0.0005M.

A solution of formaldoxime hydrochloride (British Drug Houses Ltd.), 0.5 g. in 10 ml. of glass-distilled water, was made; 0.1 ml. of this solution was diluted with 1 ml. of 10N-NaOH immediately before use.

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Procedure

Tissue samples (liver homogenates or mitochondrial suspensions) were deproteinized with trichloroacetic acid (final concn. 0.8N) or $HClO_4$ (final concn. 0.5N). The clear supernatant obtained after centrifuging constitutes the tissue extracts which were analysed. Up to 3.0 ml. of the tissue extract was placed in a test tube. With the smaller samples the volume was adjusted to 3.0 ml. with water. To ensure rapid mixing 1 ml. of formaldoxime was squirted from a 1 ml. all-glass hypodermic syringe into the test solution; mixing was completed at once by shaking the tube. Less accurate results were obtained when the mixing was not rapid. After 10 min. the optical density was measured in 1 cm. cells in the Unicam spectrophotometer SP. 600 at 450 $m\mu$ (see Fig. 1). The solutions were read against a manganese-free solution treated in the same way. The optical density of the blank solution increases slightly for the first 2 hr. and then steadily fades. However, in the test

Fig. 1. Absorption spectra of manganese and nickel complexes with formaldoxime. Solutions (3 ml.) containing 0.25 μ mole of Mn^{2+} or Ni^{2+} were treated with 1 ml. of formaldoxime solution.

solutions the colour of the manganese complex does not fade, i.e. the difference between optical density of the test and that of the blank does not vary with time. The coloured manganese complex is stable for at least 5 days. Known amounts of manganese were carried through the procedure with each set of estimations to construct a standard curve. This was linear over the range 0.025 μ mole of Mn^{2+} and 0.25 μ mole of Mn^{2+} gave an optical density of approximately 0.56 at 450 $m\mu$. Duplicate determinations agreed within 2% and therefore only single measurements were made as a rule.

RESULTS

Interfering substances

Iron. With Fe^{3+} ions formaldoxime forms a purple complex which develops much more slowly than the colour of the manganese and reaches its maximum in about 2–3 hr. The absorption when measured at 450 $m\mu$ is about half that of a manganese solution of the same concentration. Sideris (1937) recommends the addition of cyanide to overcome the interference of the Fe^{3+} ions. This was unsatisfactory under our conditions. Fe^{3+} ions in the presence of excess of ammonium thiocyanate were extractable from water solutions into ethyl acetate, whereas Mn^{2+} ions remain in the aqueous phase. This offered a possible method for the removal of iron from manganese solutions before the addition of the formaldoxime reagent.

The sample of tissue extract containing not more than 2.5 μ moles of Fe^{2+} and 0.5 μ mole of Mn^{2+} was placed in a 100 ml. separating funnel, or more conveniently in the extracting funnels described by Bartley (1953). To the sample was added 0.1 ml. of 10% aqueous ammonium thiocyanate solution, 0.1 ml. of $N-H_2SO_4$, water to 3 ml. and 10 ml. of ethyl acetate. The mixture was shaken for about 30 sec., and after a few seconds the lower aqueous layer was run into a second funnel containing 10 ml. of ethyl acetate and the extraction procedure was repeated. The aqueous layer was run off into a graduated 10 ml. tube. Water (2 ml.) was added to the first funnel and after extraction the aqueous layer was run into the second funnel. After re-extraction the aqueous layer was added to the contents of the graduated tube. The volume was adjusted to 6 ml. with water. To a 3 ml. sample of this solution was added 1 ml. of the formaldoxime reagent and the mixture was left for 24 hr. before reading. This long delay was to allow the purple colour that developed on the addition of the reagent following the extraction procedure to fade. To test the procedure tissue extracts containing known amounts of Mn^{2+} or Mn^{2+} and Fe^{3+} ions were analysed.

In Table 1 (A) are given the values obtained by the direct method when no Fe^{3+} ions were added, and in Table 1 (B) are given comparable values after extraction of the added Fe^{3+} ions.

Nickel. Ni^{2+} ions interfere with the determination as they produce a yellow-brown colour with formaldoxime with about the same molar extinction coefficient as the Mn^{2+} complex at 450 $m\mu$. It is possible that the difference in the two absorption

spectra (Fig. 1) might give a basis for the measurement of manganese in the presence of nickel. This possibility has not been further investigated.

Copper. Vogel (1948) states that Cu^{2+} ions give a blue-violet colour with formaldoxime. Under our test conditions when the amount of Cu^{2+} ions added did not exceed 0.25 μ mole the colour was transient and did not interfere with the determination of Mn^{2+} ions. With larger amounts of copper the blue colour was more permanent but could be removed by the addition of a small amount of solid sodium cyanide. As shown below NaCN does not interfere with the determination of Mn^{2+} ions.

Chromium and cobalt. Vogel (1948) also states that Cr^{3+} and Co^{2+} ions give colours with formaldoxime. The colour with chromium was found to be negligible and that with cobalt was less than 5% of the Mn^{2+} complex at the same concentration. Moreover, the cobalt complex was extracted by the procedure used for the removal of iron.

Calcium. At the alkaline pH required for the formation of the formaldoxime complex calcium salts form a precipitate. Removal of the precipitate by centrifuging results in a clear supernatant with the same optical density as a similar solution without the addition of calcium.

Phosphates. Sideris (1937) has stated that phosphate interfered with his method. At levels above

Table 1. *Manganese determinations by direct and extraction methods*

Manganese was measured either directly or after treatment with KSCN ethyl acetate as described in the text. Test samples (values A) were tissue extracts containing the amounts of Mn^{2+} ions given in column 1. To each of a further similar set of samples was added 2.5 μ moles of Fe^{3+} ions. These were analysed by the extraction procedure and gave the values B.

Added Mn^{2+} ion content (μ mole/3 ml.)	Optical density by standard method (A)	Optical density after extraction procedure (B)	100B/A
0.025	0.065	0.066	101
0.050	0.123	0.127	103
0.10	0.330	0.324	98
0.150	0.400	0.386	96
0.25	0.660	0.640	97

Table 2. *Effect of phosphates on the estimation of manganese by formaldoxime*

The amount of phosphate indicated was added to the 3 ml. of the test sample.

Phosphate added (μ moles) ...	Optical density			
	0	10	20	40
Mn^{2+} ions added (μ moles)				
0.05	0.140	0.146	0.123	0.131
0.10	0.284	0.278	0.264	0.271
0.25	0.682	0.652	0.665	0.692

40 μ moles in the test sample, phosphate also interferes in our method, but below this level no difference was found between the presence or absence of phosphate (Table 2).

Sucrose. As the fractionation of tissue homogenates is often carried out in sucrose solutions the recovery of manganese from sucrose containing suspensions was studied. Amounts of sucrose above 50 μ moles in the test sample decreased the colour formation. However, with any particular amount of sucrose the colour developed by the formaldoxime reagent was linear with respect to manganese concentration. The colour of the manganese-formaldehyde complex did not have the usual stability, some fading occurring after about 2 hr. After 24 hr. the colour had completely faded. Thus when sucrose is present in the test material at amounts greater than about 50 μ moles, the standard manganese solutions should also contain approximately the same amount of sucrose; in all cases when sucrose is present the optical density should be measured within 1 hr. of adding the formaldoxime.

If solutions containing both sucrose and Fe^{3+} ions were subjected to the extraction procedure the fading of the colour caused by sucrose did not occur. Presumably the extraction had removed or reduced this substance as well as Fe^{3+} ions to a level where interference did not occur.

Effect of metal-complex-forming agents. Many substances found in biological material or added in metabolic experiments have the property of 'binding' metal ions. The possible interference in the manganese method of some of the more common metal-complex-forming substances was therefore tested. None of the substances in the amounts given in Table 3 inhibited the reaction between manganese and formaldoxime. With cyanide the colour development was somewhat retarded, but after about 1 hr. the normal optical density was reached. The colour contribution of the cyanide solution was appreciable at the high levels. The reason for this colour is not clear but due allowance was made for it in calculating the percentage recoveries given in Table 3.

Recovery of manganese added to tissues. The recovery of manganese added to dilute mitochondrial suspensions (0.04–0.25 μ mole of Mn^{2+} added to 2.0 ml. of suspension containing 40 mg. dry wt. of mitochondria) after deproteinization and extraction with 10% trichloroacetic acid, ranged between 97 and 105%. In similar tests with unfractionated liver homogenates in 0.9% potassium chloride solution extracted by 10% trichloroacetic acid, the recoveries were usually too high and ranged between 98 and 125% recovery. When 0.5N-perchloric acid was used as the extracting fluid the recoveries (102–112%) were more consistent but were still too high. This excess of colour was due to the colour of

Table 3. *Effect of metal-binding agents on the manganese-formaldehyde reaction*

The test solution contained 0.25 μ mole of Mn^{2+} /3 ml.

Complex-forming agent added	Amount added (μ moles)	Ratio of optical densities with and without complex-forming agent $\times 100$
Cysteine	30	100
ATP	4	97
	3	100
Cyanide	1250	99
	1000	97
	750	100
	500	99
Pyrophosphate	250	99
Ethylenediamine-tetra-acetic acid	700	102

Table 4. *Recovery of Mn^{2+} ions added to a liver homogenate*

Rat liver was homogenized in 9 vol. of 0.9% KCl. Mn^{2+} ions were added to give in 0.5 ml. the amounts stated below. To the suspension was added 0.2 vol. of 2.5N- HClO_4 and, after removal of the precipitated protein by centrifuging, 0.5 ml. of the extract was analysed for Mn^{2+} ions.

Addition to	Mn^{2+} ions added (μ mole)	Mn^{2+} ions recovered (μ mole)	Recovery (%)
HClO_4 extract of rat-liver homogenate	0.1	0.099	99
	0.15	0.151	101
	0.2	0.200	100
	0.25	0.252	101
Rat-liver homogenate kept 30 sec. then HClO_4 added	0.1	0.099	99
	0.15	0.155	103
	0.2	0.197	98
	0.25	0.25	100
Rat-liver homogenate kept 90 min. then HClO_4 added	0.1	0.100	100
	0.15	0.153	102
	0.2	0.197	99
	0.25	0.248	99

the tissue extract being enhanced by Mn^{2+} ions. This enhancement occurred at low concentrations of Mn^{2+} ions (about 0.01 μ mole in the 3 ml. of test sample) and did not increase with increase of the Mn^{2+} ions added. It was therefore of significance only when the Mn^{2+} ion content of the sample was low. Table 4 gives the recoveries of various amounts of Mn^{2+} ions when allowance was made for this correction.

Estimation of endogenous Mn^{2+} ions. As expected from the previous section the method could not be applied directly to the estimation of endogenous manganese in tissue extracts prepared in 0.5N- HClO_4 or 5% trichloroacetic acid. Table 5 shows a comparison between the apparent Mn^{2+} ion content of the tissue extract and the true Mn^{2+} ion content

Table 5. *Manganese content of tissues*

The values given in the second column were calculated from the data of Skinner *et al.* (1931) for rats supplemented with 5 mg. of Mn^{2+} ions daily. The calculated Mn^{2+} ion intake of the rats used in the present work was 6–10 mg. daily. For other experimental details see text.

Tissue	Mn^{2+} ion content (μ mole/g. of fresh tissue)		
	In ashed material		In $HClO_4$ extract.
	Skinner <i>et al.</i> (1931)	This paper	
Rat liver	0.0373	0.047	0.064
Rat brain	0.0137	0.00967	0.017
Rat muscle	0.003	0.00646	0.0213
Sheep pancreas	—	0.0294	0.036
Pig pancreas	—	0.027	0.020

of the tissue extract after ashing by the method of Lorenzen & Smith (1947). A comparison is also given with the Mn^{2+} ion content of rat tissue as found by Skinner, Peterson & Steenbock (1931). These authors showed that the dietary intake of Mn^{2+} ions was reflected in the Mn^{2+} ion content of the liver and muscle. This may account for the somewhat higher values obtained with our rats, as their Mn^{2+} ion intake was about twice that of the Mn^{2+} -supplemented rats used by Skinner *et al.* (1931).

The manganese estimation gave the same value as the dry-ashed tissue if the perchloric or trichloroacetic acid extracts were submitted to a wet-ashing procedure [2.0 ml. of extract + 0.2 ml. of the ashing mixture of Hanes & Isherwood (1949) + 0.1 ml. of 5% ammonium molybdate solution boiled down to 0.2 ml.]. The wet-ashing procedure is somewhat less tedious than the dry-ashing of the whole tissue. However, it is clear that the formaldoxime method is not suitable for the routine analysis of tissues without prior ashing unless the Mn^{2+} ion content is relatively high. It is very well suited to the study *in vitro* of tissues supplemented with extra Mn^{2+} ions.

DISCUSSION

Manganese is usually estimated colorimetrically after oxidation to permanganate. The present method has the advantage over the permanganate method in that no removal of organic material is required, although it may be used on ashed material when this is necessary. No colour developed in the presence of phenols as occurs in the method of Abul-Fadl (1948). The estimation usually requires only the addition of a single solution and the great stability of the colour is also advantageous. A limitation on the method is imposed by the

absorption at 450 $m\mu$ of yellow tissue constituents.

Ni^{2+} ions seriously interfere with the estimation and the method is not applicable in their presence. When the method is used on ashed tissue or tissue extracts the iron-extraction procedure must be used as the bound tissue-iron is liberated. In most trichloroacetic acid or perchloric acid extracts of tissues the free iron content is too small to interfere and the iron-extraction procedure is unnecessary. The presence of iron in the extract may be tested for by adding ammonium thiocyanate; if a definite red does not appear the iron extraction may be omitted. The presence of thiocyanate does not interfere with the subsequent manganese determination.

The method has the merit of being insensitive to the effect of chelating agents such as ethylenediaminetetra-acetic acid and cyanide, both of which are often added to tissue preparations. The reason for the inhibitory action of sucrose is not clear as this material is not usually considered a powerful chelating substance.

SUMMARY

1. The reaction of manganese with formaldoxime, first used by Sideris (1937) as the basis of the quantitative determination of manganese, has been modified to give a simple method for the estimation of manganese in perchloric or trichloroacetic acid extracts of biological material. From 0.01 to 0.25 μ mole of Mn^{2+} in 3 ml. of tissue extract can be measured with less than 5% error. Yellow tissue constituents absorb at the same wavelength as the formaldoxime-manganese complex and reduce the accuracy of the determination when present in large amounts. The method is not suitable for the determination of endogenous tissue manganese without prior ashing, but it works well at levels (10^{-3} to $10^{-5}M$) often used in enzyme experiments.

2. Iron and nickel react with formaldoxime and interfere with the estimation. The removal of iron by extraction is described.

3. The method can be used in the presence of large amounts of metal complex-forming agents such as cyanide or ethylenediaminetetra-acetic acid. Cyanide causes some retardation in the colour development and sucrose causes the colour to fade after 2 hr.

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Application of the Carbazole Reaction to the Estimation of Glucuronic Acid and Glucose in some acidic Polysaccharides and in Urine

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A number of carbohydrates may be detected and estimated by means of a reaction with carbazole in sulphuric acid. The methods used have been summarized by Dische (1955). The present paper describes a procedure for the estimation of mixtures of glucuronic acid, glucose and xylose in pure solution, and of glucuronic acid and glucose in various polysaccharides and in urine. Work is also described indicating that the colours obtained with hexoses and pentoses are due to hydroxymethylfurfural and furfural respectively, and that the colour given by the uronic acids is due to neither of these compounds.

Dische (1947) observed that the addition of water to the coloured complex which is slowly formed after mixing carbazole and glucuronic acid in 87% sulphuric acid caused the colour to change and lighten, whereas the slight colour obtained with glucose and other sugars by the same process was intensified. In the present procedure one reading is taken with an absorptiometer 2.5 hr. after the reactants have been mixed; glucuronic acid gives an intense colour at this stage. Water is added, and two further readings are taken on the following day, one with a yellow-green and one with a violet filter. Glucose gives a more intense colour than glucuronic acid at this stage, with maximum absorption of light in the yellow-green region. Pentoses also give a colour at this stage with maximum absorption in the yellow-green region. The pentose colour is less intense than that given by glucose and other hexoses and has a secondary absorption maximum in the violet region, not shown by the hexose colour; this makes possible the detection and rough estimation of pentoses.

MATERIALS AND METHODS

Carbazole. The product from L. Light and Co. gave no colour with H_2SO_4 at 100° . Solutions (0.2 and 0.3%, w/v) of carbazole were made up in A.R. ethanol (water content

less than 2%). Both were renewed within 3 months of preparation.

Standards. The stock solution of glucuronolactone (L. Light and Co.) was 1 mg./ml. and the dilute solution 0.1 mg./ml. in water. Both were stored at 4° . The dilute standard was renewed within 1 month, and the stock within 3 months of preparation. Glucuronolactone was used in preference to glucuronic acid because of the difficulty of isolating the acid in the free state. Conversion of the lactone into the barium salt or into the free acid did not affect significantly the readings obtained in the standard procedure.

Other carbohydrates. Glucose (May and Baker Ltd.), galacturonic acid, glucosamine hydrochloride, galactono- γ -lactone and gluconic- δ -lactone (L. Light and Co.), ascorbic acid, furfural and xylose (British Drug Houses Ltd.), arabinose, lactose, galactose and fructose (Hopkin and Williams Ltd.) were used in the same concentrations as glucuronolactone. Bornyl and menthyl glucuronosides were obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.), potassium hyaluronate and chondroitin sulphate from L. Light and Co., and oxidized cellulose from Parke, Davis Ltd. 5-Hydroxymethylfurfural was prepared from sucrose by the method of Haworth & Jones (1944). Barium glucuronate and glucuronic acid were prepared from glucuronolactone by the method of Goebel & Babers (1933).

Standard procedure

Dilution. Maximum accuracy is only attained when the concentrations of both glucuronolactone and glucose in the test solution are within the range 10–100 $\mu\text{g./ml.}$ and the combined amount of the two compounds is not more than 170 $\mu\text{g./ml.}$ For estimations on urine (guinea-pig or human) suitable concentrations within this range are usually obtainable by diluting 1 ml. of urine to 20 ml.

Method. For each test, pairs of 1 ml. samples of appropriately diluted test solutions, of dilute carbohydrate standards and of water blanks are set up in 25 ml. Pyrex tubes, which are placed in water at 4° for 5 min. Then 6 ml. of ice-cold H_2SO_4 (A.R., Hopkin and Williams Ltd.) are added rapidly to each tube and mixed. All tubes are heated in boiling water for 20 min. and then transferred to water at room temperature. After 2 min., 0.2 ml. of 0.2% carbazole is mixed with one of the solutions in each pair. All tubes are kept in an oven at 26° for 2.5 hr. Light absorption is then measured in a Hilger Spekker photoelectric colorimeter