factor is not of fundamental importance, since the corrected value of $E_{1\text{ cm}}^{1\%}$ 328 m μ . is just as informative. The use of the 1800 factor would preserve continuity since it forms a link between the co-operative assays and the 1934 International Standard β -carotene.

SUMMARY

1. A re-examination of the two halibut-liver oils used in the co-operative assays of 1936 has been carried out by photo-electric spectrophotometry. The results show that the original spectroscopic data required some correction for

absorption at 328 m μ . by constituents other than vitamin A.

2. When such a correction is applied, the conversion factor relating $E_{1\text{ cm}}^{1\%}$ 328 m μ . to potency in i.u./g. is raised from 1570 to 1830, which agrees well with the values of 1770 obtained for crystalline vitamin A β -naphthoate and 1820 for cod-liver oil, and justifies a round figure of 1800.

3. The results of fractionation experiments on the oils support the method of correction for irrelevant absorption.

4. The significance of the results with respect to the spectrophotometric assay of vitamin A is discussed.

REFERENCES

- Hume, E. M. (1937). *Nature, Lond.*, **139**, 467.
 Hume, E. M. (1939). *Nature, Lond.*, **143**, 22.
 Hume, E. M. (1943). *Nature, Lond.*, **151**, 535.

- Irwin, J. O. (1944). *J. Hyg., Camb.*, **43**, 291.
 Morton, R. A. & Stubbs, A. L. (1946). *Analyst*, **71**, 348.

The Choice of a Chelating Agent for Inactivating Trace Metals

1. A SURVEY OF COMMERCIALY AVAILABLE CHELATING AGENTS

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The important part played by various heavy metals in metabolic processes is well recognized. In studies of such processes, the removal of part or all of a particular metal from active participation in a biological system may be desired. Microchemical reagents, particularly the chelating agents evolved in the last 30 years (Morgan & Drew, 1920; Morgan & Burstall, 1936), seem likely to prove valuable tools for such purposes and some such applications have already been made.

The iron-deficient medium devised by Waring & Werkman (1942) is an interesting example. These authors saturated a synthetic bacteriological medium with oxine (8-hydroxyquinoline) and then extracted it with chloroform, thus removing heavy metals in the form of oxine complexes; traces of manganese, zinc and copper were then added. This produced a medium that was deficient in iron and which supported the growth of certain bacteria in proportion as iron was added.

It is not easy to make a good choice of micro-analytical reagents in biological work because there are practically no data available as to which metals these reagents will inactivate under physiological conditions. Little help can be obtained from the analytical literature which usually stipulates that the solutions to be tested must first be boiled, or treated with strong acid or alkali, or even that all organic matter should be removed.

The present studies are intended as a contribution towards exploring this gap in knowledge. Their aim is to discover which reagents give visible signs of reacting with a series of biologically important metals at 37° and pH 7.3. The investigation is confined to those substances believed to act through chelation and hence it excludes a few reagents of comparable delicacy such as hydrogen sulphide and some oxidizing agents. The results of the present work show that several reagents which are widely used in analytical practice to remove traces of metal from solution are ineffective at pH 7.3 and 37°. Other reagents are found to retain their efficacy, even at great dilution and to show more or less specificity for the various metals.

METHOD AND MATERIALS

The metals were used in the form of sulphates (acetates in the case of calcium and lead) of analytical reagent quality. One ml. of a 0.0004M solution of the metal was mixed with 2 ml. of 0.1M-borate buffer of pH 7.3. One ml. of 0.004M-reagent was then added and the mixtures incubated in stoppered flasks at 37° for 24 hr. This made a final strength of 0.0001M-metal and 0.001M-chelating agent. Usually two molecules of chelating agent combine with one ion of metal so that there is a fivefold excess of chelating agent present in most cases. Although such a large excess may not have been necessary in every case, it was borne in mind that the

type of reaction being studied is a reversible one and that the law of mass action will come into play.

It was realized that 0.025 M-borate buffer is near the acidic limit of its usefulness, but phosphate buffer of the same strength formed precipitates with the solutions of the metals and so was unsuitable. Using the borate buffer, the final pH varied between 6.5 and 7.3 in various cases (glass electrode measurements). That the borate buffer did not enter into the complexes was confirmed by omitting the buffer in replicate experiments which were carefully neutralized to pH 7; identical results were obtained.

The choice of 0.0001 M-metal was suggested by the fact that this is the highest concentration at which two of the metals (iron and lead) are compatible with the buffer. All the tests were repeated at a tenfold greater dilution which seemed to be near the limits of visible response for even the most delicate reagents. Tables 1 and 2 record the precipitation or colour change observed. It may be assumed that the amount of precipitation or colour change provides an index of the extent of the chelation, although this may not be so in every case (see discussion).

The chelating reagents used were in the form supplied by British Drug Houses Ltd., with the following exceptions: cation was presented by its discoverer, Dr F. P. Dwyer; the two xanthates were presented by Timbrol Ltd. (Sydney) and recrystallized just before use. The reagents were dissolved in water or in ethanol (7.5% or weaker) with the following exceptions: no. 3, 10% ethanol; no. 6, 25% ethanol; nos. 9, 10 and 23, 10% ethanol plus 1 equiv. of NaOH; and nos. 15 and 28, 1 equiv. of NaOH. Blanks without metals were run for each reagent.

RESULTS

The results, given in Tables 1 and 2, record the visible changes seen after mixing solutions of the metals and chelating agents at pH 7 (approx.), and maintaining the solutions for 24 hr. at 37°. The same results were obtained at room temperature (20°). Precipitation or colour changes were often not immediate, but had usually reached completion in 5 min. The reagents are arranged in alphabetical order using the more familiar synonyms.

Because of the great practical importance of being able to remove a complex from the site being studied, it was decided to distinguish between those chelating agents which formed precipitates (whether coloured or not) with metals and those which gave only a colour change. To differentiate these types, listed in Tables 1 and 2 respectively, the terms 'hydrophobic chelating agent' and 'hydrophilic chelating agent' are suggested. The connecting link between the two classes is provided by 1-nitroso-2-naphthol (no. 12) and nitroso-*R*-salt (no. 26), the latter being derived from the former by insertion of two water-attracting sulphonic acid groups. Even no. 26 gives precipitates if used with stronger solutions of metals than is done here. Dithizone (no. 7), which gives precipitates under the conditions used here, is employed in chloroform solution by analysts so that the complexes will stay in solution and can be estimated colorimetrically.

The results of these chelation tests carried out at pH 7 and 37° show some correspondences with, and some abrupt divergences from, those obtained in normal analytical practice. Results which correspond well are given by cupferron, cupron, dimethylglyoxime, dithizone (*v.s.*), 1-nitroso-2-naphthol, 2:2'-dipyridyl and *o*-phenanthroline (nos. 1, 2, 4, 7, 12, 25 and 27 respectively).

Some results in which gross lack of correlation is observed invite closer attention. For example, picrolonic acid (no. 15) precipitates calcium, cobalt, copper and lead under analytical conditions (where stronger solutions may be used), but is without visible effect in the present tests. Again, dithiol (no. 6) and mercaptobenzthiazole (no. 11) fail to react with cadmium in the present tests; likewise chromotropic acid (no. 24) and thioglycollic acid (no. 29) do not give their usual colours with iron. These divergences may be attributed to the neutral conditions of the present tests; for example, analysts use chromotropic acid in acid solution and thioglycollic acid in the presence of ammonia.

The most striking result to be seen in the tables is the complete absence of reaction of calcium or magnesium throughout the series. Oxine (no. 13) is known to precipitate both these metals under alkaline conditions. Calcium is known to be precipitated by stronger solutions of picrolonic acid (no. 15) and sodium dihydroxytartrate osazone (no. 21) than are used here. Magnesium is commonly detected with diphenylcarbazine (no. 5), 1-hydroxy-anthraquinone (no. 8), magneson I and II (nos. 9 and 10), Titan-yellow (no. 22), cation (no. 23) and quinalizarin (no. 28), but alkaline conditions appear to be used in each case. Even traces of the fine blue precipitates commonly given by nos. 9, 10 and 23 would be obvious if formed in the present tests. These differences can only be attributed to the conditions of temperature, pH and dilution under which chelation has been sought in the present studies. Their importance will be discussed later.

Finally, it is seen that few reagents show true specificity for a metal and that a very large number of reagents will chelate copper.

The following non-chelating precipitating agents for metals were found virtually ineffectual under the conditions of our tests even when 5 equivalents of ammonium thiocyanate were added: acridine, allyl iodide-hexamine, α - and β -naphthoquinolines (i.e. benzoquinolines) and pyridine. 5:5'-Dibromosalicyl (Kuhn 3065), which might appear from its formula to be a chelating agent, was also ineffectual.

DISCUSSION

The wealth of data concerning the importance of trace metals in the economy of living cells has suggested biological experiments involving suboptimal concentrations of various metals. Sometimes this

Table 1. *Hydrophobic chelating agents: response to metals at pH 7 and 37° of chelating agents commonly used in analysis to precipitate metals*

No.	Chelating agent	Mol. wt.	Ca ⁺⁺	Mg ⁺⁺	Mn ⁺⁺	Zn ⁺⁺	Fe ⁺⁺	Fe ⁺⁺⁺	Cd ⁺⁺	Co ⁺⁺	Pb ⁺⁺	Cu ⁺⁺	Used in analysis to precipitate
1	Cupferron (ammonium nitroso-phenylhydroxylamine)	155	-	-	-	-	+	++	-	-	-	+	Fe, Cu
2	Cupron (benzoxime)	227	-	-	-	-	-	-	-	-	-	++	Cu, Mo
3	p-Dimethylaminobenzalrhodamine	264	-	-	-	+	-	-	+	-	-	++	Cu, Ag, Au
4	Dimethylglyoxime	116	-	-	-	-	-	-	-	-	-	-	Ni, Bi, Au, Pd
5	Diphenylcarbazide	242	-	-	-	++	-	-	-	-	-	++	Mg, Hg
6	Dithiol (4-methyl-1:2-dimercapto-benzene)	156	-	-	-	-	-	-	-	++	+	++	Cd, Co, Pb, Cu, Sn
7	Dithizone (diphenylthiocarbazone)	256	-	-	-	++	-	-	+	++	++	++	Zn, Cd, Co, Pb, Cu, Hg, Ag
8	1-Hydroxyanthraquinone	224	-	-	-	-	-	-	-	-	-	++	Mg, Al
9	Magneson I (p-nitrobenzeneazo-resorcinol)	259	-	-	-	-	-	-	-	-	-	-	Mg
10	Magneson II (p-nitrobenzeneazo- α -naphthol)	293	-	-	-	-	-	-	-	-	-	-	Mg
11	Mercaptobenzthiazole	167	-	-	-	-	-	-	-	-	+	+	Cd, Pb, Cu, Ti
12	1-Nitroso-2-naphthol	173	-	-	+	+	+	+	-	++	++	++	Fe, Co, Cu, Pd
13	Oxime (8-hydroxyquinoline)	145	-	-	++	++	+	+	+	+	+	+	Ca, Mg, Mn, Zn, Fe ⁺⁺ , Fe ⁺⁺⁺ , Cd, Co, Pb, Cu, Be, Al, Mo, U, V, W, Ti, Ni, Bi, Ga, In, Zr
14	Phenylthiohydantoic acid	210	-	-	-	+	-	-	+	+	+	-	Co, Ni
15	Picrolonic acid*	264	-	-	-	-	-	-	-	-	-	-	Ca, Co, Pb, Cu, Th
16	Potassium ethyl xanthate	160	-	-	-	-	-	-	+	+	+	++	Cu, Mo
17	Potassium sec.-butyl xanthate	188	-	-	-	-	-	-	+	+	++	++	Cu
18	Rubeanic acid (dithiooxamide)	120	-	-	-	+	-	-	+	+	+	++	Co, Cu
19	Salicylaldoxime	137	-	-	-	-	-	+	-	-	+	++	Cu, Pd
20	Sodium diethyldithiocarbamate	171	-	-	+	++	+	+	++	++	++	++	Zn, Cu
21	Sodium dihydroxytartrate osazone	370	-	-	-	-	-	-	-	-	-	+	Ca
22	Titan-yellow (sodium 4:4'-di-(6-methyl-2-benzthiazolyl)-diazo-aminobenzene-2:2'-disulphonate)	680	-	-	-	-	-	-	-	-	-	+	Mg

Key: - No ppt.; + trace of ppt. with 0.0001 M-metal and 0.001 M-reagent; ++ marked ppt. with 0.0001 M-metal and 0.001 M-reagent; +++ as + + but trace of ppt. also with 0.0001 M-metal and 0.001 M-reagent.

* It is not known for certain whether the metallic complexes of this reagent involve chelation.

Table 2. *Hydrophilic chelating agents: response to metals at pH 7 and 37° of chelating agents commonly used in analysis to give colours with metals*

No.	Chelating agent	Mol. wt.	Ca++	Mg++	Mn++	Zn++	Fe++	Fe+++	Cd++	Co++	Pb++	Cu++	Used in analysis to give colours with Mg, Cd
23	Cadion (<i>p</i> -nitrobenzenediazo-aminobenzene)	346	-	-	-	-	-	-	+	-	-	-	
24	Chromotropic acid (sodium 1:8-dihydroxynaphthalene-3:6-disulphonate)	364	-	-	++	-	-	-	-	-	+	+	Fe, Cr, Ti
25	2:2'-Dipyridyl	156	-	-	-	-	++	-	-	-	-	-	Fe++
26	Nitroso-R-salt (sodium 1-nitroso-2-naphthol-3:6-disulphonate)	377	-	-	-	-	++	+	-	+	-	+	Co
27	<i>o</i> -Phenanthroline	180	-	-	-	-	++	-	-	-	-	-	Fe++
28	Quinalizarin (tetrahydroxy-anthraquinone)	272	-	-	+	+	+	+	+	+	+	+	Mg, Be, Al, Ga
29	Thioglycolic acid	92	-	-	-	-	-	-	-	+	-	-	Fe++, Fe+++
30	Thiourea	76	-	-	-	-	-	-	-	+	-	+	Bi

Key: - No colour; + trace of colour with 0.0001 M-metal and 0.001 M-reagent; ++ marked colour with 0.0001 M-metal and 0.001 M-reagent; +++ as + + but trace of colour also with 0.00001 M-metal and 0.0001 M-reagent.

goal has been sought by the use of synthetic media in which chemicals of exceptional purity have been used. An alternative and less laborious approach is through the inactivation of trace metals which are naturally present, particularly in any way that permits of their removal through an induced change in solubility.

The use of chelating agents for this purpose is undergoing development. Some results of promise have already been obtained by Waring & Werkman (1942) whose iron-deficient medium was described in the introduction to this paper. No less significant was the attempted raising of oats by Piper (1942) in a copper-deficient medium obtained similarly by extraction with dithizone and chloroform. Mention must also be made of the use of thiourea and salicylaldoxime to decrease the copper available to the alga *Chlorella* and to some oxidases (Green, McCarthy & King, 1939); also an attempt to remove copper from spontaneous mammary cancer in mice (Boyland, 1946). Kindred topics are the inhibition of fungi by oxine and the reversal of this inhibition by zinc (Zentmyer, 1944), and an investigation of the connexion between chemistry and antibacterial action in a series of chelating agents (Albert, Rubbo, Goldacre & Balfour, 1947).

Although the literature of chelation is capably reviewed in various monographs (e.g. Mellan, 1941), the information most required by the biological worker is not available. First of all, the biologist wishes to know which of the reagents, that combine so readily with the various metals under the highly unphysiological conditions of an analytical procedure, will continue to do so under biological conditions. The data given in Tables 1 and 2 should go some way towards supplying this information. In particular, it is shown that many common chelating agents for calcium and magnesium do not react under these conditions. The standard conditions of the present tests (pH 7 and 37°) have, of course, special reference to mammalian physiology and the results will not necessarily apply in circumstances where the pH is widely different. However, the effects of temperature variations in the biological range will probably not be found very great.

The classification of these reagents into 'hydrophobic', i.e. those that give precipitates (Table 1) and 'hydrophilic', i.e. those that give soluble colour changes with metals (Table 2), should be of practical value because the complexes furnished by hydrophobic reagents are those most likely to be removable by extraction, e.g. with chloroform. Where hydrophilic reagents are concerned, only positive results have significance for it is quite possible for a reagent and a metal to chelate without visible colour change. This is the case with the suppression of calcium ions (in water) by calgon (hexasodium metaphosphate) and of magnesium ions by the Kom-

plexons of Schwarzenbach, Kampitsch & Steiner (1945). Again, because the division between hydrophobic and hydrophilic chelating agents is not a hard and fast one, it is possible (but unlikely) that a reagent in Table 1 which is producing precipitates with, say, four metals is forming a soluble complex with a fifth, without a colour change taking place.

Thus far the treatment has been only semi-quantitative. The questions now arise whether chelation goes on at dilutions below that at which precipitation can be recognized and to what extent the removal of a complex, e.g. by extraction, drives towards completion a reaction between a reagent and a metal for which it has little affinity. It is evident that these questions cannot fully be answered until 'stability constants' have been determined for each reagent with each metal. The stability constants are given by the expression:

$$K_s = \frac{[\text{metal complex}]}{[\text{free metal}] \times [\text{chelating agent}]}$$

Possession of a list of such constants would facilitate the choice of the chelating agent most avid for any particular metal. Conversely it would indicate the most effective metal for reversing the antibiological effects of a chelating agent. Finally, if stability constants were available for the complexes of metals with amino-acids and other cell constituents, it would bring about a better understanding of the distribution of metals in the cell and the distribution of metals between the cell constituents and any chelating reagent which had been added for experimental purposes. The fact that chelating agents added to biological material will be competing, not necessarily successfully, with chelating agents naturally present, requires some emphasis. There is evidence that trace metals can be held at a biological surface oriented in such a way that only chelating agents of appropriate architecture can have access to them (Albert *et al.* 1947).

Unfortunately, the determination of the stability constants of chelate complexes presents formidable difficulties to the physical chemist. The small literature of this subject is concerned almost entirely with a search for practicable methods (Calvin & Wilson, 1945; Calvin & Bailes, 1946; Duffield & Calvin,

1946). With two exceptions the constants that have been obtained are without absolute significance in biology since they were determined in highly unphysiological solvents (pyridine, glacial acetic acid). The two exceptions are *o*-phenanthroline (no. 27) and dipyriddy (no. 25) which have K_s values of $10^{17.7}$ and 10^{15} (approx.) respectively for their complexes with ferrous iron in water (Dwyer & Nyholm, 1946), figures indicating that these particular reactions go a very long way towards completion even though no precipitation takes place. Already it is evident from Calvin's work that stability constants can vary between wide limits and that even in a series of closely related chelating agents some will hold a given metal tenaciously whereas others, because of steric or electronic influences in the molecule, can form appreciable amounts of complexes only in the presence of a considerable excess of metallic ions.

One of the most important conclusions to be drawn from Tables 1 and 2 is that too few reagents exist with specificity for particular metals. Modern knowledge of atomic radii and residual affinities can provide some of the data that the synthetic chemist requires to fill these gaps.

SUMMARY

1. Thirty organic reagents, in particular those used in analysis to combine with traces of metallic ions by means of chelation, have been examined at 37° and pH 7. The biologically interesting metals with which they were tested were calcium, magnesium, manganese, zinc, iron, cadmium, cobalt, lead and copper.

2. It is found that in many cases the combination between reagent and metal that occurs so readily under the unphysiological conditions of an analytical procedure does not take place under the standard conditions. In other cases the correspondence is good.

3. The usefulness of such tests as a guide to planning biological experiments aimed at removing selected trace elements is discussed critically.

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REFERENCES

- Albert, A., Rubbo, S., Goldacre, R. & Balfour, B. (1947). *Brit. J. exp. Path.* **28**, 69.
 Boyland, E. (1946). *Biochem. J.* **40**, 55.
 Calvin, M. & Bailes, R. H. (1946). *J. Amer. chem. Soc.* **68**, 949.
 Calvin, M. & Wilson, K. W. (1945). *J. Amer. chem. Soc.* **67**, 2003.
 Duffield, R. B. & Calvin, M. (1946). *J. Amer. chem. Soc.* **68**, 557.
 Dwyer, F. P. & Nyholm, R. (1946). *Proc. roy. Soc. N.S.W.* **80**, 28.
 Green, L., McCarthy, J. & King, C. G. (1939). *J. biol. Chem.* **128**, 447.
 Mellan, I. (1941). *Organic Reagents in Inorganic Analysis*, Philadelphia: The Blakiston Co.
 Morgan, G. T. & Burstall, F. (1936). *Inorganic Chemistry*, Cambridge: Heffer and Sons.
 Morgan, G. T. & Drew, H. D. K. (1920). *J. chem. Soc.* **117**, 1456.
 Piper, C. (1942). *J. agric. Sci.* **32**, 143.
 Schwarzenbach, G., Kampitsch, E. & Steiner, R. (1945). *Helv. chim. Acta*, **28**, 828, 1133.
 Waring, W. S. & Werkman, C. H. (1942). *Arch. Biochem.* **1**, 303.
 Zentmyer, G. A. (1944). *Science*, **100**, 294.