$$2H_2 + HCOOCH_3 = 2CH_3OH$$
$$CH_3OH + CO = HCOOCH_3$$

Both sets of constants are of the same order of magnitude (they agree within a factor of about three), but the lowest figures obtained by calculations based on the use of specific heat data, and the estimated entropies of hydrogen and oxygen, are about 10–50 times as large as those obtained experimentally. The existence of a large discrepancy here, which was mentioned by Smith and Branting, is thus confirmed.

The data obtained by the authors at 300° and 50 atmospheres pressure agree very well with those obtained at 70 atmospheres by Newitt, Byrne and Strong, but are only about three-tenths as large as those obtained by Smith and Branting at atmospheric pressure. A discussion of possible explanations of this discrepancy is presented.

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THE NEPHELOMETRIC ANALYSIS OF ZINC

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In plant and animal tissues certain elements occur in amounts that are too small for accurate determination by the usual methods of quantitative analysis and yet comparatively gross for application of the methods of microchemistry. Zinc, which is one of these elements, is apparently important to animal life. Its wide distribution in food—particularly its concentration in the germ elements, such as the endosperm of grain and yolk of egg—points to a possible need. Furthermore, tissue zinc is susceptible to changes in condition which affect the salt metabolism in general[1] and which indicate some function as yet unknown in cellular processes.

Unfortunately, methods for the analysis of the small amounts of zinc that occur in animal tissues and fluids are difficult, and results approaching exactness are attained only with considerable skill and experience. This is owing not only to the fact that these traces of zinc are found in the presence of enormously greater quantities of other salts, but more particularly to the fact that zinc forms no useful colored salts, nor very insoluble compounds which sharply differentiate it from its milieu.

No simple, exact method for the gross estimation of zinc has yet been found in spite of the expenditure of considerable effort in this direction.[2] Methods for the analysis of *traces* of the metal are quite as unsatisfactory. Of the methods available for the analysis of amounts of zinc in the vicinity of

[1] L. T. Fairhall, *J. Biol. Chem.*, **70**, 495 (1926).

[2] F. R. Bichowsky, *J. Ind. Eng. Chem.*, **9**, 668 (1917).

a few milligrams or less, the nephelometric ferrocyanide method developed first by Mylius[3] is still apparently the most accurate, although it, too, is open to error. Not only the nephelometric determination itself, but the separation of zinc from other metals is difficult and subject to losses owing either to the frequently colloidal character of the compounds formed, or to their solubility in acids.

Iron frequently accompanies zinc and is (as in blood) much greater in amount. As a consequence it is a disturbing factor of some magnitude in the separation of zinc. It is possible to effect the separation of the two by precipitation of the iron with cupferron in acid solution,[4] but with very minute amounts of iron this has occasionally proved uncertain and in these cases has necessitated a new separation and analysis. Furthermore, it is tedious in application, as it requires destruction of the excess cupferron before the analysis is carried forward to completion.

For these reasons, an attempt was made to define more exactly the conditions under which traces of zinc could be separated and identified in the ash of biological material.

Experimental

An initial study of the effect of changes in acidity disclosed the fact that minute amounts of zinc could be recovered quantitatively as sulfide over a range of hydrogen-ion concentration at which iron is not similarly precipitated. For this purpose analysis was made of 100-cc. samples of solution each of which contained 1 mg. of zinc, 2 mg. of copper, 2 mg. of iron and 2 g. of sodium citrate. The solutions were adjusted to various degrees of acidity varying from a hydrogen-ion concentration of $C_H = 10^{-3}$ to $C_H = 10^{-6.6}$. The hydrogen-ion concentrations were measured electrometrically. The presence of copper is desirable in the precipitation of zinc as sulfide, when the latter metal is present in minute amount, in order to take advantage of the co-precipitation of the two. Furthermore, minute amounts of copper are usually present in animal tissues and must be removed in any event in the ordinary course of analysis.

The results obtained in this study are shown in Table I and in Fig. 1. These experiments indicated that with a hydrogen-ion concentration less than $10^{-4.5}$ iron becomes a factor of importance. On the other hand, at a hydrogen-ion concentration greater than $10^{-2.5}$ the recovery of zinc was poor. Over the range $C_H = 10^{-2.5}$ to $C_H = 10^{-4}$ both the recovery of zinc was good and the contamination due to iron was nil, so that there is a definite region between $C_H = 10^{-2.5}$ and $C_H = 10^{-4}$ at which minute amounts of zinc may be quantitatively separated from iron by precipitation as the sulfide.

[3] E. Mylius, *Z. anal. Chem.*, **19**, 101 (1880).
[4] L. T. Fairhall, *J. Ind. Hyg.*, **8**, 165 (1926).

TABLE I

THE OPTIMUM RANGE OF ACIDITY FOR THE SEPARATION OF ZINC AND IRON AS SULFIDE

C_H	Zinc recovered, %	C_H	Zinc recovered, %	C_H	Iron recovered, %
$10^{-1.62}$	3	$10^{-3.35}$	101	$10^{-5.0}$	12.0
$10^{-1.78}$	11	$10^{-3.60}$	104	$10^{-5.2}$	15.7
$10^{-2.23}$	40	$10^{-3.90}$	100	$10^{-5.4}$	19.2
$10^{-2.44}$	80	$10^{-4.15}$	101	$10^{-5.6}$	36.7
$10^{-2.61}$	100	$10^{-4.49}$	Iron	$10^{-5.8}$	56.0
$10^{-2.72}$	99	$10^{-5.21}$	contaminated	$10^{-5.9}$	57.7
$10^{-2.89}$	102	$10^{-6.33}$	Iron	$10^{-6.0}$	68.2
$10^{-3.02}$	100		contaminated	$10^{-6.2}$	82.2
$10^{-3.12}$	99		Iron	$10^{-6.4}$	85.5
$10^{-3.27}$	98		contaminated	$10^{-6.6}$	91.0

Fales and Ware[5] found that in zinc analysis by the usual methods and using one-gram samples of zinc ammonium sulfate, the best range for zinc precipitation lies between a hydrogen-ion concentration of 10^{-2} and 10^{-3}.

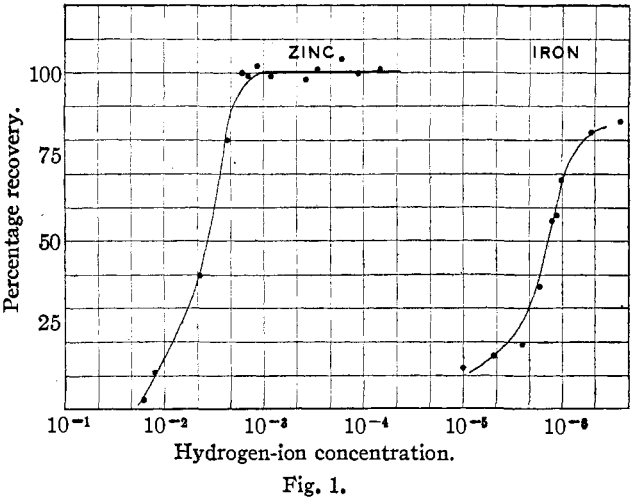

Fig. 1.

With concentrations greater than 10^{-2} precipitation is incomplete; with less than 10^{-3} the precipitate becomes slimy and difficult to filter. With minute quantities, however, losses are encountered when the hydrogen-ion concentration is greater than $10^{-2.5}$. A hydrogen-ion concentration of 10^{-3} is a convenient point of precipitation for these small amounts and may readily be adjusted by means of the two indicators thymol blue and brom chlor phenol blue. Sliminess or granularity of the zinc–copper sulfide precipitate is a factor of no consequence with minute quantities, as the filtration is always efficient.

Factors Influencing the Opalescence of Zinc Ferrocyanide.—Accuracy in estimating zinc nephelometrically as the ferrocyanide can be attained

[5] H. A. Fales and G. M. Ware, THIS JOURNAL, **41**, 487 (1919).

only by careful attention to several factors which markedly influence the degree of turbidity produced. Important among these are acidity, salt concentration and time. Very small variations in acidity produce surprisingly large changes in opacity for a given quantity of zinc. Thus a solution 1.53×10^{-5} molar with respect to zinc at an acidity of 0.002 N has an *apparent* zinc content of 7.65×10^{-5} if the acidity is increased to 0.028 N—or, in other words, the apparent zinc content is increased five-fold with a slight shift in acidity. Furthermore, the readings are subject to vagaries at the higher acid level and fluctuate more with slight differences in acid concentration than at the lower acid level. Quantities of zinc lower in amount (of the order 3×10^{-6} molar) do not respond to this degree of sensitivity to acid.

TABLE II

THE EFFECT OF ACIDITY UPON THE OPALESCENCE OF ZINC FERROCYANIDE

C_H	True concentration, moles $\times 10^5$	Apparent concentration, moles $\times 10^5$	C_H	True concentration, moles $\times 10^5$	Apparent concentration, moles $\times 10^5$
$10^{-2.49}$	1.53	1.53	$10^{-2.53}$	7.65	7.65
$10^{-2.42}$	1.53	1.53	$10^{-2.27}$	7.65	7.65
$10^{-2.26}$	1.53	1.53	$10^{-2.20}$	7.65	10.12
$10^{-1.93}$	1.53	2.75	$10^{-1.92}$	7.65	10.00
$10^{-1.68}$	1.53	3.98	$10^{-1.90}$	7.65	10.35
$10^{-1.41}$	1.53	4.50	$10^{-1.68}$	7.65	11.35
$10^{-1.03}$	1.53	7.67	$10^{-1.38}$	7.65	13.09
			$10^{-0.82}$	7.65	16.87

The effect of variation in degree of acidity upon the opalescence of zinc ferrocyanide is shown in Table II and in Fig. 2. The zinc concentrations in

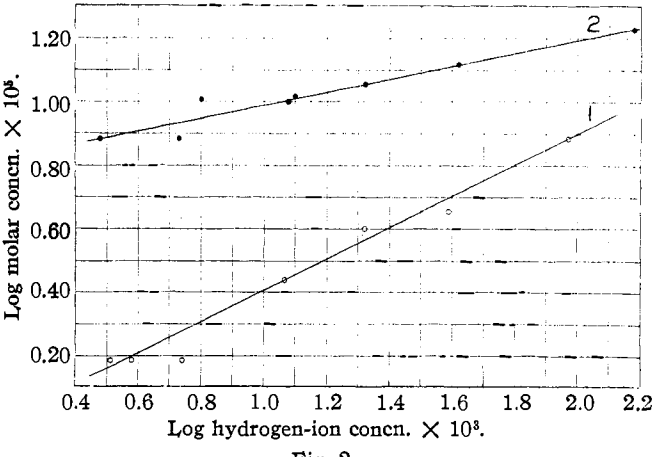

Fig. 2.

these two series were 1.53×10^{-5} and 7.65×10^{-5} molar, respectively, while the acid concentration was varied from a hydrogen-ion concentration

of $10^{-0.83}$ to $10^{-2.53}$. The apparent zinc content of each solution was determined nephelometrically against standards identical in all other respects, except that the acidity was maintained at a C_H of $10^{-2.53}$.

It is apparent from Fig. 2 that the change in opalescence with acidity takes the form $y = x^n k$, where y and x represent the degree of opalescence and hydrogen-ion concentration, respectively, and k is a constant. Decreasing the hydrogen-ion concentration to points lower than $10^{-2.50}$ affects the opalescence somewhat, but much less proportionately than at the higher acid level. Moreover, at the higher acid concentrations the Tyndall blue effect is not so apparent. A fixed hydrogen-ion concentration of $C_H = 10^{-2.3}$ was adopted as most satisfactory for the nephelometric determination of zinc as ferrocyanide.

Salt Concentration.—An excess of salt (in this case potassium chloride) is undesirable, for it not only increases the opacity of the suspension, but also causes the development of a yellowish tinge in the suspension which makes the readings difficult. In order to determine the degree of interference to which various amounts of potassium chloride will give rise, a series of experiments was made in which a constant amount of zinc in the presence of variable amounts of potassium chloride was determined nephelometrically against standards having a salt content of one gram of potassium chloride per liter of solution The acidity was the same throughout. These results are shown in Table III and in Fig. 3.

TABLE III

EFFECT OF SALT CONCENTRATION UPON THE OPALESCENCE OF ZINC FERROCYANIDE

True zinc concentration, 7.66×10^{-5} molar

Number	1	2	3	4	5	6	7	8	9
KCl, moles $\times 10^{-3}$	6.7	13.4	20.1	26.8	33.5	40.3	47.5	53.7	66.4
Apparent zinc concn., moles $\times 10^{-5}$	3.1	4.6	6.3	7.6	9.5	11.5	12.9	14.6	15.3

It will be observed that the apparent values obtained for 0.25 mg. of zinc ranged from 0.10 mg. to more than 0.50 mg. in proportion as the salt concentration varied. It was quite apparent (especially at high salt concentrations) that the increased opacity was accompanied by larger flocculates, so that while the degree of dispersion was decreased the opacity was of quite a different type from normal and therefore increased the difficulty of making satisfactory readings.

The apparent zinc content varied directly as the salt concentration. As a result of these experiments and as a convenient amount to use in the ordinary course of analysis, the quantity of salt was so adjusted that the opalescence was uniformly developed in a solution 0.0268 molar with respect to potassium chloride.

Time.—Time is always a factor of importance in the development of opalescence. After the addition of potassium ferrocyanide to a very dilute

solution of a salt of zinc, the resulting solution becomes a dispersed, unstable system. Coalescence is determined by the collision and adhesion of the dispersed particles. With zinc ferrocyanide several minutes are necessary for the attainment of maximum opalescence. The time necessary to reach this point was determined with amounts of zinc varying from 0.1 mg. to 0.4 mg. in 50 cc. of solution and was found to vary from three and one-half minutes for the lower concentration to six minutes for the upper. Maximum opalescence is certainly attained by the end of ten minutes over this range and persists at a maximum for fully twenty minutes longer. The turbidity is affected by decomposition of the ferrocyanide to some extent on exposure of the solution to direct sunlight for half an hour or more, so that it is advisable to carry out the determinations in somewhat subdued daylight. Artificial illumination is generally unsatisfactory.

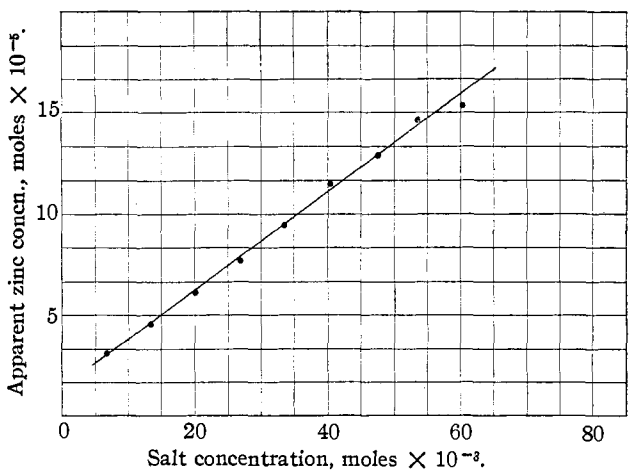

Fig. 3.

Method of Analysis.—The method of analysis based upon the foregoing experiments and which has been adapted to biological material is as follows.

Dissolve the ash of material obtained by low temperature ashing[6] in 6 *N* redistilled hydrochloric acid and hot water. For quantitities approximating the ash of 100 cc. of blood, dilute the solution to 75 cc., add 5 g. of pure sodium citrate, 2 mg. of copper as sulfate and a sufficient amount of thymol blue indicator. Add dilute potassium hydroxide solution until the solution becomes yellow and then add brom chlor phenol blue. If the solution is bluish at this point, add dilute acid until the yellow color is just restored. Saturate the cold solution with hydrogen sulfide gas, filter out the zinc and copper sulfides, washing well to free from iron salts as much as possible. Dissolve the sulfides in nitric acid and hydrochloric acid, evaporate to dryness twice with hydrochloric acid, dissolve the residue in hydrochloric acid and repeat the above procedure omitting the addition of sodium citrate. In the absence of buffer the adjustment of acidity is much

[6] P. K. Thompson, *J. Ind. Hyg.*, **7**, 358 (1925).

sharper and requires more care. Dissolve the reprecipitated sulfides as before, add 5 cc. of 6 *N* hydrochloric acid to the residue and 20 cc. of water. Slowly saturate the cold solution with hydrogen sulfide and filter. The filtrate contains the zinc as chloride. Evaporate to dryness and dissolve the residue in 4 to 5 drops of 6 *N* hydrochloric acid and a little water. The solution of the residue at this point is occasionally refractory, and it is necessary to see that it is moistened throughout with acid and warmed slightly before the addition of water. Transfer to a 25-cc. volumetric flask. To an aliquot portion (5 or 10 cc.) of this solution add 10 cc. of 0.1341 *N* potassium hydroxide and exactly neutralize the excess of potassium hydroxide with 0.10 *N* hydrochloric acid, adding exactly 1 cc. of acid in excess. Dilute almost to 50 cc., add 1 cc. of 2% potassium ferrocyanide solution, adjust exactly to a volume of 50 cc. and thoroughly mix at once. This solution is now 0.002 *N* with respect to acid and 0.0268 molar with respect to potassium chloride. The nephelometric standards should be prepared in exactly the same way in order to insure equivalent salt and acid concentrations. The most suitable range for comparison is that of standards containing 0.20 to 0.25 mg./50 cc. matched against solutions of nearly the same opacity as the unknown. If Nessler tubes are used, colorless glass tubes should be selected and the bottoms and a band about the meniscus blackened. Especial attention must also be given to the quality of light in this case. The standards for Nessler tubes should vary from 0.05 to 0.50 mg. in steps of 0.05 mg.

Analysis of biological material—particularly blood—for minute amounts of zinc is much facilitated by the above method. The conditions for exact separation and estimation are easily fulfilled and interference by other metals is minimized. In order to determine its adequacy for blood analysis, a zinc-free solution representing the relative concentration of the various inorganic salts present in blood was prepared. Five cubic centimeters of this solution represented the amount of ash from 100 cc. of blood. To 5-cc. portions of this solution amounts of zinc varying in quantity from 0.50 to 1.40 mg. were added and the solutions analyzed. In three series of experiments comprising thirty-three analyses of this type, the average error was ±0.06 mg. While this does not represent an extraordinary degree of accuracy, in view of the expediency attained it seems particularly suitable for the amounts of zinc normally present in biological material.

Summary

In the analysis of biological ash for traces of zinc, the separation of the latter from certain other metals and from iron in particular is most accurately made by co-precipitation with copper as the sulfide at a C_H of 10^{-3}. The nephelometric estimation of zinc as the ferrocyanide gives accurate results only within definite limits of acidity and salt concentration. Suitable conditions for this have been found at a C_H of $10^{-2.3}$ and at a salt concentration of 0.0268 molar with respect to potassium chloride.

BOSTON, MASSACHUSETTS