The Determination of Iron in Biological Material

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In the course of investigations on protein hydrolysates used for preparing culture media, it was found that no satisfactory method for determining small quantities of iron (0.5–10 $\mu\text{g./ml.}$) in such material existed. Wet ashing was reputed to give reliable results with biological material (Jackson, 1938; Thorp, 1941), but this method is time-consuming and the neutralization of the residual acid after digestion introduces adventitious iron, resulting in large and troublesome blanks. The alternative process of dry ashing in an electric muffle furnace offers a convenient and at first sight complete solution, but experience proves that this method introduces an unexpected chemical problem concerning iron in complex combination. It was found that recoveries of iron from such ashes were variable and low, and in addition, combination with $\alpha\alpha'$ -dipyridyl to develop a pigment suitable for colorimetric measurement was slow and incomplete.

The determination of small quantities of iron in dry ashes of material with a high P : Fe ratio has been studied by many workers, and the diverse methods which have been suggested to overcome the difficulties, attributed to complex phosphate formation, reflect the present unsatisfactory situation (Elvehjem & Hart, 1926; Elvehjem, 1930; Jackson, 1938; Howe, 1943, 1944; Bandemer & Schaible, 1944; Stugart, 1931; Koenig & Johnson, 1942). Methods which involved much manipulation after ashing, as, for instance, the complete removal of phosphates (Elvehjem & Hart, 1926), were considered unsuitable, since the main reason for using dry ashing was the low level at which blanks could be kept. The simpler methods usually involved boiling with acid or alkali for varying times (Howe, 1944; Stugart, 1931). An iron complex resistant to this procedure, presumably similar to that reported in ashes of tomato and spinach (Jackson, 1938), was found to be present in ashes of protein hydrolysates. The present communication reports a procedure overcoming this interference and the development of a simple method of good precision for estimating iron.

EXPERIMENTAL

The method as finally developed consists in (a) dry ashing the sample, (b) precipitating the iron present as sulphide by means of ammonium sulphide solution and (c) dissolving the precipitated ferrous

sulphide in acid in the presence of $\alpha\alpha'$ -dipyridyl. The resulting red colour is measured in an absorptiometer. The iron sulphide is not separated from the ash solution, and all reactions are carried out in the crucible in which the sample has been ashed. Blanks can therefore be kept low because of the very small amount of manipulation involved.

The method of ashing has been found to have a marked effect on the reproducibility of the method, and the one adopted is a modification of that used by Klumpp (1934) who damped his material with H_2SO_4 before ashing to prevent loss of iron. The H_2SO_4 acts as an effective ashing aid, and the white fluffy ashes so obtained dissolve readily and give no carbon residue of any kind.

When the search for a suitable method for determining iron was started, potassium thiocyanate was used as the chromogenic agent. This was found unsuitable owing to the necessity for strict pH control, and the poor stability of the colour formed limited the number of determinations able to be carried out at one time. $\alpha\alpha'$ -Dipyridyl was found to be a more suitable reagent, since the red-coloured complex it forms with ferrous ions is stable for a period of months (Thorp, 1941; Moss & Mellon, 1942) and its colour is not affected over the pH range 2.5–8.5 (Moss & Mellon, 1942).

It was observed that dry ashing of protein hydrolysates followed by HCl and sodium sulphite treatment for the reduction of iron gave non-reproducible results. Experimental study led to the conclusion that dry ashing resulted in a ferric complex stable to acid hydrolysis. A difficulty of the same kind was reported by Jackson (1938), who introduced the use of ammonium sulphide. This enabled ferrous sulphide to be recovered for subsequent treatment. While the application of this method in the original form, or modified by the use of adsorbents such as calcium phosphate, proved unsuccessful in our case, the use of ammonium sulphide for the purpose of bringing ferrous iron and $\alpha\alpha'$ -dipyridyl into quantitative reaction enabled the following experimental method to be successfully applied.

METHOD

Apparatus

Ashing can be carried out in platinum or silica dishes or in Royal Worcester porcelain crucibles, provided the latter are well cleaned with chromic acid before use.

Measurement of colour

A Spekker absorptiometer with 1 cm. cells and Wratten 61 green filters was used for all the colour measurements, but any other suitable instrument can be used.

Reagents

All reagents are A.R. and the distilled water is redistilled from glass.

Acids. $\text{N-H}_2\text{SO}_4$ and N and 0.1N-HCl . The hydrochloric acid solutions are prepared from glass-distilled constant-boiling (20%) HCl .

Ammonium sulphide solution. Freshly prepared for each set of determinations by bubbling H_2S through concentrated ammonia solution (sp.gr. 0.880 A.R.) for 2–5 min. The final solution should not be more than faintly yellow. A.R. ammonium sulphide is not suitable, as it tends to deposit sulphur under the experimental conditions. This deposit is most difficult to remove and makes estimation with the photoelectric absorptiometer impossible.

$\alpha\alpha'$ -Dipyridyl. A 1% (w/v) solution in Fe-free 0.1N-HCl . This solution is rejected as soon as it shows more than a slight red colour.

Sodium sulphite. A 20% (w/v) solution of $\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$ prepared just before use.

pH 4.6 buffer. This is made by mixing 200 ml. of m -sodium acetate with 100 ml. N-HCl (iron-free) and making to 1 l.

Standard Fe solution. This is made from pure iron wire or ferric ammonium sulphate. A primary standard containing $100\mu\text{g. Fe/ml.}$ is conveniently prepared by dissolving $0.8636\text{ g. of Fe}(\text{NH}_4)(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ in water, adding 100 ml. of N-HCl and making up to 1 l.

Standard curve

For the method described a calibration curve was prepared for the Spekker absorptiometer over the range $0\text{--}100\mu\text{g. Fe/10 ml.}$ Colour development of standards was carried out by reducing with sodium sulphite and HCl in the presence of $\alpha\alpha'$ -dipyridyl (Thorpe, 1941). The use of sodium sulphite and acid for reduction was considered preferable to ammonium sulphide as reduction was complete, the acid and sulphite buffered within the working pH range of $\alpha\alpha'$ -dipyridyl and the necessity for redissolving a ferrous sulphide precipitate was avoided. The standard iron solution was diluted so that it contained $20\mu\text{g. Fe/ml.}$ Suitable quantities to cover the range $0\text{--}100\mu\text{g. Fe}$ were pipetted into 10 ml. graduated centrifuge tubes and distilled water added to give a volume of 5 ml. Then $0.5\text{ ml. 20\% sodium sulphite solution, 0.5 ml. N-HCl}$ and $0.25\text{ ml. } \alpha\alpha'\text{-dipyridyl}$ solution were added. The contents of each tube were well mixed and left for 15 min., before making to 10 ml. and reading in the absorptiometer. Tubes showing any cloudiness were centrifuged before colour measurement, but in most cases this was not found necessary.

Procedure

Weigh or pipette a suitable quantity of material into a platinum, silica or Royal Worcester crucible. For hydrolysates containing between 0.5 and $10\mu\text{g. Fe/ml.}$, $5\text{--}10\text{ ml.}$ has been found satisfactory. Add $2.5\text{ ml. of N-H}_2\text{SO}_4/10\text{ ml.}$

(or 10 g.) and evaporate on a water-bath to a consistency suitable for ashing. Protect samples on the water-bath from aerial contamination by shielding them with a sheet of glass. Ash at 500° . Cool the ash, add 1.0 ml. of N-HCl and take to dryness on the water-bath. Cool, add $5\text{ ml. of distilled water}$ and $0.25\text{ ml. of ammonium sulphide solution.}$ Mix with swirling and again dry and cool. Add $2\text{ ml. water, 0.5 ml. of } \alpha\alpha'\text{-dipyridyl solution}$ and 0.5 ml. N-HCl. Place the crucible on the water-bath just off the boil and leave 5 min. with occasional swirling to assist complete solution of the ferrous sulphide. It is most important that complete solution is achieved, since this has been found to be the main cause of bad duplication of results. A black residue is sometimes seen if much copper is present and is centrifuged off at a later stage. Add $5\text{ ml. of pH 4.6 buffer}$ to bring the reaction of the solution within the range for maximum colour development. Cool and transfer the contents of the crucible to a graduated centrifuge tube. Make up to 10 ml. and centrifuge to remove any suspended matter likely to interfere with colour matching. Read in the absorptiometer.

RESULTS

Sixteen 10 ml. samples of a papain digest of horse muscle were analyzed and the following results obtained: $62.0, 63.8, 60.7, 61.9, 59.2, 61.4, 62.1, 62.0, 62.1, 63.8, 61.5, 59.9, 62.8, 61.0, 59.7, 60.5\mu\text{g. Fe/10 ml. hydrolysate.}$ The mean was $61.5\mu\text{g. Fe/10 ml.}$, and the standard deviation from the mean $1.3\mu\text{g. Fe/10 ml.}$, giving a coefficient of variation of 2.1% .

Table 1. Recoveries of iron from papain digest of horse muscle and from milk

Fe found ($\mu\text{g./5 ml.}$)			
	Papain digest	Papain digest + $10\mu\text{g. Fe}$	Papain digest + $20\mu\text{g. Fe}$
	31.0	40.0	50.0
	31.0	41.0	52.0
	30.0	41.0	49.0
Average	30.7	40.7	50.3
Fe recovered (%)		100.0	98.0
Fe found ($\mu\text{g./10 ml.}$)			
	Milk	Milk + $10\mu\text{g. Fe}$	Milk + $20\mu\text{g. Fe}$
	4.8	15.0	25.0
	4.5	15.5	25.0
	4.7	14.5	25.0
Average	4.7	15.0	25.0
Fe recovered (%)		103.0	101.5

In Table 1 are given the results of recovery experiments on 5 ml. samples of papain digest of horse muscle to which were added 10 and $20\mu\text{g. of Fe}$ and a similar series with $10\text{ ml. of fresh raw cows' milk.}$ The iron was added as a solution of ferric ammonium sulphate before ashing, and ashings were in triplicate.

DISCUSSION

The method described is based on two observations. The first, made by Jackson (1938), is that small amounts of iron can be quantitatively precipitated as sulphide from ashes of biological material with a high P : Fe ratio. The second, that the ferrous sulphide so precipitated can be redissolved without separation from the interfering ions present, and combined quantitatively and preferentially with $\alpha\alpha'$ -dipyridyl, was made while trying to apply Jackson's methods to ashes of protein hydrolysates. These ashes contain an iron complex very resistant to acid hydrolysis. It is therefore unlikely to be iron pyrophosphate, which can be hydrolyzed by acids (Jackson, 1938; Howe, 1944). The nature of the complex is not known, but it may be significant that iron metaphosphates can be formed at 200–300° and are stable up to 800° (Brasseur, 1944). Direct experimental confirmation of the presence of the metaphosphate ion is difficult; its solutions, however, are known to have marked tendencies to sequester multivalent ions. After reduction and

precipitation of iron as sulphide, one or more possible interfering ions are left in solution. It seemed that introduction of $\alpha\alpha'$ -dipyridyl and re-solution of the iron would be likely to lead to the preferential formation of stable ferrous dipyrldyl rather than any of the unwanted ferric complexes. Any tendency for the iron to revert to the ferric state would be checked by the nascent H_2S evolved from the dissolving ferrous sulphide. This appeared to be the case.

SUMMARY

1. A method for determining iron in biological materials with a high P : Fe ratio is described. It is applicable over the range 0.5–10 μg . Fe/ml. All analytical manipulations, except the final centrifuging before colour reading, are performed in the crucible in which the sample has been ashed. Blanks are therefore kept at a minimum.

2. The method has proved satisfactory with protein hydrolysates and cows' milk and may be suitable for other materials where phosphate interference is met.

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The Excretion of *l*(-)-Tyrosine in Urine

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Estimation of the *l*(-)-tyrosine content of urine is of value in the assessment of hepatic disease (Lichtman, 1934) and in the study of *l*(-)-tyrosine metabolism. The methods hitherto available lack the necessary sensitivity and specificity for the measurement of normal and pathological tyrosinuria. Thus the tyrosinase method of Lichtman & Sobotka (1929) fails to detect the tyrosine present in normal urine, and Millon's reaction lacks specificity.

In the present communication a method is described which estimates only *l*(-)-tyrosine and *l*-3,4-dihydroxyphenylalanine and is sufficiently sensitive to measure the *l*(-)-tyrosine content of normal urine. The material estimated is referred to

throughout this paper as *l*(-)-tyrosine because the presence of *l*-3,4-dihydroxyphenylalanine in normal urine is considered unlikely. The possibility that a mixture of the two substances has been estimated has not however been excluded. Data have been obtained for a small series of normal men and women and a rough assessment made of the *l*(-)-tyrosine output of a few normal domestic animals. The output of *l*(-)-tyrosine has also been measured in pregnancy and in a few pathological conditions.

Evidence for the occurrence of tyrosine in normal urine was obtained by Embden & Reese (1906), although a pure product was not isolated. Pouchet (1880) is quoted by Neubauer & Huppert (1910) as