fact that it is, suggests that phosphorylation is not the mechanism of active absorption in this case. These results confirm those of Davidson & Garry (1940) in which they found that D-xylose is absorbed as rapidly as glucose from the caudal region of the small intestine of the cat. Shapiro (1947) has suggested from results with phlorrhizin and rat kidney extracts that, although phosphorylation may play a part in the absorption of sugars, it is probably far from being the only mechanism, and this is also true of the small intestines of the rat and the cat.

SUMMARY

1. In rats the rate of absorption of 3-methylglucose has been found to be about 16 % slower than that of glucose.
2. In cats 3-methylglucose has been found to be absorbed against a concentration gradient.
3. Since 3-methylglucose is not glycogenic in the rat, it is suggested that phosphorylation is not the only mechanism of active absorption either in the rat or the cat.

REFERENCES

- Bárány, E. & Sperber, E. (1939). *Skand. Arch. Physiol.* **81**, 290.
- Campbell, P. N. & Young, F. G. (1948). Unpublished Reports.
- Cori, C. F. (1925a). *Proc. Soc. exp. Biol., N.Y.*, **22**, 497.
- Cori, C. F. (1925b). *J. biol. Chem.* **66**, 691.
- Davidson, J. N. & Garry, R. C. (1940). *J. Physiol.* **97**, 509.
- Davidson, J. N. & Garry, R. C. (1941). *J. Physiol.* **99**, 239.
- Elek, A. (1939). *Industr. Engng Chem. (Anal. ed.)*, **11**, 174.
- Hagedorn, H. C. & Jensen, B. N. (1923). *Biochem. Z.* **135**, 46.
- Shapiro, B. (1947). *Biochem. J.* **41**, 151.
- Somogyi, M. (1927). *J. biol. Chem.* **75**, 33.
- Verzár, F. & McDougall, E. J. (1936). *Absorption from the Intestine*. London: Longmans, Green and Co.
- Wilbrandt, W. & Laszt, L. (1933). *Biochem. Z.* **259**, 398.
- Young, L. (1938). *Biochem. J.* **28**, 1432.

Determination of Traces of Iron and Copper in Culture Media Prepared by Enzymic Digestion of Muscle Protein

By W. A. JONES, *Wellcome Physiological Research Laboratories, Beckenham, Kent*

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During the course of experimental work on the effect of trace metals on the toxin production of *Corynebacterium diphtheriae*, it became apparent that a very sensitive method for the determination of iron and copper was necessary. As a temporary measure, a method for iron was evolved, based on Mueller's (1938) media deferration method, consisting of adding calcium phosphate to the sample, precipitating with ammonia, and treating the precipitate with o-phenanthroline to develop a colour with the ferric phosphate adsorbed on the calcium phosphate. This method gave reliable and reproducible values for inorganic iron, but gives no indication of the amount of bound iron present. The problem thus resolved itself into two main steps: (a) incineration of the sample, and (b) the determination of iron and copper in the ash.

Dry ashing methods, particularly that due to Klumpp (1934), were investigated and rejected because of the significant amounts of iron and copper extracted from the ashing vessels. Silica, porcelain and pyrex crucibles all gave trouble, a fact noted by Braun & Scheffer (1940), but platinum was not tried since Bailey & McHargue (1945)

stated that it gave a colour with the copper reagent similar to that given by copper itself. Wet ashing with H_2SO_4 , $HClO_4$ and HNO_3 in pyrex flasks was then tried, but even after diminishing the large acid blank by redistillation of the acids, results were still erratic and high. It was not until the flasks were specially cleaned that consistent results were obtained. Wet ashing was a lengthy procedure until Smith's (1946) method was investigated, in which digestion was greatly accelerated by the use of vanadium as a catalyst. This method gave no trouble provided that the rate of heating was controlled to prevent spontaneous ignition.

Of a number of colorimetric reagents available for the determination of small quantities of iron, $\alpha\alpha'$ -dipyridyl and o-phenanthroline appeared to be the most satisfactory from the point of view of sensitivity, stability of colour and freedom from interference, and a number of methods employing both dipyridyl (Hill, 1930; Dyer & MacFarlane, 1938; Jackson, 1938; Schulek & Floderer, 1939; Thorpe, 1941; Koenig & Johnson, 1942; Moss & Mellon, 1942; Norlin, 1943; Kitzes, Elvehjem & Schuette, 1944) and phenanthroline (Saywell & Cunningham, 1937; Fortune & Mellon, 1938; Hummel & Willard, 1938; Schaefer, 1940; Mehlig & Hulett, 1942; Borei, 1943; Bandemer & Schaible, 1944; Benne & Snyder, 1944) is described in the literature. The thiocyanate complex was rejected in view of its in-

stability and sensitivity to variation of pH, etc. (Woods & Mellon, 1941; Benne & Snyder, 1944). Phenanthroline is about one and a half times as sensitive as dipyriddy, otherwise there is little to choose between the two reagents. They both form coloured complexes with ferrous iron and give practically no colour with ferric iron, so an efficient reducing agent must be used. A number of different reducing agents has been employed, of which the most satisfactory appear to be hydroxylamine hydrochloride (Fortune & Mellon, 1938; Moss & Mellon, 1942) and *p*-hydroxyphenylglycine (Dyer & MacFarlane, 1938). Sodium hyposulphite ($\text{Na}_2\text{S}_2\text{O}_4$), though efficient, is somewhat unstable, and grades obtainable contained considerable quantities of iron.

The estimation of iron in the ash was relatively simple when phosphate was absent, but in the presence of relatively large amounts (up to 10,000:1) of this ion it became difficult owing to the formation of ferric pyrophosphate during ashing. Several methods have been described to overcome this difficulty, of which the methods of Schulek & Floderer (1939), as described by Moss & Mellon (1942), and Jackson (1938) appeared to be most promising. Schulek & Floderer's (1939) phosphoric acid procedure was found to give fairly good results with tryptic digests but not with papain digests; a satisfactory explanation for this was not found. Jackson's (1938) method of precipitating the iron as sulphide was not satisfactory since filters would not hold the precipitate. Adsorption of the precipitate on $\text{Ca}_3(\text{PO}_4)_2$ and PbS , although it gave better results, was not entirely satisfactory. Attempts to prevent the formation of pyrophosphate by omitting H_2SO_4 from the digestion mixture were not successful since the presence of HClO_4 in the residue caused the precipitation of what was presumably ferrous-phenanthroline-perchlorate, for which Smith & Richter (1944) give a solubility of 0.00103 mol./l. Destruction of the excess HClO_4 with hydrazine, hydroxylamine and oxalic acid was attempted but was unsuccessful owing to the large quantities of reducing agent required. Other types of reducing agents could not be used since the decomposition products had to be volatile to limit the concentration of solids in the final solution. Hydrated silica also caused trouble by adsorbing some of the coloured iron complex on standing.

The problem was finally solved by heating the ash solution to boiling with phenanthroline and hydroxylamine, when it was found that maximum colour development was obtained in less than 30 sec. As a matter of interest, this phenomenon was more fully investigated and it was found that a similar effect was obtained in the presence of other complexes; 20 μg . of Fe^{+++} were added to c. 0.1 g. of the complexing ion (as the Na or K salt) in 5 ml. of water, followed by phenanthroline, hydroxylamine and acetate buffer. If in 5 min. the solution had not attained the same colour as a standard, it was heated to boiling. If the colours still did not match, a tiny crystal of sodium sulphide was added and the solution reheated. The results obtained are set out in Table 1.

In addition to the methods noted above for the determination of copper (Braun & Scheffer, 1940; Bailey & McHargue, 1945), a trichloroacetic acid

Table 1. *Colour development in presence of various complexing ions*

(0.1 g. Na or K salt of complex, 20 μg . Fe^{+++} , 0.25 ml. 10% (w/v) hydroxylamine hydrochloride, 0.5 ml. 0.25% (w/v) *o*-phenanthroline, 0.5 ml. 1.7 M-acetate buffer, diluted to 10 ml. with distilled water.)

Complexing ion	Maximal colour development
Benzoate	AH
Borogluconate	I
Citrate	AH
Ferricyanide*	AHS
Ferrocyanide*	AHS
Fluoride	AHS
Formate	I
Gluconate	AH
Glutamate	I
Glycerophosphate	AH
Glycollate	AH
Lactate	AH
Malate	AH
Maleate	AH
Mandelate	AH
Metaphosphate	AH
Orthophosphate	AH
Oxalate	AHS
Palmitate	Red ppt.
Phenylacetate	AH
Phthalate	I
Pyrogallate	I
Pyrophosphate	AH
Pyruvate	Incomplete
Salicylate	AH
Stearate	I
Succinate	AH
Sulphosalicylate	AH
Tannate	Pale blue ppt.
Tartrate	AH
Thiocyanate	AH
Thiodiglycollate	AH
Thiosulphate	I
Trichloroacetate	I

Key: I=immediate; AH=after heating; AHS=after heating with sulphide; * total iron liberated.

extraction method using diethyldithiocarbamate (Tompsett, 1934a; Cartwright, Jones & Wintrobe, 1945) and Eden & Green's (1940) method using amyl alcohol extraction, were investigated. Tompsett's (1934a) method proved unsatisfactory, but Eden & Green's method gave excellent results.

The determination of copper in the ash presented no difficulties since the only interfering ion likely to be encountered was iron and that was firmly bound by the phenanthroline. The use of citrate as in Eden & Green's method was continued, however, since it held calcium phosphate in solution when the solution was made alkaline with ammonia (Tompsett, 1934b). Originally, total iron and copper were determined on separate samples, but in the light of the methods of Parker & Griffin (1939) and Gerber, Claassen & Boruff (1942), both elements were determined in the same sample.

METHOD

Apparatus and anticontamination precautions

Great care had to be taken to exclude every chance of contamination by dust-borne and other extraneous iron at all stages of the procedure. Water, redistilled over AgNO_3 from a pyrex all-glass still, was used and reagents were specially purified where necessary. It was found advisable to have a dust-proof cabinet (e.g. an old balance case) where tubes, flasks, reagents, etc. could be left free from contamination at any stage of the procedure.

Pyrex narrow-necked conical flasks (250 ml.) were used for the incineration, and 10 ml. graduated centrifuge tubes for the solutions after the development of the iron colour. The copper colour was developed in 40 ml. pyrex glass-stoppered tubes. When not undergoing heating, the flasks were covered with 50 ml. pyrex beakers. The flasks were specially cleaned to prevent contamination from the glass. When new, they were treated with boiling conc. H_2SO_4 for c. 30 min., and then with boiling aqua regia. Before use, they were treated each time with boiling 20% (w/v) NaOH to dissolve deposited silica (which appeared to cause erratic results) and then with boiling aqua regia. After acid treatment, the flasks were rinsed successively in tap, distilled and redistilled water and then inverted on a sheet of clean glass and allowed to drain. Tubes and pipettes were cleaned by immersing in chromic-nitric acid cleaning mixture (2.5% (w/v) CrO_3 in conc. HNO_3) overnight and rinsing as above. (Chromic-nitric acid mixture was found preferable to the conventional chromic-sulphuric acid mixture. When the oxidizing properties of the acid were spent, the HNO_3 was recovered by distillation.)

For the incineration stage, a heater to accommodate six flasks was constructed from two 1 kW. electric fire elements placed end to end and mounted on a suitable fire-proof base. The elements were wired in parallel and rate of heating was controlled by a 'Variac' transformer. The elements were surrounded by a 'fence' of 'Poi-lite' which projected to a height of approximately 3 in. above their surface. Along one side were cut six slots into which the flask necks were placed when tilting was necessary.

To prevent contamination by dust while the flasks were on the heater, a stream of completely dust-free air was blown into them. The filter system was of the utmost importance and after experiments the following train was found to be the most efficient: main air supply, 5 lb./sq.in.; glass wool column; P. 16 'Aerox' filter; P. 27 'Aerox' filter in water; H_2SO_4 wash bottle; trap. The system was connected to a manifold with six outlets, these being connected by rubber tubing to pieces of pyrex glass tubing bent so that they could be hooked on to the neck of the flask, with the outlet about 2 in. from the bottom.

Reagents

Sulphuric acid. A.R. grade acid was distilled *in vacuo* over AgNO_3 to prevent volatilization of traces of FeCl_3 .

Nitric acid. A.R. grade acid was purified as above.

Perchloric acid. The 60% (w/v) acid was purified as above and collected as the constant boiling acid of 72% (w/v) strength.

Ammonium vanadate (0.25%). This (0.25 g.) was dissolved in 75 ml. of redistilled water with heating, cooled, a few drops of conc. NH_4OH solution added and the solution

diluted to 100 ml. The solution at first assumed a yellowish tint which soon faded.

Digestion mixture. The acids purified as above were mixed in the proportions by volume: H_2SO_4 (1), HClO_4 (1.5), HNO_3 (7.5). Vanadate solution (2.5 ml.) was added to 1 l. of this mixture, giving a concentration of c. 5 $\mu\text{g./ml.}$ The mixture was prepared immediately before use since it tended to extract iron from glass on standing.

Phenolphthalein (0.01%). The indicator (0.01 g.) was dissolved in 50 ml. of ethanol and the solution diluted to 100 ml. with redistilled water with constant stirring. It was filtered (Whatman no. 44 paper) before use.

Ammonium hydroxide (sp.gr. 0.880). A.R. grade material was satisfactory after filtration through a Whatman no. 44 paper.

Hydrochloric acid (2N). A.R. grade acid was purified by distillation. The constant boiling mixture obtained (20-22% at 760 mm.) was diluted to 2N (360 ml. diluted to 1 l.).

Hydroxylamine hydrochloride (10%). The hydrochloride (20 g.) was dissolved in 100 ml. distilled water together with 1 g. of $\text{Ca}_3(\text{PO}_4)_2$. The solution was adjusted to pH 8 with NH_4OH solution, warmed to flocculate the precipitate, then filtered through a Whatman no. 44 paper. The solution was then adjusted back to pH 2.5 with purified HCl and diluted to 200 ml.

o-Phenanthroline (0.25%). This was made up in 0.1N-HCl.

Acetate buffer, pH 4.6. Glacial acetic acid (50 ml.) was slowly neutralized with 10N-NaOH (phenolphthalein indicator), the flask being well cooled with running water during the reaction. A further 50 ml. of glacial acetic acid were added and the solution diluted to 500 ml. The reagent was purified by the extraction method of Waring & Werkmann (1943) as follows. To the solution in a pyrex separating funnel (with an ungreased tap) was added a solution of 10-20 mg. of 8-hydroxyquinoline in 5 ml. of redistilled metal-free chloroform, and the mixture shaken vigorously for 1 min., then rotated for 30 sec. to cause the droplets to coalesce and sink. The chloroform was drawn off and the procedure repeated until this layer had only a slight yellow tint, two to three times usually being sufficient. Finally, the solution was washed with 10 ml. portions of chloroform until the chloroform extract caused no change in the colour of two to three drops of 20% ferric alum solution after vigorous shaking. The purified solution was then warmed to expel excess chloroform and finally filtered through a Whatman no. 44 paper.

Ammonium citrate (50% w/v). The reagent was purified by the extraction procedure described above for the acetate buffer.

Sodium diethyldithiocarbamate (2%). This was dissolved in redistilled water with gentle heat, and filtered through a Whatman no. 44 paper each time before use to remove deposited sulphur, etc.

isoAmyl alcohol. A.R. grade primary isoamyl alcohol could be used after one distillation, but commercial 'amyl alcohol' could only be used after purification by two to three distillations. It was readily recovered after use by drying over CaO followed by distillation.

Standard iron solution. A solution containing 100 $\mu\text{g. Fe}^{+++}/\text{ml.}$ was prepared from A.R. $\text{NH}_4\text{Fe}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ (0.8636 g./l. 0.1N-HCl).

Standard copper solution. This solution contained 100 $\mu\text{g. Cu}^{++}/\text{ml.}$ (0.3928 g. of dry, recrystallized A.R. $\text{CuSO}_4 \cdot 5\text{H}_2\text{O/l.}$ 0.1N-HCl).

* BP=papain digest of beef muscle.

phenanthroline complex at 508 m μ . Using 2 cm. cells, the working range was 0–20 μ g. Fe, but could be extended to 40 μ g. with 1 cm. cells and to 80 μ g. with 0.5 cm. cells. Beer's law was obeyed and the molar extinction coefficient obtained under these conditions was 11,150.

After reading, the coloured solutions were quantitatively transferred to the glass-stoppered tubes using 5 ml. of wash water. The following reagents were then added with mixing to these solutions in the order given: ammonium citrate (1 ml.), concentrated ammonium hydroxide (1 ml.) and diethyldithiocarbamate (0.25 ml.). Exactly 10 ml. of amyl alcohol were then added and the tubes shaken for not less than 30 sec. in order to extract the copper complex completely. This colour was read after 15 min. under the same conditions as for iron, but using two Ilford 601 (spectrum violet) filters and an amyl alcohol blank. The colour obeyed Beer's law giving a molar extinction coefficient of 11,650. The ranges of working could be extended to 80 μ g. as with iron.

The transmission values obtained for both colours were converted to concentration of the respective metal from a standard curve which was prepared by taking a set of standards through the whole process, excluding ashing. A blank on all the reagents was carried out with each set of determinations.

RESULTS

Table 2 gives an indication of the accuracy of the method.

SUMMARY

1. A colorimetric method is described for the determination of the iron and copper (0–20 μ g.) of meat digests in the same sample. Organic matter is destroyed by rapid wet ashing with a mixture of sulphuric, perchloric and nitric acids, using vanadium as a catalyst. Special precautions against dust and reagent contamination are necessary.

2. A novel feature is the decomposition of the ferric pyrophosphate complex by heating the ash solution with phenanthroline and a reducing agent, maximum colour being developed in less than 30 sec.

3. Copper is determined as the diethyldithiocarbamate complex, after extraction with isoamyl alcohol.

4. Mean recoveries obtained are 98 % (mean deviation ± 1.5 %) for iron and 99 % (mean deviation ± 2.5 %) for copper.

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REFERENCES

- Bailey, L. F. & McHargue, J. S. (1945). *Plant Physiol.* **20**, 79.
 Bandemer, S. L. & Schaible, P. J. (1944). *Industr. Engng Chem. (Anal. ed.)*, **16**, 317.
 Benne, E. J. & Snyder, A. J. (1944). *J. Ass. off. agric. Chem., Wash.*, **27**, 526.
 Borei, H. (1943). *Biochem. Z.* **314**, 359.
 Braun, L. & Scheffer, L. (1940). *Biochem. Z.* **304**, 397.
 Cartwright, G. E., Jones, P. J. & Wintrobe, M. M. (1945). *J. biol. Chem.* **160**, 593.
 Dyer, W. J. & MacFarlane, W. D. (1938). *Canad. J. Res. B*, **16**, 91.
 Eden, A. & Green, H. H. (1940). *Biochem. J.* **34**, 1202.
 Fortune, W. B. & Mellon, M. G. (1938). *Industr. Engng Chem. (Anal. ed.)*, **10**, 60.
 Gerber, L., Claassen, R. I. & Boruff, C. S. (1942). *Industr. Engng Chem. (Anal. ed.)*, **14**, 364.
 Hill, R. (1930). *Proc. Roy. Soc. B*, **107**, 205.
 Hummel, F. C. & Willard, H. H. (1938). *Industr. Engng Chem. (Anal. ed.)*, **10**, 13.
 Jackson, S. H. (1938). *Industr. Engng Chem. (Anal. ed.)*, **10**, 302.
 Kitzes, G., Elvehjem, C. A. & Schuette, H. A. (1944). *J. biol. Chem.* **155**, 653.
 Klumpp, T. G. (1934). *J. biol. Chem.* **107**, 213.
 Koenig, R. A. & Johnson, C. R. (1942). *J. biol. Chem.* **143**, 159.
 Mehlig, J. P. & Hulett, H. R. (1942). *Industr. Engng Chem. (Anal. ed.)*, **14**, 869.
 Moss, M. L. & Mellon, M. G. (1942). *Industr. Engng Chem. (Anal. ed.)*, **14**, 862.
 Mueller, J. H. (1938). *J. Bact.* **36**, 499.
 Norlin, G. (1943). *Acta path. microbiol. Scand. Suppl.* **50**, 1.
 Parker, W. E. & Griffin, F. P. (1939). *Canad. J. Res. B*, **17**, 66.
 Saywell, L. G. & Cunningham, B. B. (1937). *Industr. Engng Chem. (Anal. ed.)*, **9**, 67.
 Schaefer, K. H. (1940). *Biochem. Z.* **304**, 417.
 Schulek, E. & Floderer, I. (1939). *Ber. ungar. pharm. Ges.* **15**, 210.
 Smith, G. F. (1946). *Industr. Engng Chem. (Anal. ed.)*, **18**, 257.
 Smith, G. F. & Richter, F. P. (1944). *Phenanthroline and Substituted Phenanthroline Indicators*, p. 54, 1st ed. Columbus, Ohio: G. F. Smith Chem. Co.
 Thorpe, R. H. (1941). *Biochem. J.* **35**, 672.
 Tompsett, S. L. (1934a). *Biochem. J.* **28**, 1544.
 Tompsett, S. L. (1934b). *Biochem. J.* **28**, 2044.
 Waring, W. S. & Werkmann, C. H. (1943). *Arch. Biochem.* **1**, 303.
 Woods, J. T. & Mellon, M. G. (1941). *Industr. Engng Chem. (Anal. ed.)*, **13**, 551.