be absent from the mitochondrial outer membrane.[2] It may be speculated that the outer mitochondrial membrane arises from endoplasmic membranes, which, after undergoing this specialization, lose some of their enzymatic functions; in fact, the three last-mentioned enzymes are well documented to be subject to vigorous control by substrates and hormones. Alternatively, the two types of membrane may arise from a common origin, after which each of them may acquire special additional enzymatic complements according to its physiological function.

Some of the enzyme distribution patterns described above are strikingly different from those recently reported by Green and associates[23] for beef heart mitochondria (cf. also this volume [72a]). These authors have concluded that all enzymes involved in the citric acid cycle and in fatty acid metabolism are associated with the outer mitochondrial membrane. It appears unlikely that there would exist such fundamental differences in intramitochondrial enzyme topography between two animal species or organs. More probably, these discrepancies reflect differences in the separation methods employed and/or in the criteria chosen for the morphological identification of the resulting subfractions.

[23] E. Bachmann, D. W. Allmann, and D. E. Green, *Arch. Biochem. Biophys.* **115**, 153 (1966); D. W. Allmann, E. Bachmann, and D. E. Green, *Arch. Biochem. Biophys.* **115**, 165 (1966); D. E. Green, E. Bachmann, D. W. Allmann, and J. F. Perdue, *Arch. Biochem. Biophys.* **115**, 172 (1966).

[73] Determination of Nonheme Iron, Total Iron, and Copper

By PHILIP E. BRUMBY and VINCENT MASSEY

Introduction

An extensive variety of methods has been described for the determination of iron and copper in biological materials,[1-3] some of which have been covered in a previous volume in this series.[4] However, most of them require amounts of these elements prohibitively large for the

[1] E. B. Sandell, "Colorimetric Determination of Traces of Metals," 3rd ed. Wiley (Interscience), New York, 1959.
[2] L. M. Melnick, *in* "Treatise on Analytical Chemistry" (I. M. Kolthoff and P. J. Elving, eds.), Vol. 2, Part II, Section A, p. 247. Wiley (Interscience), New York, 1962.
[3] W. C. Cooper, *in* "Treatise on Analytical Chemistry" (I. M. Kolthoff and P. J. Elving, eds.), Vol. 3, Part II, Section A, p. 1. Wiley (Interscience), New York, 1961.
[4] R. Ballentine and D. D. Burford, Vol. III, p. 1002.

analysis of a number of oxidizing enzymes or other samples available in very small quantities. For this reason, methods of analysis more appropriate for use with such small samples have been developed, and some of them are presented below. In addition to methods chosen for their sensitivity, others have been selected for use in determinations of the valency state of the elements in the sample.

As a good account of the general techniques and precautions used in colorimetric methods for metal analysis is already available,[1] this topic will not be considered here. It must be emphasized however, that a test for interference should always be made by adding copper or iron to the sample and evaluating its recovery, before the validity of any determination can be accepted. Also, the metal content should always be determined by reference to a calibration curve prepared under the same conditions. Values obtained by extraction techniques should be compared with those by a total digestion method for each type of sample when analyses are first undertaken.

Iron Determinations

Stable, strongly colored complexes of the hexacovalent type $Fe^{++} L_3$ are formed in a weakly acidic, neutral, or weakly alkaline medium between iron and 1,10-phenanthroline and various derivatives of this compound. The high absorbance of these complexes makes the phenanthrolines useful in the determination of trace amounts of iron. A number of elements, especially divalent metals, also form complexes and cause interference. Fortunately most of these elements, e.g., cadmium, silver, bismuth, are not likely to be present in biological samples. Interference is also caused by groups which form complexes with iron, e.g. phosphate and pyrophosphate. By careful control of pH and by adopting certain other precautions, many interferences may be minimized or eliminated[1,2,5,6] (see the table). Solutions of phenanthroline are stable for several weeks at 0°. They should be discarded if at all colored.

Nonheme Iron

Nonheme iron may be extracted quantitatively from most materials with trichloroacetic acid, e.g. NADH dehydrogenase,[7] succinic dehydrogenase,[8] and the liberated iron determined as described in method A. For materials from which all the nonheme iron is not extracted by tri-

[5] W. B. Fortune and M. G. Mellon, *Ind. Eng. Chem., Anal. Ed.* **10**, 60 (1938).
[6] G. F. Smith, W. H. McCurdy, and H. Diehl, *Analyst* **77**, 418 (1952).
[7] H. R. Mahler and D. G. Elowe, *J. Biol. Chem.* **210**, 165 (1954).
[8] V. Massey, *J. Biol. Chem.* **229**, 763 (1957).

INTERFERENCES IN THE DETERMINATION OF IRON BY 1,10-PHENANTHROLINE[a-d]

Element or ion	Tolerance limit for 2 ppm iron (ppm)	pH	Notes
Cd	50	—	Form slightly soluble complexes with 1,10-phe-
Zn	10	—	nanthroline and reduce the intensity of the
Hg (II)	1	—	color. Interference is diminished by using a
Hg (I)	10	3–9	larger excess of reagent
Be	50	3.0–5.5	Below pH 3.0 a stable complex is formed. Above pH 5.5 the hydroxide is precipitated
Mo (VI)	100	5.5–9.0	Produces turbidity below pH 5.5
W	5	—	Decreases color intensity
Cu	10	2.5–4.0	Interference reduced by using 4,7-diphenyl-1,10-phenanthroline
Ni	2	—	Produces change in color and increase in absorbance below 540 mμ
Co	10	3.0–5.0	Produces yellow color
Sn (II)	20	2.0–3.0	—
Sn (IV)	50	2.5	—
Zr	10	—	—
Cr	25	—	—
Mn (II)	200	—	—
Ag	—	—	Precipitates formed
Bi	—	—	—
Oxalate	500	6.0–9.0	—
Tartrate	500	3.0–9.0	—
Fluoride	500	4.0–9.0	—
Perchlorate	—	—	Forms precipitate if present in more than small amounts. This may be overcome by the addition of pyridine which forms a complex with perchlorate[e]
Pyrophosphate	50	6.0–9.0	Interference reduced by standing 1 hour or more
Phosphate	20	2.0–9.0	In the presence of aluminum with phosphate, iron is carried down with an aluminum phosphate precipitate. Addition of citrate after addition of 1,10-phenanthroline and reducing agent, and before pH adjustment overcomes this difficulty

[a] E. B. Sandell, "Colorimetric Determination of Traces of Metals," 3rd ed. Wiley (Interscience), New York, 1959.

[b] L. M. Melnick, in "Treatise on Analytical Chemistry" (I. M. Kolthoff and P. J. Elving, eds.), Vol. 2, Part II, Section A, p. 247. Wiley (Interscience), New York, 1962.

[c] W. B. Fortune and M. G. Mellon, Ind. Eng. Chem., Anal. Ed. 10, 60 (1938).

[d] G. F. Smith, W. H. McCurdy, and H. Diehl, Analyst 77, 418 (1952).

[e] B. F. Cameron, A Comparative Study of Hemoglobin M, Ph.D. Thesis, University of Pennsylvania, Philadelphia, 1962.

chloroacetic acid, ethanol after dithionite may be used (method B). By carrying out the color reaction in the presence and the absence of a reducing agent, method A may be used to determine the valency state of the iron in the trichloroacetic acid extract. It should be noted, however, that liberation of protein groups during the extraction process may cause changes in the oxidation state of the iron, so that the valency in the extract may be quite different from that in the sample. For example, hydrogen sulfide produced upon acidification of nonheme iron proteins containing inorganic or labile sulfide[8,9] will reduce iron to the ferrous state. This may be overcome by treatment of the sample with trichloro-acetic acid containing a mercurial. The concentration of mercurial required may vary from sample to sample and should be ascertained by using a range of conditions. A concentration of $0.05 M$ p-chloromercuriphenylsulfonic acid in the trichloroacetic acid precipitant is often satisfactory.

Method A (20–300 millimicromoles)

The method described is that presented by Massey.[8]

Reagents

Trichloroacetic acid, 20% (w/v) $\pm$ $0.05 M$ p-chloromercuriphenyl-sulfonic acid
1,10-Phenanthroline, 0.1% (w/v)
Ascorbic acid, $0.06 N$
Acetic acid, $0.06 N$
Ammonium acetate, saturated solution
Standard iron solution, $1.78 \times 10^{-2} M$: 1.0 g of electrolytic Fe or Fe wire is dissolved in 50 ml 1:3 HNO_3, boiled to expel oxides of nitrogen, and diluted to 1 liter. This solution is diluted to 10^{-3} to $10^{-5} M$ with 5% (w/v) trichloroacetic acid.

Procedure. Into a centrifuge tube, pipette 1.5 ml of sample containing 20–300 millimicromoles of iron and 0.5 ml of 20% trichloroacetic acid. Mix and allow to stand 10 minutes at room temperature, then centrifuge at 5000 g for 10 minutes. Withdraw the supernatant and pipette 0.4-ml aliquots of this extract or standard iron solution into 1-ml cuvettes containing 0.36 ml of water and 0.15 ml of 1,10-phenanthroline, or 0.51 ml of water (sample blank). For estimation of ferrous iron only, add 0.05 ml of acetic acid or for ferrous + ferric iron, 0.05 ml of ascorbic acid. Finally add 0.04 ml of saturated ammonium acetate, mix, and read the absorbance at 510 mμ against distilled water.

[9] R. W. Miller and V. Massey, *J. Biol. Chem.* **240**, 1453 (1965).

A modification of this method which gives better iron recoveries under some conditions has been described.[9] The reagents are added directly to the sample in trichloroacetic acid, the colored complex is extracted into n-amyl alcohol, and the absorbance is read at 510 mμ against n-amyl alcohol.

Method B (2–30 millimicromoles)

This method was developed by Doeg and Ziegler[10] for estimation of iron in mitochondrial preparations.

Reagents

Sodium dithionite, 0.2% (w/v) prepared immediately before use by dissolving 20 mg of sodium dithionite in 10 ml of water through which pure N_2 is bubbled vigorously before and during solution

Ethanol, 95%

4,7-Diphenyl-1,10-phenanthroline, 0.2% (w/v) in 95% ethanol

Sodium acetate, 1 M, pH 4.6, prepared by adjusting 1 M sodium acetate to pH 4.6 with N acetic acid and made iron free by repeated extraction with 4,7-diphenyl-1,10-phenanthroline (0.083%, w/v, in isoamyl alcohol) in the presence of thioglycolic acid (1.0 ml per liter) and washing afterward with several portions of isoamyl alcohol

Standard iron solution. The solution prepared as above may be used, diluting to 10^{-3} to 10^{-5} M with water.

Procedure. Pipette 0.1-ml aliquots of sample or standard containing 2–30 millimicromoles of nonheme iron into stoppered test tubes. Add 0.1 ml dithionite solution and 0.7 ml 95% ethanol. Stopper the tubes and mix vigorously on a mechanical vortex shaker. Add 0.05 ml of diphenyl-phenanthroline or 0.05 ml of 95% ethanol (sample blank) and 0.05 ml of sodium acetate, pH 4.6. Stopper the tubes and mix vigorously once again, then place in a 38° water bath for 5 minutes to permit maximum color development. Centrifuge the tubes, and read the absorbance of the supernatant at 535 mμ against a reagent blank.

Total Iron

For the determination of total iron in biological samples, the material for analysis is most commonly subjected to an acid digestion prior to carrying out the color reaction. A number of different methods for di-

[10] K. A. Doeg and D. M. Ziegler, *Arch. Biochem. Biophys.* **97**, 37 (1962).

gesting the sample and developing the color are described (methods A, B, and C). A method is also described for the determination of total iron using an extraction procedure in place of the acid digestion (method D).

Method A (5–50 millimicromoles)

This method, developed by Beinert,[11] employs wet oxidation of the sample by nitric acid followed by evaporation to dryness. The absence of chloride in samples to be ashed by this procedure is desirable since dry heating in the presence of chloride results in loss of iron by volatilization. The dissolved digest is reduced with ascorbic acid and the color is developed with 1,10-phenanthroline.

Reagents

Nitric acid, concentrated or redistilled
Hydrochloric acid, 2 N
Ammonium acetate, saturated solution
Ammonia, 12.6% (w/v), concentrated ammonia diluted with equal volume of water
Ascorbic acid, 1% (w/v), prepared freshly for each determination
1,10-Phenanthroline, 0.1% (w/v)
Standard iron solution, prepared as above, diluting to 10^{-3} to $10^{-5} M$ with water

Procedure. Pipette sample or standard containing 5–50 millimicromoles of iron into a 150 × 17 mm Pyrex test tube (previously cleaned with boiling HCl and glass-distilled water), add 0.2 ml concentrated HNO_3, and evaporate the contents to dryness taking care that none of the sample is lost by spattering. As soon as drying is complete, cool the tube, and add another 0.2 ml portion of nitric acid. Repeat the digestion until the dried residue is completely colorless. Cool the tube and add 0.2 ml of 2 N HCl. Dissolve the residue very thoroughly by gently warming and swirling the tube. Low recoveries result if this operation is inadequate, but care must be taken to prevent evaporation of water. Add 0.65 ml of water, warm, and swirl again, then add 0.15 ml of 1,10-phenanthroline, 0.05 ml of ascorbic acid, 0.02 ml of saturated ammonium acetate, and 0.03 ml of ammonia. Mix the contents well and read the absorbance at 510 mμ against a reagent blank. In the presence of certain ions such as phosphate and pyrophosphate, reading of the absorbance may be delayed several hours until the net difference between the blank and the sample no longer increases.

[11] H. Beinert, personal communications, 1965.

Method B (50–500 millimicromoles)

The following method is based on that of Cameron[12] with modification by Smith.[13] The sample is ashed with perchloric acid and hydrogen peroxide at 100°, excess perchlorate is complexed with pyridine and the iron is reduced with sodium dithionite for the color reaction with 1,10-phenanthroline.

Reagents

Perchloric acid, 60% (w/w)
Hydrogen peroxide, 30%
Pyridine
Sodium dithionite. The solution prepared as described above may be used.
Standard iron solution, prepared as above diluting to 10^{-3} to 10^{-5} M with water
1,10-Phenanthroline, 0.2% (w/v)

Procedure. Weigh out or pipette the sample containing 50–500 millimicromoles of iron into a 10-ml Pyrex volumetric flask. Concentrate if necessary to a volume of about 0.1 ml. Add 0.2 ml of 60% perchloric acid and immerse in a boiling water bath, adding hydrogen peroxide dropwise as necessary until the digest is completely colorless. When this occurs, heat for 30 minutes more to effect complete breakdown of hydrogen peroxide, then cool to 40–50° (15–20 minutes at room temperature), add 0.5 ml of pyridine, 1.0 ml of sodium dithionite, and a further 0.5 ml of pyridine, mixing after each addition. Add 1.0 ml of 0.2% phenanthroline and make up to 10 ml with water. Read the absorbance at 510 mμ against a reagent blank.

Method C (2–30 millimicromoles)

The following method is a modification of that of Peterson.[14] The sample is ashed with sulfuric, nitric, and perchloric acids and the iron is reduced with thioglycolic acid, complexed with 4,7-diphenyl-1,10-phenanthroline, and extracted into isoamyl alcohol.

Reagents

Sulfuric acid, concentrated
Nitric acid, concentrated or redistilled

[12] B. F. Cameron, A Comparative Study of Hemoglobin M, Ph.D. Thesis, University of Pennsylvania, Philadelphia, 1962.
[13] M. H. Smith, personal communication, 1965.
[14] R. E. Peterson, *Anal. Chem.* **25,** 1337 (1953).

Perchloric acid, 60% (w/w)

Thioglycolic acid, 1% (v/v)

Sodium acetate, saturated solution adjusted to pH 6.0 with glacial acetic acid, and made iron-free as above

4,7-Diphenyl-1,10-phenanthroline, 0.083% (w/v) in isoamyl alcohol

Standard iron solution, prepared as above diluting to 10^{-3} to 10^{-5} M with water

Procedure. Pipette samples or standards containing 2–30 millimicro-moles of iron into 150 × 17 mm stoppered Pyrex test tubes (previously cleaned by mock ashing when first used, or by boiling in HCl for reuse, finally flushing with water). Add 0.1 ml of concentrated H_2SO_4 and 0.1 ml of concentrated HNO_3 and heat to fumes of sulfuric acid. Cool, add another 0.1-ml portion of HNO_3, and heat as above. Cool again and add 0.05 ml of 60% perchloric acid and heat (without boiling) for 3–5 minutes. Cool and add 0.5 ml of water, 0.25 ml of 1% thioglycolic acid, 1.5 ml of saturated sodium acetate, and 1.0 ml of 4,7-diphenyl-1,10-phenanthroline. Stopper the tubes and shake on a mechanical vortex mixer for 3 minutes, then centrifuge to separate the layers. Transfer the upper layers to cuvettes and read the absorbances at 535 mμ against a reagent blank. Between readings cuvettes may be rinsed with 100% ethanol, then air dried.

Method D (2–30 millimicromoles)

This method was developed by Doeg and Ziegler[10] for estimating iron in mitochondria. Iron is released into solution by treatment of the sample with thioglycolic and acetic acids and extracted as the complex with 4,7-diphenyl-1,10-phenanthroline into isoamyl alcohol.

Reagents

Thioglycolic acid, 5% (v/v)

Acetic acid, glacial

Sodium acetate, saturated pH 6.0, prepared and made iron free as above

4,7-Diphenyl-1,10-phenanthroline, 0.083% (w/v) in isoamyl alcohol

Standard iron solution, prepared as above diluting to 10^{-3} to 10^{-5} M with water

Procedure. Pipette 0.1-ml portions of the sample or standard con-taining 2–30 millimicromoles of iron into stoppered test tubes, and add

0.1 ml of 5% thioglycolic acid and 0.2 ml of glacial acetic acid. Stopper the tubes and agitate vigorously on a mechanical vortex mixer for several minutes. Recovery is low if mixing is inadequate. Add 0.28 ml saturated sodium acetate, 0.32 ml water and 1.0 ml diphenylphenanthroline (or in the case of the sample blank, 1.0 ml isoamyl alcohol). Stopper the tubes, agitate them vigorously as above, centrifuge to separate the phases, transfer the upper layers to cuvettes, and read the absorbances at 535 mμ against isoamyl alcohol.

Copper Determinations

There are a number of reagents which form more or less specific colored complexes with copper, so that interference can usually be overcome by selection from the methods of determination available. This is especially so for biological samples which are generally free from interfering elements, and preliminary separations are seldom required. If a separation is unavoidable, Sandell[1] and Cooper[3] may be consulted for information about useful techniques.

As for iron determinations, the color reaction with copper is usually preceded by digestion of the sample (methods A and B). An extraction method has also been described and may be more convenient in some cases. It has also been used to determine the valency state of the copper in the sample.[15, 16]

Method A (5–50 millimicromoles)

This method is a micro adaptation of that of Martens and Githens[17] and uses zinc dibenzyldithiocarbamate for complexing with the copper. This reagent is superior to the more commonly used sodium diethyldithiocarbamate since the copper complex is more light stable and the extraction may be made from a digest of higher acidity. This feature reduces inteference by ferric iron, nickel, and cobalt. Antimony, bismuth, mercury, and silver combine with the reagent and inhibit copper extraction.

Reagents

> Sulfuric acid, concentrated
> Nitric acid, concentrated or redistilled
> Hydrogen peroxide, 30%
> Zinc dibenzyldithiocarbamate, 0.01% (w/v) in reagent grade carbon tetrachloride

[15] G. Felsenfeld, *Arch. Biochem. Biophys.* **87**, 247 (1960).
[16] D. E. Griffiths and D. C. Wharton, *J. Biol. Chem.* **236**, 1850 (1961).
[17] R. I. Martens and R. E. Githens, Sr., *Anal. Chem.* **24**, 991 (1952).

Copper standard, $10^{-3} M$. Dissolve 0.2497 g of clear uneffloresced crystals of $CuSO_4 \cdot 5 H_2O$ in water, add 100 ml of N HCl, and make up to 1 liter with water. When required dilute to 10^{-4} to $10^{-6} M$ with 0.1 N HCl.

Procedure. Weigh out or pipette sample and standards containing 5–50 millimicromoles of copper into stoppered 150×17 mm Pyrex tubes (previously cleaned by mock ashing, washing with dibenzyldithiocarbamate solution, and rinsing with CCl_4). Add 0.1 ml of concentrated sulfuric acid and heat carefully until charring begins. Cool, add 0.1 ml of concentrated nitric acid and continue heating, making further additions of nitric acid as charring recurs, until upon heating to fumes of sulfuric acid no charring takes place. Cool and add 1 drop of hydrogen peroxide and heat again to fumes of sulfuric acid, repeating this process until the digest is colorless. Cool again, add 2 ml of water and boil to fumes of sulfuric acid. Cool and dilute with 3.5 ml of water. Add 1.0 ml of dibenzyldithiocarbamate, stopper the tube, and agitate vigorously on a vortex shaker for 3 minutes. Centrifuge to separate the layers, withdraw the lower layer with a fine-tipped pipette into a 1-ml cuvette, and read the absorbance at 435 mμ against a reagent blank.

A modification of this method in which copper is extracted by boiling the sample in N sulfuric acid instead of ashing has also been described.[18]

Method B (1–10 millimicromoles)

In spite of its high color and poor stability, dithizone is frequently used in copper determinations because of its extreme sensitivity. It also has the advantage of complex formation in dilute mineral acid. Silver, gold, palladium, bismuth, mercury, and iron interfere, but nickel, cobalt, lead, zinc, and cadmium are without effect unless present in high concentrations. If interference is encountered, preliminary extraction of the digest with dithizone in carbon tetrachloride must be made. The organic layer containing the copper is then washed twice for 2–3 minutes with an equal volume of 0.1 N HCl with bromide added for removal of mercury, or iodide for silver and bismuth. The organic solvent is then evaporated, and the residue is redigested.[1] The procedure described below was developed by Beinert.[11]

Reagents

Sulfuric acid, concentrated
Hydrogen peroxide, 30%
Dithizone 0.001–0.0012% (w/v) in carbon tetrachloride. The latter

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[18] I. Stone, R. Ettinger, and C. Gantz, *Anal. Chem.* **25**, 893 (1953).

must be reagent grade and have passed the dithizone test. The 0.001% solution is advantageously prepared immediately before use by diluting a 0.01% (w/v) solution. The concentrated reagent is quite stable if kept cold and dark.

Sulfuric acid, 0.05 N

Standard copper solution, $10^{-3} M$, prepared and diluted as above

Procedure. Use pipettes and tubes cleaned in hot aqua regia, washed with water followed by dithizone, then rinsed thoroughly with carbon tetrachloride, and finally air dried. Pipette samples containing about 1–10 millimicromoles of copper into 150 × 15 mm stoppered Pyrex tubes. Add 0.1 ml of concentrated H_2SO_4 and evaporate to fumes of sulfuric acid, taking care to avoid excessive foaming. Cool, add 1 drop of hydrogen peroxide, heat to fumes of sulfuric acid again and reflux until the walls of the tubes are free from particles. Repeat this process until, after refluxing, the digest is clear and colorless. Heat more strongly and drive off all the sulfuric acid. As soon as this is complete, cool the tubes and add 5 ml 0.05 N H_2SO_4. Because the dithizone complex is unstable, it is essential to exclude light as much as possible and process each sample quickly (if necessary, independently). Add 1.0 ml of 0.001% (w/v) dithizone solution, stopper the tube, and agitate vigorously on a vortex mixer for 3 minutes. Centrifuge to separate the layers, withdraw the lower layer with a fine-tipped pipette, and place in a 1-ml cuvette. Read the absorbance at 510 mμ against carbon tetrachloride.

Method C (10–50 millimicromoles)

A method has been devised for extraction and estimation of copper with glacial acetic acid and biquinoline. Since the biquinoline forms the colored complex only with cuprous copper, the method may be useful for estimating the valency state of the copper in the native material, provided certain precautions are taken, by carrying out the determination in the presence and absence of a reducing agent.[15, 16] The determination is remarkably free from interference; only cyanide, thiocyanate, and oxalate interfere appreciably and must be absent. The complex is stable for several days. The method presented is taken from those of Felsenfeld,[15] Griffiths and Wharton,[16] and Fowler *et al.*[19]

Reagents

2,2'-Biquinoline, 0.1% (w/v) in glacial acetic acid

Hydroxylamine hydrochloride, 10% (w/v)

[19] L. R. Fowler, S. H. Richardson, and Y. Hatefi, *Biochim. Biophys. Acta* **64**, 170 (1962).

1-Hexanol

Standard copper solution, $10^{-3}\,M$. Prepared as previously described, but diluting to 10^{-4} to $10^{-6}\,M$ with the medium in which the biological sample is dissolved.

Procedure. If the valency of the copper in the sample is to be determined, cupric ions must be protected from reduction by sulfhydryl groups in the sample by the additions of ethylenediaminetetraacetic acid, disodium salt, to $0.02\,M$. Alternatively, the sulfhydryl groups may be blocked by titration with *p*-chloromercuribenzoate before the analysis is made.[15] Pipette 1 ml of the sample or standard containing 10–50 millimicromoles of copper into stoppered Pyrex test tubes and add 1.0 ml of biquinoline reagent, or 1.0 ml of glacial acetic acid (for sample blank). For total copper add also 0.05 ml of hydroxylamine hydrochloride, or for cuprous copper only, 0.05 ml of water. Mix, allow to stand 5 minutes, then add 2 ml of 1-hexanol. Stopper the tubes and agitate vigorously on a vortex shaker for 1 minute. Separate the phases by low speed centrifugation, then transfer a portion of the organic phase to a cuvette and read the absorbance at 540 mμ against 1-hexanol.

A modification of the method not involving extraction of the complex into an organic solvent has also been used.[16] Ethanol, 0.95 ml, is added in place of the hexanol and the absorbance is read at 535 mμ.

[74] The Fluorometric Determination of Mitochondrial Adenine and Pyridine Nucleotides

By RONALD W. ESTABROOK, JOHN R. WILLIAMSON, RENE FRENKEL, and PABITRA K. MAITRA[1]

Spectrophotometric methods have been developed[1a] to measure the extent of reduction of the cytochromes or pyridine nucleotides of mitochondria during reactions of oxidative phosphorylation. Such methods, however, cannot distinguish between DPNH and TPNH, nor can they evaluate modifications in the intramitochondrial balance of ATP, ADP, and AMP. Determination of the concentrations of oxidized and reduced diphospho- and triphosphopyridine nucleotides, as well as adenine nucleotides, can only be accomplished by rapidly terminating mitochondrial reactions with acid or alkali followed by extraction and assay of the various forms of the nucleotides.

[1a] B. Chance, see Vol. IV, p. 273.