Guide to diagnosis of inborn errors of metabolism in district general hospitals

Clinical and laboratory awareness of inherited metabolic disorders has increased due to advances in diagnostic tests and treatment. In the United Kingdom the only inherited disorders which are routinely screened for in the neonatal period are phenylketonuria and congenital hypothyroidism. For the rest, correct diagnosis depends firstly on the appropriate tests(s) being requested, and secondly, on the analysis being carried out reliably and with correct interpretation and action. The laboratory has a responsibility to both patient and clinician to ensure that this second step is provided.

Because there are large numbers of different, although rare disorders, paediatricians in district general hospitals have little experience of them and may require advice on which tests to request. It is in this respect that the local laboratory is so important because of the way in which it can guide investigations. If the simpler more easily available tests are done first useful clues are often given as to which of the more specialist (and often more expensive) investigations would then be most appropriate.

Each laboratory must decide if particular tests are to be carried out locally or referred to a specialist laboratory. This will vary, depending on available manpower and expertise and workload, proximity and ease of transport to a more specialist laboratory. Another important consideration is the volume of work; laboratories with small workloads are not likely to have gained the necessary experience of these "rare" disorders.

It is also important to remember that some tests must be instantly available and often out of normal laboratory hours. In the case of an acutely sick baby or child first-line investigations should be completed within 24 hours.

The most important role for the local laboratory is good communication with the clinical team and specialist laboratories; without this the latter may waste time and effort, not to mention money on inappropriate tests, added to which the diagnosis may be missed.

Definitive diagnoses of inborn errors of metabolism are vital if families are to benefit from the advancing technology of molecular biology, and in particular, if they are to take advantage of new treatments and earlier prenatal diagnosis by chorionic villus sampling.

Clinical presentation(s) and investigation

The clinical presentation of these disorders is variable and diagnosis is not easy because most of the presentations are non-specific and the disorders are rare. Most babies with inborn errors of metabolism seem to be normal at birth. Symptoms that may develop during the first week of life include lethargy, poor feeding, vomiting, hypotonia, excessive weight loss and tachypnoea. Severe hypotonia is particularly suggestive of non-ketotic hyperglycinaemia or a peroxisomal disorder, especially if associated with liver dysfunction. Hyperventilation secondary to an unexplained metabolic acidosis suggests an organic acid disorder: unheralded and early onset of fits may be the first sign. The onset of symptoms in relation to feeding is relevant. Dysmorphic features, although more suggestive of a chromosome abnormality, are, however, characteristic of some inborn errors. To decide on the most appropriate investigations it is useful to consider the clinical presentation under the following categories, depending on whether it is acute or chronic.

ACUTELY ILL NEONATES OR INFANTS

The possibility of an inborn error in a neonate or infant with an unexplained acute illness should always be considered, particularly if there are previous unexplained deaths or illnesses in the family. Such an event may occur in the neonatal period or after several months of apparently healthy childhood. It is essential to investigate patients with a "Reye-like" encephalopathy as amino acid, urea cycle, organic acid or fatty acid oxidation defects can present in this way.

Consanguinity should increase the index of suspicion and a history of "male deaths" in maternal relatives suggests an X-linked disorder. In many cases there is rapid deterioration, and treatment must be started quickly. Even if there is no effective treatment it is important to make a diagnosis to enable genetic

counselling and possible prenatal diagnosis.

Clinical signs which can be important clues in the acute situation include: abnormal smell—sweet, sweaty, cabbage-like (amino acid organic acid disorders); cataracts with jaundice (galactosaemia); neurological depression with respiratory alkalosis (urea cycle disorders).

It is particularly important to remember that diagnosis of an infection does *not* preclude an inherited metabolic disorder; *Escherichia coli* septicaemia is often associated with classic galactosaemia.

For further details on inherited metabolic disorders which present in the neonatal period and the more common clinical presentations, the reader is referred to more comprehensive texts.¹⁻³

Whenever possible, investigations should proceed in a logical manner; the initial tests as detailed below should be carried out before proceeding to specific tests for metabolic disorders. It is, however, essential to collect both blood and urine specimens *during an attack* and store for further investigation. If, however, the baby deteriorates rapidly and is likely to succumb (or has already died) before results of these initial tests are available, more rigorous sampling should proceed (see Appendix: guide for emergency specimen collection).

Initial biochemical investigations

The following investigations should be undertaken locally before proceeding to more specific metabolic tests.

Urine:

Reducing substances (Clinitest or Benedict's test)

Glucose (Clinistix, or BM test strip)

Ketones (Acetest, Ketostix or Boehringer Mannheim test strip)

Blood:

Hydrogen ion, Pco₂, bicarbonate, base excess

Sodium, potassium, chloride (calculate Anion gap):

$(\text{Na} + \text{K}) - (\text{HCO}_3 + \text{Cl}) = (\text{normally less than } 20 \text{ mmol/l})$

Glucose (fasting, if hypoglycaemia not a presenting feature)

Calcium, magnesium

Bilirubin, alkaline phosphatase, alanine amino transferase or aspartate amino transferase

Creatinine (or urea)

Further investigations should then proceed according to the clinical problems and biochemical abnormalities identified. These can be categorised into five groups (table 1). The investigations listed are those which should be readily available and would either be diagnostic or would provide a useful guide to other investigations. Primary disorders of the immune sys-

tem, gastrointestinal tract, and haemolytic disorders have not been included.

If more than one of the problems listed (table 1) is present then a metabolic disorder is more likely.

Specimens for more specific investigations (such as enzymes) usually need not be taken until results of the investigations suggested are available (table 1)—the exception is that if the child deteriorates rapidly or dies (see Appendix) or unless a particular disorder is suspected on clinical grounds or family history, in which case investigations should be discussed with workers in a specialist laboratory before taking any specimens.

NON-ACUTE PRESENTATION

Investigation of a patient with a chronic problem is usually more difficult as clinical presentation may be even more variable and many of the investigations more complex. A brief guide to the initial biochemical investigation of patients with these types of chronic presentations is provided below:

Liver disease

Metabolic disorders which may present in this way include: galactosaemia; tyrosinaemia (type I); fructosaemia/hereditary fructose intolerance; Wilson's disease; α -1-antitrypsin deficiency; glycogen storage disorders; Niemann Pick type C; cystic fibrosis.

Biochemical investigations which should be considered include: *fasting* glucose (plasma); amino acids (urine and plasma); sugars (urine); α -1-antitrypsin (plasma); α -fetoprotein (plasma); urate (plasma); cholesterol (plasma); lactate (plasma); sweat test. If neonate: galactose-1-phosphate uridyl transferase (blood). If more than 5 years old: copper and caeruloplasmin (plasma).

Unexplained failure to thrive

Disorders of amino acid, urea cycle, and organic acid metabolism can present in this way. Investigations which should be considered are amino acids (urine and plasma), plasma ammonia, and urine organic acids. Decision to investigate will be influenced by family history and presence of any suggestive clinical or metabolic abnormalities.

Mental retardation—with no specific signs

Decision to investigate patients in this group for metabolic disorders will be influenced by family history and results of other investigations. The reader is referred to other discussions on this topic.^{4,5}

Neurological degenerative disorders

These include the lysosomal storage and peroxisomal disorders. Most patients present non-acutely with neurological dysfunction, often with associated liver

Table 1 Guide to further investigations in acutely ill neonates or infants

Presentation	Possible metabolic disorders	Suggested investigations
<i>Unexplained hypoglycaemia:</i>	Organic acid disorders Amino acid disorders Glycogen storage disease (type I) Disorders of gluconeogenesis Congenital adrenal hyperplasia	Organic acids (U) Amino acids (U, P) Lactate (P) Insulin (P) Cortisol (P) 17-hydroxyprogesterone (P)
<i>Acid base imbalance:</i> —metabolic acidosis exclude primary cardiac and respiratory disorders —respiratory alkalosis	Organic acid disorders Congenital lactic acidosis Urea cycle disorders	Organic acids (U) Lactate (P) Amino acids (U, P) Ammonia (P), Orotic acid (U), Amino acids (U, P)
<i>Liver dysfunction:</i> often associated with hypoglycaemia and galactosuria	Galactosaemia Fructose 1-6, diphosphatase deficiency Fructose intolerance Tyrosinaemia (type) Glycogen storage (type I) Disorders of gluconeogenesis	Galactosaemia screen (B) Sugars (U) Amino acids (U, P) Succinyl acetone (U) Alpha-fetoprotein (P) Lactate (P) Oligosaccharides (U) Organic acids (U) Alpha-1-antitrypsin (P)
<i>Neurological dysfunction:</i> —seizures —depressed consciousness —hypotonia	Non-ketotic hyperglycinaemia Glyceric acidaemia Urea cycle disorder Xanthine/sulphite oxidase deficiency	Amino acids (U, P) Organic acids (U) Orotic acid (U) Ammonia (P) Urate (P, U) Sulphite (U) Lactate (P)
<i>Cardiomyopathy:</i>	Glycogen storage type II (Pompe's) Fatty acid oxidation disorders Tyrosinaemia (type I)	Lactate (P) Oligosaccharides (U) Organic acids (U) Carnitine (P) Amino acids (U, P)

U = urine; B = whole blood; P = plasma

disease, eye abnormalities, and sometimes dysmorphic features.

Other non-biochemical investigations, especially radiological and testing for chromosomal abnormalities are important in this group. Although the diagnosis of these disorders is usually outside the scope of the district general hospital laboratory, investigations which should be considered are: amino acids (plasma and urine); vacuolated lymphocytes on peripheral blood film, oligosaccharides/sialic acid (urine); glycosaminoglycans (urine); organic acids (urine); bile acids (urine).

For all types of chronic presentation, specific leucocyte or tissue enzymes may be indicated either from the results of these initial tests or on clinical presentation alone. Before embarking on these, detailed discussion with the clinician and specialist biochemist is required.

Laboratory methods for district general hospitals

SPECIMEN REQUIREMENTS

Appropriate specimens are crucial if diagnoses are not to be missed. The following information should be provided with all specimens:

- 1 Details of feeding regimen *at the time of and before sampling* (especially if amino acids, organic acids, or sugars are requested).
- 2 Details of all drugs or other treatment (including blood transfusion).
- 3 Full clinical details (including information on any previous sibs and results of any preliminary investigations). Indication of the degree of urgency is required.

The acute attack

In some cases biochemical "markers" of the disease may be detected only during an acute attack. Therefore, the diagnosis can be missed if specimens are not obtained at that time. The following specimens should be collected as a minimum. If the child is severely ill, more rigorous specimen collection is indicated (see Appendix, guide for emergency specimen collection).

- 1 *Urine:* 5 ml (smaller quantities are useful if there is difficulty). Store deep frozen (-20°C or lower) immediately with no preservative.
- 2 *Blood:* Collect at least 1 ml blood (preferably 5 ml) into heparin if possible before the child is given glucose or saline or any other treatment. Store plasma deep frozen (-20°C or lower) and red cells

at +4°C. If there is sufficient specimen deproteinise 1 ml whole blood with 1 ml 1M perchloric acid and store supernatant deep frozen.

As most investigations cannot be done locally it is important that contact with a specialist laboratory is made at the earliest opportunity. For details on availability of and specimen requirements for specialist investigations, the reader is referred to *UK Directory of laboratories diagnosing inborn errors of metabolism*.⁶

METHODS

As a minimum, every laboratory supporting neonatal and paediatric patients should have the following investigations available: urine screening tests for reducing substances, glucose, and ketones; plasma (or whole blood) lactate; plasma ammonia (quantitative). The following may also be appropriate for some laboratories: plasma amino acids (qualitative); urine amino acids (qualitative); urine sugars (TLC); urine methylmalonic acid (TLC).

Chromatography for methylmalonic acid is not a substitute for a full organic acid profile by gas chromatography-mass spectrometry. As the methylmalonic acidemias are one of the commoner groups of organic acid disorders, however, it can be useful to include this as an extension to amino acid chromatography.

A decision to provide these optional tests must be a local one, depending on expertise, workload, and availability of assays being provided elsewhere. Where speed is essential, expert technique and interpretation is necessary and local laboratories with insufficient experience should not be tempted to provide these additional investigations.

Tests outside the remit of most district general hospital laboratories are: urinary organic acids (gas chromatography-mass spectrometry); urinary glycosaminoglycans (mucopolysaccharides—electrophoresis and quantitation); urinary oligosaccharides (chromatography). Although the techniques may not be particularly difficult and equipment may already be available, interpretation is difficult. Sufficient expertise is unlikely to be available in a local laboratory and it is therefore inappropriate that these tests be carried out.

URINE SCREENING TESTS

Most of these tests are non-specific but a positive result may focus attention on a particular area for investigation (table 2). For more details the reader is referred to *Selected screening tests for genetic metabolic diseases*.⁷

pH and nitrite

The quality of urine specimens can be assessed by checking the pH and using the "nitrite" pad on N-

Table 2 Some causes of positive urine screening tests

Metabolic disorder/cause	Abnormal colour
Ferric chloride:	
Phenylketonuria (Phenylpyruvic acid)	Dark green
Tyrosinaemia	Light green
Maple syrup urine disease	Grey
Histidinaemia (imidazole pyruvic acid)	Blue-green
Alcaptonuria (homogentisic acid)	Green/brown
Azide	Red
Salicylate	Purple
Bilirubin	Green
Acetoacetate	Red
Cyanide nitroprusside:	
Cystinuria	
Homocystinuria	
Beta-mercaptolactate cysteine disulphiduria	
Concentrated urine	
Dinitrophenylhydrazine:	
Maple syrup urine disease	
Methioninaemia	
Valproic acid treatment	

labstix. An alkaline pH (greater than 7.0) suggests bacterial contamination. The nitrite test will be positive only if the contaminating bacteria are "nitrite producers".

Ferric chloride test

This comprises 1 g ferric chloride and 1 g ferrous ammonium sulphate ($\text{Fe}(\text{NH}_4)_2 (\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$) in 100 ml of 0.02 M hydrochloric acid.

Add 10 drops of urine to 1 ml of ferric chloride reagent. Ferric chloride gives a characteristic green colour with phenylpyruvic acid and suggests a raised phenylalanine. It is useful for the detection of missed or maternal phenylketonuria. It is unreliable with alkaline urine specimens and may also be subject to interference from drugs and phosphates.

Cyanide/nitroprusside (CNP) test for cystine/homocystine

To 1 ml urine add 2 drops concentrated ammonia solution followed by 0.5 ml of 10% (w/v) sodium cyanide (care needed). Leave for at least five minutes and then add 5 drops of 5% (w/v) sodium nitroprusside. Positive samples give a distinct magenta colour. It is advisable to run a positive control in parallel. If an authentic urine is not available a normal urine spiked with cystine (0.3 mmol/l) is suitable; aliquots can be stored at -20°C. Any compound that contains sulphhydryl groups or yields sulphhydryl groups on reduction will give a positive reaction. This test is sensitive to 0.25 mmol/l cystine and 0.25 mmol/l homocystine. Concentrated normal urine samples (creatinine greater than 7 mmol/l) may give a misleading positive result.

Dinitrophenylhydrazine (DNPH) test for keto compounds

The reagent comprises 0.7 g 2,4-dinitrophenylhydrazine in 250 ml 1 M hydrochloric acid. Warm to dissolve. When cool, filter reagent and store at room temperature. Cloudy urine samples should be centrifuged before analysis. Mix an equal volume of urine and DNPH reagent. Observe after five minutes. DNPH forms insoluble hydrazones with keto compounds (such as α -ketoisocaproic acid) which result in the formation of a heavy yellow-white precipitate. The presence of these keto compounds may indicate an underlying metabolic defect such as maple syrup urine disease. Patients receiving valproic acid may give a positive result.

Other useful tests to include are Clinitest, Acetest, Clinistix and Albustix or the Boehringer Mannheim test strip range. A false positive Albustix result may be obtained in a urine with an alkaline pH. These results should be checked with the sulphosalicylic acid test for urine protein.

PLASMA LACTATE

Lactate can be reliably measured using the Boehringer Kit assay (No 149993) *without* deproteinisation). The principle of the method is oxidation of L-lactate to L-pyruvate using lactate dehydrogenase. The pyruvate formed is then removed by reaction with ϵ -glutamate using alanine amino transferase.

The method is performed manually according to the manufacturer's instructions or can be adapted for automation—for example, by using a centrifugal analyser.⁸

Specimen collection for lactate

Collection tubes are prepared by placing 20 μ l of the (Boehringer Corporation, London) anticoagulant (fluoride/edetic acid solution) in each tube and evaporating to dryness. Small plastic tubes, such as Sarstedt bullet tubes 72-690PP, 1.5 ml capacity, are suitable for this. Alternatively, commercially available fluoride/oxalate tubes used for the measurement of blood glucose can be used.

For most purposes fasting blood (four hours or overnight) should be collected. Venous blood should be drawn without stasis and about 500 μ l placed into the special tube. Plasma should be separated as soon as possible and always within two hours. If analysis cannot be undertaken immediately the plasma should be stored at -20°C .

For quality assurance there are several lyophilised plasmas commercially available: BCL Precinorm and Precipath or Merz and Dade QAP Chemistry Controls level 1 and 2.

For automated methods using a Cobas Bio, inter-

batch precision of about 4% can be achieved at lactate concentrations of 1–2.5 mmol/l.

PLASMA AMMONIA

Ammonia can be quantitated either by a specific flowthrough electrode system or by one of the commercially available enzymatic kit methods (spectrophotometric or fluorimetric).

The ion specific electrode is best used as a flowthrough system.⁹ Since this method was published, further modifications have been introduced (Hjelm M, Jenkins P, personal communication). In the author's experience this provides an undemanding and robust method suitable for routine use. Further details can be obtained from the author.

The Swiftest screening test (DIC blood ammonia test kit, Clandon Scientific Instruments, Aldershot, Hampshire), although useful as a screening method is not a substitute for a quantitative method.

The specimen required is 500 μ l venous (or arterial) plasma anticoagulated with lithium heparin (*beware of ammonium heparin tubes*). Each batch of specimen tubes should be checked for ammonia contamination before use. The blood must be centrifuged within 15 minutes of collection. Whole blood should not be stored, even at $+4^{\circ}\text{C}$. Centrifuge at $+4^{\circ}\text{C}$ for five minutes at 2000 g and separate plasma, avoiding the buffy coat. Haemolysed samples are unsuitable as higher concentrations of ammonia occur in erythrocytes. Plasma ammonia should be assayed at once, but if storage is unavoidable then it should be at -70°C or lower. Storage of plasma at -20°C is unreliable.

AMINO ACIDS (PLASMA AND URINE)

The reader is referred to a discussion of the technical aspects and interpretation of amino acid investigations before deciding to provide this service.¹⁰

URINARY METHYLMALONIC ACID BY THIN-LAYER CHROMATOGRAPHY

Methylmalonic acid is separated by thin-layer chromatography on cellulose and stained with fast blue B salt.^{11 12}

The specimen should comprise fresh random urine with no preservative. Store at -20°C until analysis. A standard should be prepared by dissolving 100 mg methylmalonic acid (Sigma Chemicals) in 100 ml 0.5M hydrochloric acid. Store at $+4^{\circ}\text{C}$. The thin-layer plates should be DC—alufolien cellulose 20 $\times$ 20 cm (Merck, product No 5552). These are cut to form 10 cm $\times$ 20 cm plates. The Solvent should comprise n-butanol/acetic acid/water (5:1:2v/v). This is prepared freshly for each run. Fast blue B (0.25 g, BDH, product No 341162N) is "dissolved" in a mixture of 38 ml absolute ethanol and 12 ml water (note that the dye will not completely dissolve).

Glacial acetic acid (2 ml) is added and mixed. The stain is not stable and must be prepared freshly for each run.

Chromatography

Samples (2 μ l neat urine) and standard (2 μ l) are applied as a series of spots 1.55 cm apart and 1.5 cm from the bottom (20 cm edge). Standard applications should be included at both edges. Chromatograph until solvent is 1 cm from top edge. Dry the plate at room temperature, spray with freshly prepared stain, and develop in an oven at 100°C for one minute. Methylmalonic acid is fast running (Rf 0.83) and stains as a bright magenta spot within a minute. The

chromatogram should be viewed immediately as there is rapid colour change and fading. In normal urine several minor spots appear (figure). Beware of excess acetoacetate which stains an orange colour and may mask methylmalonic acid. The detection limit by this technique is 25 pg. A detectable spot is clinically important.

Urine sugars (mono and disaccharides) by thin-layer chromatography

It is useful to "screen" urines for reducing substances and glucose with Clinitest and Clinistix before performing sugar chromatography. The most important urine sugars to identify accurately are galactose and fructose. Several suitable one-dimensional thin-layer chromatographic techniques have been described.¹³

It is essential that glucose and galactose separate reliably. It is useful to set up duplicate plates so that a specific stain for fructose, sucrose, lactose and raffinose—for example, naphthoresorcinol—can be used in addition to p-aminobenzoic acid. Other stains, such as aniline-diphenylalanine phosphate reagent may be used as a further aid to identification.

Interpretation and further investigations

LACTATE

A plasma lactate concentration greater than 2.5 mmol/l is clinically important provided the specimen has been collected without stasis. Increased concentrations occur in some types of glycogen storage disease, defects of pyruvate metabolism, mitochondrial myopathies and some organic acid disorders, as well as liver or heart disease not associated with a metabolic disorder. In some disorders the

Figure One-dimensional chromatography for urine methylmalonic acid.

Urine	Spot	Compound	R _f	Comments
1 Normal urine	—			Normal urines with a high creatinine concentration may show several grey-brown spots
2 Normal urine	—			
3 Patient with tyrosinaemia type I	A	P-hydroxyphenyl pyruvate	0.91	Purple spot running just ahead of methylmalonic acid
4 Ketotic patient	B	Acetoacetate	0.85	Orange spot running in the same position as methylmalonic acid
5 Patient with methylmalonic aciduria	C	Methylmalonic acid	0.85	Intense magenta spot
6 100 mg% methylmalonic acid standard	D			

increase may be intermittent or exacerbated by a carbohydrate meal or by exercise. A chronic increase of plasma lactate concentration may be associated with increased plasma and urine alanine, plasma proline, and urate concentrations. Plasma pyruvate is usually increased but does not aid differential diagnosis.

Increased urinary lactate only occurs if the renal threshold (about 7.0 mmol/l) is exceeded. Further investigation of a clinically important lactic acidosis includes fasting glucose, urine and plasma amino acids, and urine organic acids.

AMMONIA

Plasma ammonia in healthy adults and children (1 month to 14 years) is less than 40 $\mu\text{mol/l}$ and is not affected by fasting. Healthy term infants in the first few days of life may have concentrations up to 100 $\mu\text{mol/l}$. In preterm infants and sick neonates concentrations may reach 200 $\mu\text{mol/l}$. Hyperammonaemia is not a diagnosis and requires further biochemical investigation.¹⁴

URINARY METHYLMALONIC ACID

Excess urinary methylmalonic acid occurs as a result of cobalamin deficiency (including a vegan diet) or one of the several inherited defects of methylmalonic acid metabolism. The finding of methylmalonic aciduria together with homocystinuria occurs in some of the cobalamin metabolic defects. This is a complex area and the finding of methylmalonic acid in the urine must be fully investigated.

URINARY SUGARS

Clinically important galactosuria—that is, greater than g/100 ml—in a neonate suggests galactosaemia, but may also occur in patients with other causes of liver disease—for example, tyrosinaemia type I. The baby should be taken off lactose immediately while further investigations (particularly erythrocyte galactose-1-phosphate uridyl transferase and urine/plasma amino acids) are undertaken.

In older children without liver disease galactosuria suggests the possibility of galactokinase deficiency. Fructosuria requires investigation for the possibility of fructose intolerance or fructosaemia.

Beware of xylose following tolerance tests. Several minor bands—for example, lactulose, raffinose—can result from dietary sources. Heavily staining bands at or near the origin require further investigation of urinary oligosaccharides as they may indicate mucopolidoses or possible glycogen storage disease.

More detailed interpretation of these tests and a guide to further investigations is beyond the scope of this paper, and advice from a specialist centre must be sought.

Appendix

GUIDE FOR EMERGENCY SPECIMEN COLLECTION

In life-threatening situations, where an inherited metabolic disorder is thought to be likely (either from family history, results of preliminary investigations, or clinical presentation), the following specimens should be taken. At the earliest opportunity contact a specialist laboratory to discuss appropriate investigations. If possible, urine and blood specimens should be taken before death. Skin and tissue specimens should be taken as soon as possible after death.

If any of the samples are taken after death it is extremely important to record accurately both the time of death and when the samples were taken. Appropriate storage as detailed below is *essential*.

1 Urine

Ideally at least 120 ml of random urine. Collect into a bottle with no preservative and store deep frozen (-20°C or lower). If the sample is contaminated with blood centrifuge to remove cells before freezing supernatant.

2 Blood

Collect 10–20 ml of heparinised blood, separate plasma as soon as possible and store plasma deep-frozen (-20°C). Store the packed red cells at $+4^{\circ}\text{C}$ (do not freeze). If DNA analysis is likely to be required, store a further 120 ml whole blood (edetic acid) in a plastic tube deep frozen (at least -20°C).

3 Skin (for fibroblast culture)

Skin taken up to 24 hours after death is likely to be viable *provided it is not infected*. Take a skin sample and place it in suitable transport medium* (obtainable from most virology or cytogenetics departments). In an emergency sterile isotonic saline can be used, *but do not use agar*. The specimen should be stored at $+4^{\circ}\text{C}$ before despatch. *Do not freeze*.

Sterility is of *paramount* importance when taking skin biopsy specimens, especially at necropsy.

If indicated:

4 Tissue samples (liver, heart muscle, skeletal muscle)

These should only be taken if there is a strong clinical suspicion of a primary defect in one of these tissues. *It is very important that blood and urine specimens are also taken and not just tissue specimens*. Necropsy tissue samples are only suitable for biochemical analysis if taken *within two hours of death*. Two or three needle biopsy specimens of tissue should be taken, wrapped in aluminium foil, and snap frozen in liquid nitrogen (or solid CO_2). Store the sample deep frozen, as cold as possible.

Please note that these samples are required for biochemical analyses only. Appropriate fixed samples may also be required for histological investigation.

- 5 *Cerebrospinal fluid* Sometimes a cerebrospinal sample may be useful. Collect a sample and store deep frozen (-20°C).

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