Archives of Disease in Childhood, 1975, **50**, 264.

Screening for inherited metabolic disease in Wales using urine-impregnated filter paper

D. M. BRADLEY

From the Department of Medicine, Welsh National School of Medicine, Heath Park, Cardiff

Bradley, D. M. (1975). *Archives of Disease in Childhood*, **50**, 264. **Screening for inherited metabolic disease in Wales using urine-impregnated filter paper.** Urine specimens from 135 295 infants have been collected on filter paper and tested for 7 abnormal urinary constituents using spot tests and paper chromatography. The method has detected 5 infants with phenylketonuria, 4 with histidinaemia, 5 with cystinuria, 5 with diabetes mellitus, and one with alcaptonuria. Transient abnormalities such as tyrosyluria, generalized aminoaciduria, cystinuria, and glycosuria have been noted. 2 phenylketonuric infants failed to excrete a detectable quantity of *o*-hydroxyphenylacetic acid at the time of testing. The findings show that the detection of this compound in urine is an unreliable method of screening for phenylketonuria.

Early detection of phenylketonuria became essential when it was found that the severe mental retardation associated with this disorder could be prevented by introducing a low phenylalanine diet in the first months of life (Bickel, Gerrard, and Hickmans, 1953). The Medical Research Council Working Party on Phenylketonuria (1968) recommended that the Phenistix test should be replaced by the microbiological test for phenylalanine in blood (Guthrie and Susi, 1963). This was because Phenistix, when used routinely between 4 and 6 weeks of age, had been found to pass a substantial proportion of patients with the disease as normal. Though the method of screening for phenylketonuria by detecting *o*-hydroxyphenylacetic acid in urine (Woolf, 1967) had not been used on a sufficient scale to assess its reliability, the Working Party concluded that it compared favourably with the Guthrie method and had the advantage of being able to detect some other disorders apart from phenylketonuria.

The newborn population of Cardiff has been screened by this method since 1962. During 1970 the programme was extended to cover the whole of Wales with about 40 000 births a year. This has given considerable experience and allows an assessment to be made of its reliability, advantages, and disadvantages.

Received 14 October 1974.

Method

Collection of urine specimens. The recommended time of testing is between the 10th and 14th day of life. In practice, 53% of all specimens are collected by the 14th day, rising to 98% by the 28th day (Table I). Occasionally samples have been taken on the 9th day, but none have been noted as being taken earlier than this.

TABLE I

Age distribution of infants when screened

Age (d)	% Newborns screened
10–14	53·1
15–20	35·1
21–28	9·7
>28	2·1

Since the test is performed on or after the 10th day of life, the majority of specimens are collected at home by parents. The health visitor explains the purpose of the test to the parents and provides them with three pieces of filter paper (Whatman No. 1, 6 cm × 3 cm) and a small cartridge paper envelope (Meritor, 121 mm × 73 mm). A urine specimen is collected on the filter paper by placing one of the pieces into the folds of the infant's napkin. The urine-soaked filter paper is allowed to dry off naturally and is put in the envelope. Should the paper be soiled with faeces it is discarded and a fresh specimen is collected on one of the spare pieces. When the health visitor returns she

records the infant's details on a duplicate filing card and posts the card and the specimen to the laboratory. On the card is recorded the infant's surname, forenames (if known), sex, date of birth, address, date of sample, name and address of the family doctor, and name of the local authority.

Analytical procedure. Each specimen is tested for 7 conditions (Table II). 4 strips (approx. 0·8 cm × 3 cm) are cut from each filter paper specimen and 3 of these are used for spot tests: cystine and homocystine are detected using cyanide-nitroprusside; Benedict's reagent is used to detect reducing substances; and protein is detected using the potassium salt of tetrabromophthalein ethyl ester and a citrate buffer, *p*H 3·7. The fourth strip of filter paper is stapled to a sheet of chromatography paper (Whatman No. 1, 25·4 × 25·4 cm), 10 samples per sheet. These are chromatographed overnight in isopropanol:ammonia: water (200:10:20), dried at room temperature with a fan, and then sprayed first with sodium carbonate solution (10%) followed after 10 minutes by a solution of diazotized *p*-nitroaniline (equal volumes of *p*-nitroaniline, 0·1% in N HCl and sodium nitrite, 5%). The whole procedure is complete within 24 h of receiving the specimen.

TABLE II

Disorders detected

Phenylketonuria	Proteinuria
Tyrosyluria	Glycosuria
Histidinuria	Cystinuria
Homocystinuria	

Specimen problems. A minor difficulty in using urine collected on filter paper is that the paper may not have in fact been wetted. The presence of histidine on the chromatogram is taken to indicate that urine is present, but should any doubt remain, the specimen may be tested for urea. Otherwise variations in urine concentration did not create problems. Contamination of the sample with faeces, talc, and nappy rash cream may occur and then a fresh specimen is requested. In all, only about 1 in 2000 samples have been rejected because of contamination or lack of specimen.

Reporting results. Negative as well as positive results are reported. When the results have been entered on the duplicate filing card, the top copy is returned to one of the 25 participating local authorities. If a test is positive, a repeat specimen is requested and the general practitioner is informed. This is collected in liquid form by the health visitor and is sent to the laboratory in a universal container. The original report form is sent with the specimen so that the result of the repeat test may be entered on the same form. Except in the case of tyrosyluria (see below), no delay is requested before taking repeat specimens which are generally sent to the laboratory within 5–10 days of their being requested. If phenylketonuria is suspected, the request for a repeat specimen is made by telephone. All communication with patients is through the health visitor who is informed of the significance of any positive test.

Results

The results of screening 135 295 newborns over the 4-year period, 1970 through 1973, are shown in Table III. During the earlier part of this period, assessment of both the spot tests and chromatograms was oversensitive. During the first 18 months, repeat specimens were requested from 4% of infants tested; this figure has now dropped to 1%. This increased discrimination applies to all the tests performed with the exception of that for phenylketonuria.

Phenylketonuria. During the 4 years 6 phenylketonuric infants were born in Wales. One of these gave a negative test at 7 weeks of age. Screening of this child was delayed because of a

TABLE III

Results of newborn screening in Wales between January 1970 and December 1973 (number tested: 135 295)

Test	Result					
	1st specimen	% Total	Repeat specimen	% Total	% 1st specimen	Confirmed cases*
Phenylketonuria	26	0·02	5	0·004	19·3	6
Tyrosyluria	549	0·40	93	0·07	17·0	0
Histidinuria	1025	0·76	72	0·05	7·1	4
Cystinuria/ homocystinuria	656	0·48	109	0·08	16·6	5
Glycosuria	1046	0·77	14	0·01	1·3	4
Proteinuria	493	0·36	59	0·04	12·0	2
Total	3795	2·79	352	0·26	9·3	21

*See text.

history of illness, including suspected malabsorption. A diagnosis of phenylketonuria was finally made at 6 months after emergency hospital admission with failure to thrive and a poor physical condition. One cannot be certain that other phenylketonuric infants have not been missed. However, at the present time, no other undiagnosed cases of the disease have come to the attention of the paediatric services in Wales. Only 21 infants (1 in 6400) gave weakly false positive tests, all of which were negative when repeated. A small quantity of *o*-hydroxyphenylacetic acid may be detected in normal urine (Armstrong, Shaw, and Robinson, 1955) and the Medical Research Council Working Party noted that about one nonphenylketonuric infant in 5000 gave a weak positive reaction.

Tyrosyluria. Tyrosine metabolites (*p*-hydroxyphenylacetic and *p*-hydroxyphenyl-lactic acids) were frequently present in the urine of the newborn. During the first part of the programme a second specimen was simply requested, but for the last 12 months we have recommended giving the infant ascorbic acid 100 mg daily for one week before collecting the second urine specimen. Such treatment appears to make little difference to the rate of disappearance of tyrosine metabolites from the urine, reducing the percentage of infants still showing tyrosyluria on repeat from 19% to 16%. This is in agreement with the observation of Raine *et al.* (1972) regarding neonatal tyrosinaemia. No cases of tyrosinosis or tyrosine transaminase deficiency have so far been detected. All infants still excreting tyrosine metabolites on repeat tests (normally 1 to 2 weeks after the first test) have returned to normal by 6 to 10 weeks of age.

Histidinuria. During the early part of the screening programme, assessment of the urinary chromatograms in respect of histidine was oversensitive. Normally the histidine spot at the origin is the only compound seen on the *p*-nitroaniline-stained chromatograms. Repeat specimens requested because of apparent excessive histidinuria still account for about one-quarter of all positive first tests. If excessive histidinuria is still present in the second specimen, thin layer chromatography for urinary amino acids is performed. On this basis, those infants in whom histidinaemia is not subsequently confirmed are either normal or have a moderate aminoaciduria which generally disappears by 3 months of age. 4 cases of histidinaemia have been confirmed by plasma histidine determinations. 2 of these infants have been treated and 2 have not; the development of all 4 is regarded as normal at the present time.

Cystinuria, homocystinuria. No cases of homocystinuria have been detected. Most of the infants giving a positive first test were negative on repeat. When a repeat specimen is positive, the urine is subjected to thin-layer chromatography, and if necessary, column chromatography. On this basis, of the 109 infants who repeatedly gave a positive cyanide-nitroprusside test, 5 are regarded as having cystinuria. The remainder were found to be normal or had transient generalized aminoacidurias. The results of the Massachusetts Metabolic Disorders Screening Programme (Levy, Madigan, and Shih, 1972) showed that neonates excrete larger quantities of cystine and lysine than older children and adults. These authors observed that after tyrosyluria-tyrosinuria, the most common transient urinary amino acid abnormalities were generalized aminoaciduria, iminoglycinuria, and cystine-lysinuria.

Glycosuria. At first glucose and galactose oxidases (Glucostat and Galactostat, Worthington Biochemical Corp.) were used to test for glucosuria and galactosuria. This was expensive and produced a large number of positive results: 1·2% of all specimens. No repeat test for galactosuria was positive, while only 1·9% of those for glucosuria were found positive on repeat. Benedict's reagent has been perfectly adequate and using this we regard 0·3% of all specimens as positive on the first test. Under the present system, any repeat specimen which is still positive for reducing sugars is subjected to thin-layer chromatography for identification of the sugar present. No case of galactosaemia has been detected by screening, but galactosaemia was diagnosed in one infant in hospital who died, aged 5 days, before being screened. 4 infants with diabetes mellitus have been detected; 2 of whom died within the first months of life. The other infants with repeated glucosuria have subsequently become normal.

Proteinuria. During the early part of the programme, transient mild proteinuria (approx. 100 mg/100 ml) was often found: 0·7% of all tests. From a follow-up survey of infants whose urines were repeatedly positive for any of the tests, proteinuria (>300 mg/100 ml) was found in 8% of those infants whose original tests were negative for protein. Glycosuria (>500 mg/100 ml) was also found in the same proportion of infants whose

original sugar tests were negative. All these babies were thriving.

Mild proteinurias are now ignored and only those infants with proteinuria >500 mg/100 ml are followed up. This is 0·01% of first tests. Only one case of gross proteinuria has been observed and the infant subsequently died of septicaemia. Apart from a moderate proteinuria associated with renal infection, all those infants with a positive repeat test later became normal.

Miscellaneous. 2 infants with alcaptonuria have been detected. One of these was the sib of a known case. Both these children gave a positive test with Benedict's reagent and the urinary chromatogram had a large diffuse brownish spot at the origin. The presence of homogentisic acid and the absence of sugars was confirmed by thin-layer chromatography.

During the early period when assessment of the tests tended to be oversensitive, about 0·5% of urines gave a positive result with more than one test (most commonly cystine and protein). The urines of all these infants have become normal.

Discussion

The use of urine-impregnated filter paper as a screening procedure for detecting phenylketonuria has proved acceptable to both the parents and the health visitors responsible for collecting the specimens; the laboratory has been notified of only two refusals. During the earlier part of the programme some strain was put on the paediatric services in the rural areas particularly because of the oversensitive assessment of urinary histidine in infants who were subsequently shown to be normal. It is difficult to assess accurately how many infants were missed by the screening programme. However, relating the number screened in any year to the number of live births recorded by the Registrar General in Wales showed that not less than 97% were included in the programme.

Although only 53% of newborns were tested at the recommended time of 10–14 days, 98% of infants were screened by 5 weeks of age. Thus, a phenylketonuric infant should have started dietary treatment by 6 weeks of age at the latest. In terms of ultimate intellectual capacity, there is no evidence that these children would be distinguishable from those treated from 3 weeks of age. The largely rural nature of Wales makes communication between local authorities and patients more difficult than in an urban community. This, and the uncertainty which may be experienced in obtaining a urine specimen on demand from a newborn, probably contributes to the reduced number of tests at the recommended time. Preliminary experience with testing blood specimens for raised phenylalanine levels (see below) indicates that a larger proportion of infants are screened at the recommended time, presumably because blood specimens, obtained by heelprick, are available on demand. The test for reducing substances in the urine of 10 to 14-day-old infants could detect galactosaemia. However, newborns should ideally be tested before 10–14 days since the galactosaemic infant may be severely ill or even dead by this age. On the other hand, where the condition is mild, galactose may not be present in the urine (Segal, 1972). Galactosaemia therefore may only be satisfactorily detected at birth by determining galactose-1-phosphate uridyl transferase activity in cord blood (Beutler and Baluda, 1966). In spite of the ease and acceptability of the method described of screening for phenylketonuria, there are a number of possible inherent sources of error. The presence of *o*-hydroxyphenylacetic acid in the urine of a phenylketonuric infant is dependent upon the following factors: (i) That the plasma phenylalanine level is sufficiently high for the minor pathway of phenylalanine catabolism, via transamination, to become of major importance; (ii) that transamination does occur; and (iii) that the products of transamination are excreted.

Screening methods which rely upon measurement or chromatographic observation of blood phenylalanine levels may give a false negative result in the case of a phenylketonuric infant who is a poor feeder or who vomits excessively, resulting in a slow rise in the blood phenylalanine level. Detecting phenylketonuria by the presence of the products of transamination in urine carries the additional risk of a negative test if maturation of phenylalanine transaminase is delayed or if the infant's renal threshold for *o*-hydroxyphenylacetic acid is abnormally high.

As mentioned above, one phenylketonuric child did not excrete a detectable amount of *o*-hydroxyphenylacetic acid at 7 weeks; unfortunately his blood phenylalanine level was not measured at that time. However, this case and the other drawbacks mentioned above prompted us to look at the suitability of other screening methods. The thin-layer chromatographic method using an eluate from blood collected on filter paper (Ireland and Read, 1972) appeared to be the most suitable. During 1973 all newborns in Cardiff were screened by both this and the previous method. In this period 2 cases of transient hyperphenylalaninaemia (<10 mg/100 ml) were detected by the blood chromatography test and as expected these babies did not excrete metabolites.

Additionally, a sib of a known phenylketonuric was born and serial samples of blood and urine were collected. This infant's blood phenylalanine level was noticeably raised on the third day of life and metabolites were detected in his urine on the fifth day. Since extending this method to cover most newborns in South Wales, another 3 phenylketonuric infants have been detected. 2 of these, tested at 6 and 10 days respectively, were excreting *o*–hydroxyphenylacetic acid and had markedly high blood phenylalanine levels. The third infant, however, whose blood level was grossly raised when tested at 11 days of age, did not have a detectable quantity of *o*-hydroxyphenylacetic acid in her urine and would therefore have given a false negative test if urine chromatography alone had been used. From these experiences we conclude that relying upon the presence of *o*-hydroxyphenylacetic acid in the urine of 10- to 14-day-old newborns is an unreliable method of screening for phenylketonuria.

The method of blood chromatography will become the primary screening procedure for phenylketonuria in the whole of Wales during 1974. Urine specimens will still be collected, primarily to be tested for reducing substances, cystine, and homocystine. From the point of view of the local authorities, this will mean a considerable change; not only because a different type of specimen is required, but since the recommended time of testing is 6–10 days after birth, the collection of specimens now becomes the responsibility of the domiciliary midwives.

A neonatal screening programme is a search for disease in a symptomless individual. The unsolicited detection of such disease carries with it the responsibility of treating the disorder as effectively as possible. Screening for other aminoacidaemias grew out of programmes established for the early detection of phenylketonuria. Unlike phenylketonuria, however, no established treatment was available and at the present time treatment remains largely experimental and in some cases the need to treat is yet to be established. In histidinaemia, probably the most common of the other aminoacidaemias, with an incidence similar to phenylketonuria, the necessity for treatment is not satisfactorily established since there is a 0·6 probability of an untreated newborn histidinaemic escaping impaired intellectual development (Popkin, *et al.*, 1974). In the absence of established lines of treatment, the screening programme makes it ethically possible to assess the natural history of some of these disorders. Any new forms of treatment may then be evaluated critically against this knowledge.

The author is indebted to Miss V. Evans for technical assistance and to the Medical Officers of Health and their staffs for co-operation; to Professor R. F. Mahler and Dr. P. S. Harper for constructive criticism throughout the programme; and to Mrs. L. Mace for typing the manuscript.

References

Armstrong, M. D., Shaw, K. N. F., and Robinson, K. S. (1955). Studies on phenylketonuria. II. The excretion of *o*-hydroxyphenylacetic acid in phenylketonuria. *Journal of Biological Chemistry*, **213**, 797.

Beutler, E., and Baluda, M. C. (1966). A simple spot screening test for galactosemia. *Journal of Laboratory and Clinical Medicine*, **68**, 137.

Bickel, H., Gerrard, J., and Hickmans, E. M. (1953). Influence of phenylalanine intake on phenylketonuria. *Lancet*, **2**, 812.

Guthrie, R., and Susi, A. (1963). A simple phenylalanine method for detecting phenylketonuria in large populations of newborn infants. *Pediatrics*, **32**, 338.

Ireland, J. T., and Read, R. A. (1972). A thin layer chromatographic method for use in neonatal screening to detect excess amino acidaemia. *Annals of Clinical Biochemistry*, **9**, 129.

Levy, H. L., Madigan, P. M., and Shih, V. E. (1972). Massachusetts metabolic disorders screening program. 1. Technics and results of urine screening. *Pediatrics*, **49**, 825.

Medical Research Council Working Party on Phenylketonuria. (1968). Present status of different mass screening procedures for phenylketonuria. *British Medical Journal*, **4**, 7.

Popkin, J. S., Clow, C. L., Scriver, C. R., and Grove, J. (1974). Is hereditary histidinaemia harmful? *Lancet*, **1**, 721.

Raine, D. N., Cooke, J. R., Andrews, W. A., and Mahon, D. F. (1972). Screening for inherited metabolic disease by plasma chromatography (Scriver) in a large city. *British Medical Journal*, **3**, 7.

Segal, S. (1972). Disorders of galactose metabolism. In *The Metabolic Basis of Inherited Disease*, 3rd ed., p. 174. Ed. by J. B. Stanbury, J. B. Wyngaarden, and D. S. Fredrickson. McGraw-Hill, New York.

Woolf, L. I. (1967). Large-scale screening for metabolic disease in the newborn in Great Britain. *Phenylketonuria and Allied Metabolic Diseases*, p. 50. Ed. by J. A. Anderson and K. F. Swaiman. U.S. Department of Health, Education, and Welfare, Washington.

Correspondence to Dr. D. M. Bradley, Department of Medicine, University Hospital of Wales, Heath Park, Cardiff CF4 4XN.