

A SIMPLE PHENYLALANINE METHOD FOR DETECTING PHENYLKETONURIA IN LARGE POPULATIONS OF NEWBORN INFANTS

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PRINCIPLE

THE INHIBITION of growth of *Bacillus subtilis* ATCC 6051 by B-2-thienylalanine in a minimal culture medium is prevented by phenylalanine, phenylpyruvic acid, and phenyllactic acid.^{1*} This finding has permitted the development of a convenient agar diffusion microbial assay for phenylketonuria (PKU), employing small filter paper discs, impregnated with blood or urine, placed upon the surface of the agar culture medium.

The method was first used in screening an institution for mental retardates and detected 21 cases of PKU among 3,118 residents; 4 more than were detected by simultaneous ferric chloride testing of urine specimens.² This assay has been used successfully in the determination of blood phenylalanine levels during low-phenylalanine diet treatment of 22 patients with phenylketonuria (PKU) at this hospital by Dr. Robert Warner during the past 5 years. More recently, the method has been adapted for testing newborn infants before leaving the hospital, as described below.

PROCEDURE

A. Preparation of Specimens

1. A small amount of fresh blood obtained

* This publication stated that proline was also active in this assay. However, subsequently proline has been *inactive* in repeated tests. We have no explanation as to why proline was found active initially.

by heel puncture is applied immediately to a piece of thick, very absorbent filter paper (Schleicher & Schuell #903). The blood spot when air dried should be at least $\frac{3}{8}$ inch in diameter (but not more than $\frac{1}{2}$ inch) and close enough to the edge of the paper to facilitate punching out the disc. This paper is so absorbent that even very viscous blood from a young infant spreads through the paper, so that the appearance of the blood spot is similar on both sides of the paper. These conditions must be met to obtain a uniform blood sample by means of the paper punch.

2. Before assay, the individual filter papers are numbered with a pencil, placed on pieces of metal screening or wire test tube racks and autoclaved at 15 pounds pressure for 3 minutes with dry steam. This prevents blood pigments from later diffusing from the paper discs into the agar during incubation, which tends to mask possible growth zones. Prolonged autoclaving will destroy the phenylalanine.

3. A disc, $\frac{1}{4}$ inch in diameter, is then punched from the center of the blood spot.

4. It has been found convenient to place the discs as they are punched in rows on a clean piece of paper, in the same sequence that they will be placed on the agar dish for assay. The discs are placed within numbered squares printed on the paper. A plastic sheet with rows of depressions is also very convenient for this purpose and

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TABLE I
DEMAIN'S MEDIUM* (MODIFIED) FOR *BACILLUS*
SUBTILIS SPORE GERMINATION

Substance	Grams	Grams/Liter
Dextrose	10.0	
K ₂ HPO ₄	30.0	
KH ₂ PO ₄	10.0	
NH ₄ Cl	5.0	
NH ₄ NO ₃	1.0	
Na ₂ SO ₃	1.0	
Glutamic acid	1.0	
Asparagine	1.0	
L-Alanine	0.5	
Salt solution (10 ml)		
MgSO ₄ ·7H ₂ O		10.0
MnCl ₂ ·4H ₂ O		1.0
FeCl ₃ ·6H ₂ O		1.0
CaCl ₂		0.5

* Demain, A. L.: Minimal media for quantitative studies with *Bacillus subtilis*. *J. Bact.*, **75**:517, 1958.

prevents the tiny paper discs from being displaced by an air current.

B. Preparation of Assay Medium*

The formula is a modification of that of Demain (Table I). The components in the amount shown in Table I are dissolved in 900 ml of distilled water. The pH will be 6.8–7.0. A 90-ml volume of the solution is dispensed in each of 10 8-oz prescription bottles and sterilized by autoclaving. A 10% solution of dextrose is sterilized separately, to be used as a 10-ml supplement per bottle. Similar bottles are prepared containing 100 ml of 3% agar and sterilized. For use, the dextrose solution is added, and 100 ml of medium is mixed with 100 ml of agar which has been previously melted and cooled to 55°C in the water bath.

* It has also been possible to prepare the culture medium as a dry powder and include it in a "test kit." Several thousand "laboratory test kits" have been prepared for shipment to State Health Laboratories for use in a screening program currently sponsored by the Children's Bureau, Department of Health, Education, and Welfare. The other components in the "kit" are in the dry state, including the spore powder inoculum, and the phenylalanine control blood spots on a filter paper strip.

C. Preparation of the Inoculum

The use of a dried spore powder as the inoculum is very convenient and helps assure uniform results. This is prepared as follows: bottles or Petri dishes containing a potato infusion agar medium (Difco Bacto B51 is satisfactory) are prepared. The agar surface is inoculated heavily with a *Bacillus subtilis* ATCC 6051 cell suspension from overnight cultures grown on Difco Heart Infusion agar slants. During incubation for one week at 30°C, the growth is examined microscopically at intervals for presence of spores. These are scraped and washed off the agar into 0.9% NaCl, and washed three times with 0.9% NaCl by centrifuging (11,000 rpm in a Serval Model SS3). Final suspension is made in distilled water at an optical density of 0.9, measured at 550 mμ wave length in a colorimeter; 0.3 ml of this suspension is dispensed in each of a large number of small screw-capped vials, dried on a shaking machine at 60°C, and stored in the refrigerator with the cap tightly closed. For use, 1 or 2 ml of Demain's medium is added to the vial, the dried powder scraped off the glass wall with aid of a plastic stick, and decanted into 200 ml of culture medium.

D. B-2-Thienylalanine

This compound (California Biochemical Corp.) is made up in a 0.01M solution, and 0.3 ml is pipetted into each of a number of small screw-capped vials, dried, and stored at room temperature until needed. A vial is then rinsed with 1 or 2 ml of medium into 200 cc of Demain's medium. This provides a final concentration of $1.5 \times 10^{-5}M$ of the inhibitor.

E. Preparation of Controls

Out dated blood is obtained from the hospital blood bank and assayed for phenylalanine content by any available procedure, such as that of LaDu.³ L-phenylalanine is then added to a series of aliquots of blood to make concentrations of 2, 4, 6, 8, 10, 12, and 20 mg/100 ml. With a pipette, the blood is spotted on S. & S. #903 filter

TABLE II
PHENYLALANINE LEVELS IN BLOOD IN 682 INFANTS
4 DAYS OF AGE

Phenylalanine Level (mg/100 ml)	Infants	
	Number	Per Cent
4 or >4	0	0.0
2-4	56	8.2
2	113	16.6
<2	513	75.2
Total	682	100.0

paper, to make spots of between $\frac{3}{8}$ and $\frac{1}{2}$ inch in diameter. After drying, these control spots are kept in a dessicator at 2° to 5°C. The controls are autoclaved simultaneously with the "unknowns" before discs are punched out of each spot for use.

An alternative, and perhaps more satisfactory, set of eight control discs may be used for routine screening in the following sequence: 2,4,4,4,6,6,6, and 8 mg/100 ml. This sequence is based upon the data reported here, which suggest that a level of 6 mg/100 ml, or above, should be considered a positive screening result.

F. Procedure for Assay

After combining the culture medium, agar, B-2-thienylalanine, and the spore powder, all are thoroughly mixed by pouring back and forth, and poured into a suitable flat dish. We have used 8 by 12 inch Pyrex baking dishes, but a styrene plastic tray has also been found suitable for routine use. It is very important that the table top be perfectly level. This insures an agar layer of uniform thickness.

After the agar is hardened, the dish is placed over a paper pattern sheet with a printed grid containing an appropriate number of intersecting lines (50 to 100) and a suitable space approximately one inch wide with positions marked for the control discs. With forceps, the control discs and the discs from unknown specimens are placed in position. The agar dish is then placed in an incubator at 35° to 37°C overnight for

observation the following morning (a period of 16 hours).

RESULTS

The results for 682 infants are shown in Table II. Note that all values were below 4 mg/100 cc, and only 8% of the values were above 2 mg/100 cc.

It is important to point out that 96% of these infants were 4 days old when the specimens were collected.

COMMENT

The results presented here are the first data obtained in our own laboratory with a routine phenylalanine test used in a screening program proposed for phenylketonuria detection in hospital nursery infants. This program will include testing of two kinds of filter paper specimens obtained on every infant: (1) dried blood spots collected on the day before discharge from the hospital and (2) dried urine-impregnated filter paper, collected at 2 or 3 weeks by the mother and mailed back to the laboratory. Only data for blood specimens are presented here.

These data permit adoption of a result of 6 mg/100 ml or above as "positive" by this screening method, without encountering frequent "false-positives" among the 10,000 to 20,000 tests that may be necessary to find a case of phenylketonuria, providing previous estimates of frequency are correct.

These data are not in disagreement with data for blood phenylalanine concentrations in young infants obtained by other methods. Howell and LaDu,⁴ using LaDu's method, found a range of 0.98 to 2.5 mg/100 ml in infants 1 to 2 days old, but 6 of 30 older infants 2 to 7 days of age had levels above 3 mg/100 ml, with one level of 7.5. Of 30 premature infants studied by these workers, half had values above 3 mg/100 ml, with one value of 5.8. However, the older infants studied by Howell and LaDu were infants kept in the hospital because of such problems as physiologic jaundice, and were interpreted by the authors as reflecting a delay in the development of the liver

enzyme systems responsible for metabolism of phenylalanine.

Our own infants were unselected, which perhaps explains the slightly lower values obtained by us, as compared to Howell and LaDu, if we can assume this difference is not due to the different methods employed. However, this latter assumption is not warranted until a direct comparison of the different methods is carried out on the same specimens. Recently, Hsia has reported⁵ the results of his own determination of phenylalanine blood levels in 100 full term newborn infants, all of whom were also found to have values below 4 mg/100 ml, as in the case of our 682 infants.

Results of blood screening for phenylketonuria by the inhibition assay technique among residents of an institution for mental retardates have already been published.² However, the "inhibition assay" screening technique used for that purpose (and not described in detail in that brief "Letter to the Editor") was different from the procedure now described, principally in the manner of collecting the dried blood specimen.

The filter paper used for screening the institution was Whatman No. 3MM. This paper was not absorbent enough to allow for uniform spotting. The variability in the specimens was not important in screening a population of older children and adults for phenylketonuria, since in these cases the blood phenylalanine levels are usually more than 20 mg/100 ml.

However, the problem of detecting this disease during the first few days of life is very different. The blood phenylalanine level is in the normal range at birth, and its rise in an infant with phenylketonuria is completely dependent upon protein intake.

Scheel and Berry⁶ reported a high incidence of "false positives" among 95 young infants tested by the "inhibition assay" method in their laboratory: 20% gave values of 8 mg/100 ml, or higher. Therefore, they recommended not using this test method for screening. While it is not possible to account with certainty for the discrepancy between their results and ours, one likely

source of error is the type of filter paper used in their study (Whatman 3MM).

Armstrong *et al.*⁷ have published blood phenylalanine levels on six infants with phenylketonuria which indicated that all six had levels above 6 mg/100 ml on the fourth day of life, and therefore presumably would have been detected at this time by the screening technique presented by us.

Two PKU infants (newborn siblings of known cases) have been found by us to have blood phenylalanine levels above 6 mg/100 ml on the second day of life. Blood spots on filter paper was collected daily. Dr. George Jarvis has reported⁸ similar results on the second day of life for three additional PKU newborn infant siblings of known cases. In addition, three confirmed cases of PKU have been detected in infants upon the second day of life during the newborn screening program with the "inhibition assay" (by the Massachusetts, Nevada, and Michigan State Departments of Health).^{9,10}

However, the available published data on blood phenylalanine concentrations in very young infants with PKU is still very scanty. Certainly, no claim is made by us at this time that the "inhibition assay" screening technique described herein can detect all cases of phenylketonuria from blood specimens collected on filter paper before infants leave the hospital. One formidable limitation is that presented by some hospitals in the United States which now discharge their nursery infants after 1 or 2 days. In such cases, perhaps a visiting nurse could collect the dried blood specimen during a home visit. Alternatively, an "inhibition assay" for urine phenylalanine on urine-impregnated paper mailed in by the mother at 2 or 3 weeks presumably would also detect such cases. This latter possibility remains to be determined by the screening program we have initiated.

It should be emphasized that any posi-

⁹ Private communication.

¹⁰ A summary of this program, which is sponsored by the Children's Bureau, is to be published at its completion.

tive test by this method should be confirmed by repeated tests upon new blood specimens, and also by at least one independent method of determining blood phenylalanine. In our laboratory we subject blood and urine specimens to one dimensional paper chromatography and carry out "inhibition assays" using narrow paper strips cut from the chromatograms. Thus, the Rf value, chemical reaction (ninhydrin or ferric chloride) and "inhibition assay" response can all be related to the same "spot" on the chromatogram. It is strongly recommended that under no circumstances should diet treatment be initiated without such confirmatory testing by more than one method.

With the present methods of detection, employing ferric chloride testing of urine after one and two months of life at well baby clinics or in a physician's office, only a small fraction of PKU infants have been placed on diet treatment within the first 3 months of life. Usually such infants are new siblings of known PKU cases. Therefore, what we do claim is that the "inhibition assay" technique described herein, if applied widely to test hospital nursery infants as late as practical during the first week of life, could make possible a substantial increase in the percentage of PKU infants placed on the therapeutic diet within the first or second month of life, with subsequent prevention of mental retardation.

ADDENDUM

Since this manuscript was prepared, a case of phenylketonuria was detected by the method described above, in the Niagara Falls City Health Department Laboratory, by Miss Ruth Bitter, technician, and reported to us by Dr. Gerald A. Campbell, City Health Officer.^o This laboratory has tested 1,384 infants, with three "false-positives" value of 6 mg/100 ml). The infant was in the fifth day of life when its blood was spotted on filter paper. The test result was interpreted as 20 mg phenylalanine per 100 ml. We confirmed this case in our own laboratory by paper chromatography of blood serum and urine, in addition to the "inhibition assay." It is of interest that this infant was totally breast fed from birth.

^o Private communication.

This method has also been used by the Massachusetts Department of Health's Diagnostic Laboratory, under Dr. Robert A. MacCready⁷ to detect successfully three cases of phenylketonuria among the first 8,000 newborn infants tested. These cases were confirmed by blood phenylalanine determinations, using the LaDu method.

Total results of the trial of this method by 30 State Health Departments to date (June 1, 1963) and (1) total number of blood tests—185,681; (2) total "presumptive positive" tests—160, and (3) total cases confirmed as PKU—18. Thus, the true frequency of this disease among newborns appears to be greater than previous estimates of 1:20,000.

SUMMARY

A new method is described for rapid and economical screening of large numbers of hospital nursery infants for elevation in blood phenylalanine associated with phenylketonuria. Results are presented for 682 infants, 96% of whom were 4 days of age. None of the blood phenylalanine values were found to be as high as 4 mg/100 ml, and only 8% were above 2 mg/100 ml. These values appear to be in agreement with values obtained by other methods, and indicate that a very low rate of "false-positives" will be encountered during screening of the 10,000 or more infants that may be necessary to detect a case of phenylketonuria. It is recommended that any result of 6 mg/100 ml or above be considered positive, and require confirmation by phenylalanine determination of a second blood specimen.

Editor's Note:

Because of wide lay publicity given to phenylketonuria and the screening test described above, the results of a conference on both subjects called May 16, 1963, by the California State Department of Public Health were awaited with interest. Knowing that a report was to be forthcoming, we asked Dr. Guthrie if we might hold his description of a procedure already in wide use until it could appear with the comments of the consultants assembled in California. Dr. Guthrie promptly agreed. We are grateful to him and to Mr. David S. Kleinman, Assistant Chief (Administrative), Bureau of Maternal and Child Health, who prepared the report (see p. 344).

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