psychosis, pyramidal, extrapyramidal or muscular disease or by congestive heart failure or a number of drugs. In these 321 cases, seven false hyperthyroid but no false hypothyroid curves were recorded.

The results suggest that this technique is a convenient and reliable diagnostic aid in the estimation of thyroid function.

Our appreciation is due to the Departments of Endocrinology, Thyroid Division, of the Detroit Receiving Hospital and the Radioisotope Laboratories of Detroit Memorial and Detroit Receiving Hospitals for their collaboration in this study.

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Case Finding in Phenylketonuria:
II. The Guthrie Test

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ABSTRACT

Experience with over 6000 Guthrie tests is presented. This test is a screening procedure for phenylketonuria using small amounts of blood spotted on a filter paper which are tested by a bacterial "inhibition assay". Certain technical aspects of the test (e.g. relation between the concentration of phenylalanine in the blood and extent of the bacterial growth zones produced, type of filter paper, size of the blood spot on the paper) were investigated. It was shown that the Guthrie test clearly distinguishes between subjects with normal plasma phenylalanine levels and patients with untreated phenylketonuria.

Applications of the Guthrie test in screening a mental hospital population, admissions to a penitentiary and newborn babies are described.

SOMMAIRE

L'auteur rapporte l'expérience acquise et portant sur plus de 6000 épreuves de Guthrie. Cette épreuve, destinée au diagnostic de la phénylcétonurie, consiste à répartir de petites quantités de sang sur un papier filtre et à procéder à l'examen biologique de ces taches par "inhibition bactérienne." L'auteur étudie certains éléments techniques de l'épreuve (par ex. la relation existant entre la concentration de phénylalanine dans le sang et les dimensions des zones de prolifération bactérienne obtenues, le type de papier filtre à utiliser, les dimensions des taches de sang sur le papier). Il conclut à la valeur du test de Guthrie qui permet de distinguer nettement les sujets dont les concentrations plasmatiques de phénylalanine sont normales de ceux qui sont atteints de phénylcétonurie non traitée.

L'article passe en revue les applications diagnostiques de l'épreuve de Guthrie parmi les malades mentaux hospitalisés, au moment des entrées au pénitencier et chez les nouveau-nés.

SURVEYS for cases of phenylketonuria have usually been carried out by means of urine tests.[1-4] As time has gone by the limitations of this method have become apparent. In the first place the patient (often a baby or a severely retarded child or adult) may not produce a specimen of urine at the time or in the place that it is wanted. Despite the demonstration that urine may be tested with ferric chloride or Phenistix (Ames Co. Ltd.) on a wet diaper, bedding or even the floor, lack of a specimen at the right time is still a serious handicap.[5-7] In the second place, the urine may contain substances which give a colour change with ferric chloride or Phenistix, but which are not phenylketones. These substances may be drugs (e.g. salicylates, phenothiazines) or the metabolic products of some other disease (e.g. histidinemia).[8] In the third

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place, newborn babies with phenylketonuria, in spite of high blood phenylalanine levels, may not excrete detectable amounts of phenylpyruvic acid in the urine for the first few weeks or even months of life, that is, at the age when diagnosis is most urgent.

For these reasons and because a gross increase in the concentration of phenylalanine in the blood is pathognomonic of phenylketonuria, the ideal screening test should be a measure of the blood phenylalanine level. Several methods of estimating the concentration of phenylalanine in blood are available but they are expensive and time-consuming. What is needed for case-finding programs is a simple, cheap and reliable test which can be done *en masse*. In 1960, Guthrie and Tieckelmann[9] presented details of a bacterial "inhibition assay" for phenylalanine which seemed to fulfil these requirements. This report presents our experience with the so-called Guthrie test.

METHODS

Estimations of phenylalanine in plasma and serum were made by the enzymatic method of Udenfriend and Cooper.[10]

The Guthrie Test

Several accounts of this test have been published which are similar in principle but differ in detail.[9, 11-15] The method depends on a bacterial "inhibition assay". A standard culture of *B. subtilis* is incubated on agar in the presence of a phenylalanine antagonist which limits the growth of the organism. Blood-soaked filter paper discs are placed on the agar. Phenylalanine reverses the effect of the inhibitor, and the extent of the bacterial growth surrounding the disc is taken as a measure of the phenylalanine content of the blood. The procedure followed in our studies was essentially that described by Guthrie.[13]

Preparation of samples.—(1) Blood (whole blood, plasma or serum) was applied directly to filter paper (Whatman No. 3 or Schleicher and Schuell No. 903). The blood was allowed to soak completely through the filter paper over an area at least one-half inch in diameter. The filter paper was labelled, dried in air and stored at room temperature until assayed, usually within two weeks.

2. Just before the assay, the filter papers were placed on racks and autoclaved at 15 pounds per square inch pressure in order to prevent subsequent diffusion of the blood pigments on to the agar. This step was omitted with plasma or serum.

3. One-quarter-inch discs were punched out of the blood-soaked areas of the filter papers, labelled with a pencil and laid out in rows on a clean Petri dish prior to transfer to the agar plate.

Materials for assay—(1) Culture of *B. subtilis* (Strain ATCC 6051, American Type Culture Collection, 2112 7th Street, N.W., Washington 7, D.C.).

2. Suspension of *B. subtilis* spores prepared as described by Guthrie.[13]

3. Double strength, minimal, modified Demain's[16] medium, sterile.

4. 3% Difco Bacto-agar, sterile.

5. M/100 β-2-thienylalanine solution (Sigma Chemical Company), kept frozen until needed.

6. Sterile disposable Petri plates, 100 x 100 x 15 mm.

7. Whole-blood standard discs punched from dried, autoclaved blood-soaked filter paper in the following phenylalanine concentrations: Control (blood to which no L-phenylalanine was added), 2, 4, 6, 8, 10, 12, 20 and 40 mg. %

Procedure.—(1) A bottle containing 90 ml. of double-strength Demain's medium and one containing 10 ml. of 10% dextrose were put in a water bath at 55° C. A bottle containing 100 ml. of 3% agar was placed in a boiling water bath until the agar was completely melted and was then transferred to the water bath at 55° C.

2. The 90 ml. of medium and 10 ml. of 10% dextrose were combined in a 250 ml. flask. To this was added 0.2 ml. of M/100 β-2-thienylalanine and 0.2 ml. of *B. subtilis* spore suspension. The 3% agar was then added, the solution mixed well and approximately 20 ml. poured on to each of nine Petri dishes.

3. The agar was allowed to cool and solidify. The blood-soaked filter paper discs were laid on the surface of the agar, using a paper pattern sheet to facilitate uniform placement. Although there were minor variations from time to time, the general procedure was to run one set of phenylalanine blood standards ("control", 2, 4, 6 and 8 mg. %) on each plate together with 11 unknowns. The placement of the standard discs was systematically varied from plate to plate in order to offset the effect of position on the plate on the size of the growth zone. One plate was reserved for the 10, 12, 20 and 40 mg. % phenylalanine standards. In practice, the discs were placed on the agar at about 12 noon; the plates were kept at room temperature until 5.00 p.m. and were then put in the incubator at 30° C. Incubation was carried out overnight for 16 hours and the growth zones were assessed.

4. The diameters of the circular bacterial growth zones surrounding the filter paper discs were measured with a vernier caliper. Measurements between two observers on any one growth zone were usually within 2 mm., but repeated observations by the same observer were within 1 mm. All the measurements in this series were made by one observer.

Comment.—As the trial progressed, a number of modifications of the procedure were suggested by Guthrie.[11, 13] Some of these (e.g. type of filter paper, size of blood spot on the paper) were tested. Others (e.g. incubation temperature, preparation of spore suspension) were not tested or introduced into our routine, since the procedure was giving reproducible results and we wished to make our data comparable over the whole trial period.

Pheniplate.—Some tests were carried out using a "kit" for the Guthrie test put out by Ames Co., Indiana. This kit closely embodies the version of the Guthrie[11] test published in 1963. The medium (with the inhibitor) is packaged in powder form and reconstituted by adding water. A separate vial contains the organism, and blood standards of 2, 4, 4, 6, 6, 8, 12 and 20 mg. of phenylalanine/100 ml. on Whatman No. 3 filter paper are provided.

This report concerns tests carried out over a 20-month period between August 1962 and March 1964. A total of 6348 tests were performed. The

TABLE I.—SOURCES OF BLOOD FOR GUTHRIE TESTS, AUGUST 1962 - JANUARY 1964

Source of blood	*Number of observations*
Standards: whole blood	1290
plasma	108
Ontario Hospitals and Hospital Schools	957
Penitentiaries (serum)	1083
Kingston General Hospital	
Newborn babies	2400
Patients	30
Miscellaneous	18
Standards, newborn babies, etc. using Pheniplate	462
Total	6348

sources of blood for these tests are shown in Table I. In most instances, tests were done on whole blood but in certain situations plasma or serum was used. The diameters of the growth zones were not measured routinely for the first few months of the trial period; systematic measurements were started in December 1962.

RESULTS

Standards

"Outdated" blood from the blood bank—containing approximately 380 ml. of blood and 120 ml. of a dextrose/citrate anticoagulant—was used to make up standards for the Guthrie test. Sixteen milligrams of L-phenylalanine were added to 40 ml. of blood. This mixture was diluted with more blood from the same bottle to give a series of standards containing approximately 2, 4, 6, 8, 10, 12, 20 and 40 mg. of phenylalanine/100 ml. These are referred to loosely as the "2-mg. standard", the "4-mg. standard" and so on. Three separate standards were made up during the trial period (Standards I, II and III).

The plasma phenylalanine of the original blood and of each of the standards was estimated enzymatically. As expected, the phenylalanine levels were somewhat higher than 2 mg./100 ml., 4 mg./100 ml. and so on. This may be explained by (a) the fact that the original plasma contained phenylalanine and (b) the assumption that the added phenylalanine was not distributed throughout the entire volume of the blood but only through the plasma and the cellular water.[14]

Standard I was used for an eight-month period from December 1962 to July 1963. Table II summarizes the

TABLE II.—GUTHRIE TEST, STANDARD I. ESTIMATED PLASMA PHENYLALANINE LEVELS AND MEANS AND STANDARD DEVIATIONS OF CORRESPONDING GROWTH ZONES

Whole blood standard ("mg.%")	*Plasma phenylalanine (mg./100 ml.)*	*Number of observations*	*Growth zones (mm.) Mean*	*Standard deviation*
"Control"*	1.1	1	12.0†	—
" 2 mg."	2.8	91	16.1	0.90
" 4 mg."	5.9	91	18.1	1.10
" 6 mg."	7.5	91	19.2	1.10
" 8 mg."	9.7	108	20.8	1.00
"10 mg."	13.0	16	23.9	1.46
"12 mg."	16.0	16	24.9	0.90
"20 mg."	26.0	16	27.3	1.13
"40 mg."	48.6	16	30.6	1.32

*"Control"—no added phenylalanine.

†One observation; multiple observations were made on the "control" of Standards II and III.

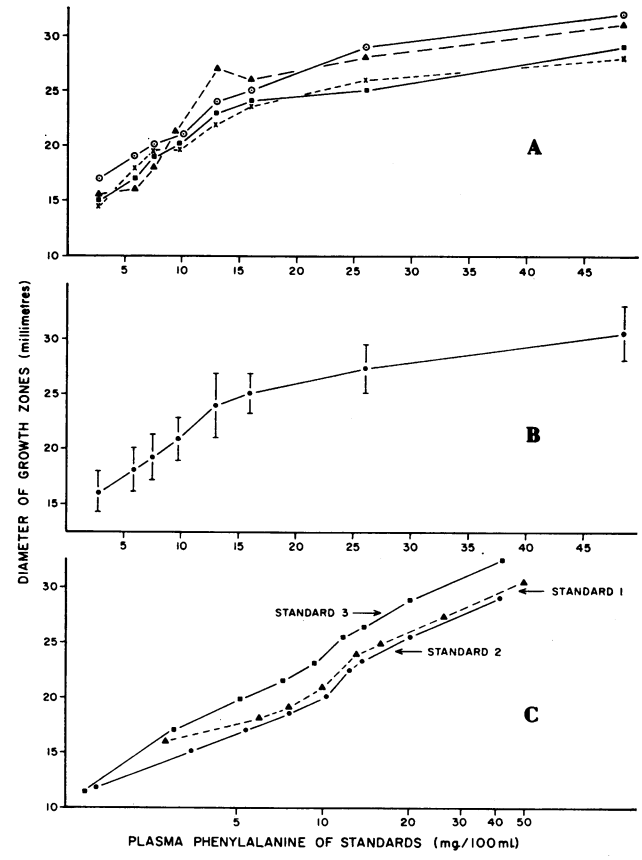

Fig. 1.—Relation between the plasma phenylalanine level of the whole blood standards and the diameter of the growth zones on the Guthrie test. A: Four typical sets of standards from four separate batches of tests (Standard I). B: Means (± twice the standard deviation) of the growth zones for all observations on Standard I (see Table II for numbers of observations [N]). C: Mean growth zones for all observations on Standards I, II and III related to the log. plasma phenylalanine level. For Standard II, N for each mean was 90 up to a plasma level of 10 mg./100 ml.; above this level N was 23. For Standard III the corresponding values for N were 45 and 8.

variations in the bacterial growth zones over this period. The means and standard deviations of the diameters of the growth zones are given for each of the plasma phenylalanine levels. Four representative curves relating the plasma phenylalanine level of the standard to the diameter of the growth zones from four separate batches of tests are shown in Fig. 1A. It can be seen that, in general, the extent of the bacterial growth increased with increasing concentration of the phenylalanine standard. There was some variation in the curves from one batch of tests to another. There was no evidence that the standard deteriorated over the eight-month period, since the extent of the growth zones at each particular phenylalanine level was no less at the end of the period than at the beginning.

In Fig. 1B the phenylalanine standards are related to the means of the diameters of the corresponding growth zones for all the observations on Standard I. The limits of plus or minus twice the standard deviation are shown. The slope of the curve was found to be steepest at the lower concentrations of phenylalanine. There was an inflection in the curve corresponding to a plasma phenylalanine level of about 12 mg./100 ml., and at higher phenylalanine levels the slope was much less steep. Fig. 1C shows the same mean measurements related to the logarithm of the concentration of the phenylalanine standards. Similar values for Standards

II and III are also shown. It can be seen that the relationship was approximately linear.

It was concluded that the day-to-day variations in the growth zones were such that at least one set of standards should be included in each batch of tests. It was also shown that the general shape of the curve relating extent of growth to phenylalanine concentration favoured the detection of changes in the phenylalanine level from about 1 to 12 mg./100 ml. but that this method was far less accurate at higher concentrations.

Type of Filter Paper

In the first accounts of his test, Guthrie[9, 11] advised the use of Whatman No. 3 filter paper. In later accounts, and in response to criticism of his methods, Guthrie[12, 13] laid great stress on the improved results obtained with a filter paper made by Schleicher and Schuell, No. 903 (S. and S.). The latter paper is said to be more highly absorbent.

Both types of filter paper were used in the present study. We were unable to find an advantage of one filter paper over the other. There was no evidence that the more absorbent S. and S. paper facilitated the collection of blood from the patients. In a series of direct comparisons of growth zones from the same sample of blood (Standard II) soaked on to each type of filter paper, it was found that the diameter of the growth zones at all phenylalanine concentrations were slightly larger with the S. and S. paper. However, the differences were small (the maximum difference in diameter at any phenylalanine concentration was 2 mm.), and seemed to be of no particular advantage, since the slope of the curve relating phenylalanine concentration to the diameter of the growth zone was not altered. For example, in Fig. 1C, Whatman filter paper was used for Standards I and III, and S. and S. paper for Standard II.

In another experiment of this type, using a single sample of blood from the same subject spotted on to the two types of filter paper, no difference was found in the size of the growth zones referable to the type of filter paper in 20 comparisons. Furthermore, the variations in the growth zones at different phenylalanine concentrations of the standards were comparable for each type of paper, and the variations in the size of the growth zones from newborn babies were no different when Whatman or S. and S. paper was used.

Size of the Spot

Another technical consideration, stressed by Guthrie[12] is the size of the blood spot on the filter paper. According to Guthrie, this should be between ⅜ and ½ inch in diameter and the punched-out disc ¼ inch in diameter.

A sample of blood was taken from one subject and spotted on to filter paper in 20 places according to the specifications in the previous paragraph. Other filter papers were more liberally soaked with the same sample of blood to give areas of blood-staining more than 3 inches in diameter. In 20 comparisons of subsequent Guthrie tests, the diameters of the growth zones from the more generously soaked filter papers were slightly bigger than those from the spotted papers. However, the differences were small; the diameters were the same in seven pairs, 1 mm. larger in 12 pairs and 2 mm. larger in one pair.

In many of the blood samples collected from newborn babies, the blood spot on the filter paper was so small that it was not possible to punch out a disc of blood-soaked paper a full quarter of an inch in diameter. In other samples the area of blood-staining was large enough on one side of the paper but the blood had not soaked completely through to the other side. These were referred to as "poor" samples. It was decided to test poor samples if it were judged that one-half or more of the paper discs was soaked in blood. In practice, the majority of poor samples were at least three-quarters blood-soaked.

The distribution of the diameters of the growth zones from 888 poor samples from full-term newborn babies was compared with that of 1224 "good" samples also taken from full-term babies (Table III). The growth zones from the poor samples were significantly smaller than those from the good samples ($\chi^2 = 39.4$; d.f. = 6; $p < 0.01$) but the differences were small. Similar differences were found between the good and poor samples from premature babies (Table III).

This matter was investigated further. The usual quarter-inch discs were punched out from the whole blood phenylalanine standards. A quarter segment was cut from one disc. Another disc was cut in half. A half disc was then halved and halved again. Guthrie tests were run with these various fragments. The resulting growth zones were approximately circular regardless of the shape of the fragment of paper; the diameters of the growth zones are shown in Fig. 2, which also

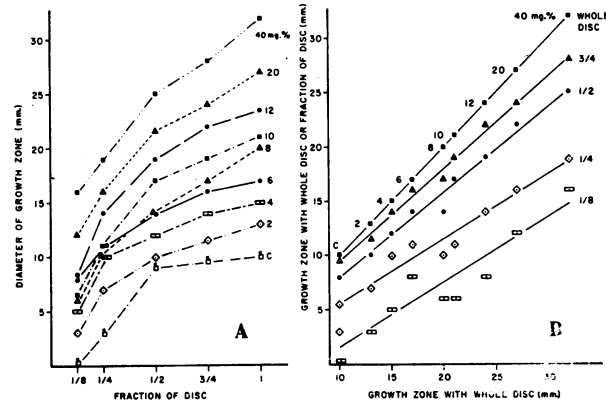

Fig. 2.—A: Relation between bacterial growth zones on the Guthrie test and blood phenylalanine standards, using various fractions of the filter paper discs (see text). B: Diameters of bacterial growth zones with whole discs related to growth zones with the various disc fractions for each blood standard.

shows the relation between the size of the growth zones from the various fractions of the disc and the size of the growth zones from the whole disc for each of the phenylalanine standards. There was a good correlation between the growth from the half and three-quarter discs and that from the whole disc at all levels of the phenylalanine standard. This seemed to justify the testing of "poor" samples provided that at least half the disc was soaked in blood, since a "corrected" phenylalanine blood level could be estimated by reference to the standards run on the same day. By assuming that all poor samples were only half soaked in blood, a maximal correction was applied to guard against underestimating the phenylalanine content of the sample.

TABLE III.—THE GUTHRIE TEST: DISTRIBUTION OF DIAMETERS OF BACTERIAL GROWTH ZONES ACCORDING TO VARIOUS GROUPS OF BLOOD SAMPLES.

Blood samples	Number of observations	Diameter of Growth Zones (mm.) 9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
Standards I, II, II,																											
"Control"	117	—	7	49	46	14	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
" 2 mg.%"	227	—	—	—	—	4	19	64	63	64	12	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
" 4 mg.%"	208	—	—	—	—	—	—	3	24	57	58	39	19	7	1	—	—	—	—	—	—	—	—	—	—	—	—
" 6 mg.%"	208	—	—	—	—	—	—	—	2	6	47	68	52	20	8	3	2	—	—	—	—	—	—	—	—	—	—
" 8 mg.%"	251	—	—	—	—	—	—	—	—	—	5	33	67	27	36	25	10	3	—	—	—	—	—	—	—	—	—
"10 mg.%"	51	—	—	—	—	—	—	—	—	—	—	—	—	5	13	15	7	2	5	3	1	—	—	—	—	—	—
"12 mg.%"	51	—	—	—	—	—	—	—	—	—	—	—	—	1	5	9	13	14	5	2	2	—	—	—	—	—	—
"20 mg.%"	51	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	5	6	14	11	8	5	2	—	—	—	—
"40 mg.%"	54	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	3	1	4	9	10	4	14	7	2
Normal subjects.	78	—	10	36	24	8	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Phenylketonurics	66	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	3	1	8	12	18	19	5	—	—
Ontario Hospital (Mowat Division)	318	—	22	161	114	19	1	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—
Penitentiary (serum)	340	—	—	7	23	105	129	59	15	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Newborn																											
Full term—																											
good samples	1224	—	43	263	571	294	45	8	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
poor samples	888	6	73	226	386	159	34	4	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Premature—																											
good samples	168	—	9	27	56	50	19	5	1	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
poor samples	108	1	3	24	48	26	2	2	1	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—

Clear Zones

With some blood samples the agar beneath and around the filter paper disc showed no perceptible bacterial growth at all. Since there was some background bacterial growth throughout the rest of the agar on the plate, the area around these particular blood samples appeared as a clear zone. This phenomenon was seen with six blood samples, all taken from newborn babies.

In two cases it was thought that the clear zones were explained by the fact that the babies were being treated with antibiotics at the time the blood sample was taken. It was established that this strain of *B. subtilis* was sensitive to these particular antibiotics. In an older child being treated with massive intravenous doses of tetracyclines, penicillin and sulfonamides for pyogenic meningitis, the Guthrie test showed a clear zone some 30 mm. in diameter. However, this effect was not consistent. A number of other babies and older subjects who were taking antibiotics at the time the blood samples were taken had normal growth zones on the Guthrie test. Furthermore, in four cases, clear zones were seen from blood samples of babies who had had no antibiotics; previous or subsequent Guthrie tests from two of these babies were normal. No satisfactory explanation for these clear zones was found.

Serum, Plasma and Blood

In one group of subjects (Kingston Penitentiary), Guthrie tests were performed with serum rather than whole blood. It was found that the diameters of the bacterial growth zones from this group were consistently larger by about 2 mm. than those from the whole blood control standard or whole blood samples from normal subjects, patients in the Ontario Hospital, or newborn babies (Table III). In order to show that this was not just due to a fundamental difference between the penitentiary population and the other groups, multiple comparisons were made between Guthrie tests done with serum and blood from the same sample from a normal subject (Table IV). The average diameter of 35 growth zones from whole blood was 12.7 mm. whereas that from serum was 14.2 mm. The differences between blood and serum were highly significant.

Whole blood was treated differently from serum in that it was autoclaved prior to testing and serum was not. For this reason, in the previous experiment, 35 Guthrie tests were done on serum which had been autoclaved. The mean growth zone diameter (12.6 mm.) was almost identical with that from whole blood. Further observations showed that plasma also gave larger growth zones than whole blood and that this was abolished by autoclaving.

In the same way, larger growth zones were found with unautoclaved fresh blood than with autoclaved fresh blood but exact measurements of the growth zones from unautoclaved blood were difficult because of the blood pigments which had diffused out from the paper disc on to the agar. Similar experiments showed that this difference between autoclaved whole blood and unautoclaved serum was lost slowly over a period of weeks if the serum was stored on filter paper at room temperature. A slight but definite loss of activity was found if the serum was stored frozen for several months.

If these observations were due to the destruction of phenylalanine by autoclaving or storage of the blood, serum or plasma on filter paper at room temperature for a period of weeks, one would expect that (a) the whole-blood standards stored on filter paper for several months would produce successively smaller growth zones and (b) that autoclaving plasma or serum stored on filter paper for months should further reduce the phenylalanine content, as judged by the size of the bacterial growth zones. In fact, neither of these phenomena occurred. It was concluded that, in addi-

TABLE IV.—COMPARISON BETWEEN GUTHRIE TESTS ON WHOLE BLOOD, SERUM AND AUTOCLAVED SERUM FROM A SINGLE SAMPLE OF BLOOD

	Number of observations	Diameter of growth zone (millimetres) 12	13	14	15
A Whole blood (autoclaved)	35	17	11	7	0
B Serum (not autoclaved)	35	1	5	16	13
C Serum (autoclaved)	35	17	6	2	0

χ^2 between A and B = 27.92; d.f. = 3; $p<0.01$.

tion to phenylalanine, fresh serum, plasma and probably whole blood contain a growth-promoting factor (or factors) for *B. subtilis* under the conditions of the Guthrie test, which is destroyed by autoclaving or storage on filter paper at room temperature. The identity of this factor was not established.

ADMINISTRATION AND COST

In general, the cost of the materials used in the Guthrie test was low and the test was easy to carry out and interpret. Since there is currently great interest in whether this test should be applied routinely to all newborn babies before discharge from hospital, we have described our routine in some detail.

A preliminary explanation of the purpose of the test and the technique of collecting blood samples was given to the nursing and medical staff. Printed instruction sheets were posted in all of the newborn nurseries, and each nursery was supplied with a stock of filter papers (9 cm. in diameter). Blood was collected by the nurses from a heel prick on all babies on the day of discharge from hospital. In the case of premature babies, blood was collected at the age of one week and at weekly intervals thereafter until discharge home. The baby's name, date and hour of birth, and the date and hour of blood sampling were recorded on the filter paper. The filter paper blood samples were stored on the ward at room temperature and every two weeks these were delivered to the laboratory. Guthrie tests were run every two weeks, each batch containing about 88 samples from newborn babies. Negative tests were not reported, and positive or borderline tests were followed up through the baby's physician or directly to the patient at home.

The laboratory time required for this routine was about four hours every two weeks. Given access to the usual laboratory facilities, including the use of an autoclave and incubator, the cost of 1000 tests on newborn babies was approximately $100, or about 10 cents per test (Table V).

TABLE V.—APPROXIMATE COST OF 1000 GUTHRIE TESTS ON NEWBORN BABIES

Culture of *B. subtilis* (ATCC 6051)	$ 5.00
Filter paper	6.75
Disposable Petri dishes	7.00
Chemicals and agar	2.75
Technician time: 42 hours (Collection and delivery of filter paper and blood samples; preparation of solutions and media; setting up and reading nine runs of test)	84.00
Total	$105.50

PATIENTS WITH PHENYLKETONURIA

Blood samples were taken from 66 patients with untreated phenylketonuria. The diagnosis had been established by the clinical picture, urine tests and demonstration of a grossly elevated plasma phenylalanine level (14-42 mg./100 ml.) by the enzymatic method of Udenfriend and Cooper.[10] The growth zones on the Guthrie test from these samples were compared with those from blood samples from 78 subjects who were known not to have phenylketonuria because of normal plasma phenylalanine levels (less than 2.5 mg./100 ml.). The results are shown in Fig. 3. There was a clear-cut separation of normals from phenylketonurics with no overlap.

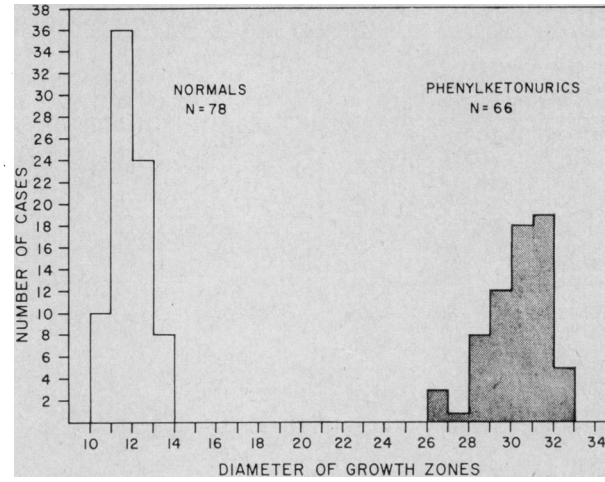

Fig. 3.—Comparison between diameters of bacterial growth zones in Guthrie tests from subjects with normal plasma phenylalanine levels and patients with untreated phenylketonuria.

APPLICATIONS

(a) *Ontario Hospitals*

In August 1961, a urinary survey for cases of phenylketonuria was carried out among patients of the Rockwood Division of the Ontario Hospital, Kingston. The urine of each of the 516 patients was tested once with Phenistix by the nurses on the wards. The urines of 58 patients were reported as showing some colour change on the test. For administrative reasons, it was more convenient to measure the plasma phenylalanine levels of all these patients than to discontinue whatever drugs they were taking and retest the urines. Out of the 58 patients, six were shown to be cases of phenylketonuria. From other information, it was known that a further patient with phenylketonuria was in this division of the hospital at the time, so that it was clear that she had been missed by this survey.

In October 1962, the same division of the Ontario Hospital was resurveyed for cases of phenylketonuria using Guthrie tests. Out of the 539 patients tested, nine were suspected of having phenylketonuria and the diagnosis was confirmed in all of these by plasma phenylalanine measurements. Of these nine patients, six were patients found by the urinary survey of 1961 and the seventh was the patient known to have been missed at that time. The eighth case of phenylketonuria was entirely unsuspected; he had also been tested but missed in the urinary survey of 1961. The ninth patient had been admitted to the hospital between the times of the two surveys.

In contrast to the urinary survey, there were no "false positives", that is, patients in whom the screening test indicated phenylketonuria but in whom the diagnosis was not confirmed by enzymatic measurement of the plasma phenylalanine level. It was, of course, impossible to know whether there were any "false negatives".

In this group of Guthrie tests the diameters of the growth zones were not measured. In a later, similar survey of 348 patients in the Mowat Division of the same hospital, the growth zones were measured. The results are shown in Table III. As can be seen, one case of phenylketonuria was discovered; the diagnosis was confirmed by measuring the plasma phenylalanine level. No "false positives" were found.

(b) *Penitentiary*

There are reasons for believing that there may be more persons with untreated phenylketonuria and normal or slightly subnormal intelligence in the general population than is indicated by urinary surveys.[17] Untreated phenylketonurics with comparatively high intelligence tend to have relatively low blood phenylalanine levels when compared to untreated phenylketonurics in general. The excretion of phenylpyruvic acid in phenylketonuria largely depends on the blood phenylalanine level and it is possible for the patient with relatively high intelligence to have a negative urine to the ordinary screening tests.

On the probably erroneous assumption that the population of a federal penitentiary would contain a high proportion of persons with subnormal intelligence and, therefore, a higher proportion of unrecognized phenylketonurics than the general population, it was decided to run a pilot survey of blood samples from such an institution. All inmates on admission to Kingston Penitentiary have blood drawn and the serum tested for syphilis. Part of the serum was soaked on to filter papers and Guthrie tests were carried out.

A total of 1083 sera were tested in a 14-month period. The growth zones of 340 of these samples were measured and the results are shown in Table III. As described previously, the diameters of the growth zones were somewhat larger than those found for whole blood from comparable populations. However, no very large growth zones were observed and no cases of phenylketonuria were discovered.

The points of interest about this investigation were: (a) that it was a workable proposition to test sera *en masse*,[18] (b) that no "false positives" were found and (c) that larger growth zones were discovered with sera than with whole blood.

NEWBORN BABIES

The general routine of collecting blood samples from the newborn nurseries has been described. No cases of local sepsis or other complications of the heel prick were encountered. Several mothers expressed spontaneous interest in and appreciation of the test. The major defect of this program was the comparatively high proportion of "poor" blood samples. It was thought that this could be corrected by more frequent demonstration and encouragement to the nurses and by the use of printed circles on the filter paper to indicate the size of blood spot desired, as described by Guthrie and Susi.[11] The rationale for testing "poor" samples has been explained.

In the period from December 1962 to January 1964, 2400 blood samples were tested from 2198 babies born in the Kingston General Hospital. Approximately three-quarters of these tests were performed on the fourth to sixth day of life. Premature babies kept in hospital longer than a week were tested on the seventh day of life and weekly thereafter until the time of discharge.

Table III shows the distribution of the measurements of the Guthrie test from these samples. The group was divided into full-term and premature babies and "good" and "poor" blood samples. The great majority of the growth zones from the "good" samples from both premature and full-term babies were no larger than those of the control blood standards or those of normal subjects; about 1% of the growth zones were very slightly larger. Except for one case, the largest growth zones from the "poor" samples were about the same. Even if a maximal correction were applied to the "poor" samples, by assuming that all of them were only half soaked in blood, there was no indication that any baby might have abnormally high blood phenylalanine levels, save one.

This one patient was a premature baby and his blood sample gave rise to a growth zone some 22 mm. in diameter. This was well outside the normal range and compatible with phenylketonuria. However, on the day this blood sample was taken, his plasma phenylalanine level was only 3.1 mg./100 ml. Further Guthrie tests were normal. This child was subsequently diagnosed as having idiopathic renal acidosis, but we were unable to explain the abnormal Guthrie test on this basis. This sample was the only "false positive" in the entire series.

We did not find a case of phenylketonuria among the newborn babies during this period by means of the Guthrie test. However, in another hospital a baby was born to a family which had already had two children with phenylketonuria. At the age of 3 days, a blood sample gave a bacterial growth zone of 24 mm. on the Guthrie test. The plasma phenylalanine level was 20.5 mg./100 ml. on the same day. Observations on subsequent days are shown in Table VI. It is reasonable to assume that, had this baby been an unsuspected case in our newborn series, she would have been detected if blood had been taken at any time from the third day of life onward.

TABLE VI.—GUTHRIE TESTS AND PLASMA PHENYLALANINE LEVELS ON A NEWBORN BABY WITH PHENYLKETONURIA

Age	*Guthrie test (diameter of growth zone in mm.)*	*Plasma phenylalanine (mg./100 ml.)*
3 days	24	20.5
4 "	28	—
5 "	27	—
6 "	28	—
8 "	28	46.3
13 "	18	3.9
4 weeks	11	0.8
6 "	11	0.7
2 months	16	2.0
3 "	12	0.8
4 "	15	2.2
5 "	20	6.6

The baby was breast-fed for the first week of life and started on a low phenylalanine diet at the age of 8 days.

PHENYLKETONURIC PATIENTS ON DIETARY TREATMENT

Guthrie[12] has claimed that this test has been used successfully for measuring the blood phenylalanine level during treatment of phenylketonuria with a low phenylalanine diet. Our experience in this respect is limited to two patients. The first case has been described already and the Guthrie tests are shown in Table VI in relation to the plasma phenylalanine levels while the baby was being treated with a low phenylalanine diet.

The second child, a 10-month-old boy, was referred because his elder brother had been admitted to an Ontario Hospital School where phenylketonuria was diagnosed. Urine tests on the younger boy suggested that he had phenylketonuria also. The diagnosis was confirmed and treatment with a low phenylalanine diet was started in hospital. Fig. 4 shows the effects of the diet on the plasma phenylalanine level and the Guthrie test.

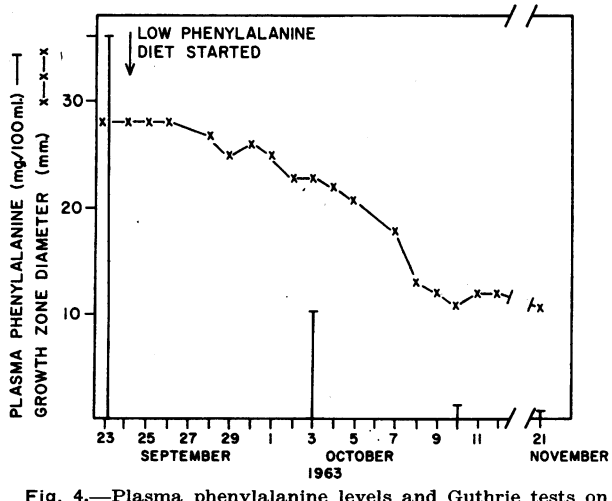

Fig. 4.—Plasma phenylalanine levels and Guthrie tests on a 10-month-old boy with phenylketonuria after treatment with a low phenylalanine diet.

In both cases, the Guthrie tests reflected the plasma phenylalanine levels with fair accuracy when the levels were less than 10 mg./100 ml. At higher phenylalanine levels, the Guthrie test was less accurate, as was predicted by the observations on the standards (Fig. 1).

DISCUSSION

Guthrie has claimed that the inhibition assay test gives a moderately accurate measure of the phenylalanine level in the blood and that the test is sensitive enough to use as a screening procedure for phenylketonuria on all newborn babies. Many objections have been raised to these claims.[19] For example, Scheel and Berry[14, 20] have severely criticized the Guthrie test on technical grounds. These workers compared estimates of the blood phenylalanine level by Guthrie tests with estimates of the serum phenylalanine level obtained by the method of La Du and Michael[21] and by paper chromatography. The results suggested that whereas all three methods gave roughly comparable results when the serum phenylalanine level was between 20 and 40 mg./100 ml., at lower serum phenylalanine levels the Guthrie test gave considerably higher results than the other two methods. In newborn babies, Scheel and Berry found that in 21% of the samples the Guthrie test indicated a blood phenylalanine level of 8 mg./100 ml. or more when the serum level was 2 mg./100 ml. or less by paper chromatographic estimations. If the blood phenylalanine level was 8 mg./100 ml. or more in the first few days of life the most likely explanation would be that the subject had phenylketonuria. In other words, Scheel and Berry found that the Guthrie test gave a very high proportion of "false positive" results.

Our experience has been quite different. We found one "false positive" Guthrie test in 2400 blood samples from newborn babies and none in a further 1734 from other sources. MacCready,[22] from an experience of 50,000 tests on newborn babies, found that about one in 700 gave borderline results suggesting a blood phenylalanine level of between 6 and 12 mg./100 ml.; repeat Guthrie tests were normal. A recent progress report[23] of the PKU Screening Program at present being conducted in the United States by Guthrie and the Children's Bureau (which includes MacCready's series) states that in the first 238,161 tests on newborn babies there were 186 in which the diagnosis of phenylketonuria was suspected. Twenty-two babies were subsequently shown to have phenylketonuria. This gives an incidence of about one "false positive" test in 1450. Brandon and Ashley[18] reported an approximately similar incidence (1 in 1650) of "false positives" in Guthrie tests on 11,556 samples of blood serum.

It has been suggested[12, 18, 22] that the anomalous findings by Scheel and Berry might have been due to their use of Whatman instead of Schleicher and Schuell filter paper for collecting blood samples. Our observations with these two types of filter paper do not support this explanation.

In contrast to Scheel and Berry, we have found that the Guthrie test reflects the blood phenylalanine concentration more accurately when it is below 10 mg./100 ml. than when it is above this level. This favours the detection of slight increases in the blood phenylalanine level such as may be found in phenylketonuria in the newborn period. The Guthrie test clearly distinguishes between normal subjects and older patients with untreated phenylketonuria (Fig. 3). Like Guthrie,[24] we were able to find, by means of this test, cases of phenylketonuria in a mentally retarded population that had been missed by urine tests. In the Rockwood series six patients with phenylketonuria were found by urine tests and two further patients by Guthrie tests. The reason may be either the irregular excretion of phenylpyruvic acid in the urine of older patients with untreated phenylketonuria or the difficulty in collecting urine samples from grossly retarded patients.

We encountered remarkably few technical difficulties with the test itself. The occasional appearance of clear zones with little or no bacterial growth around the filter paper disc has been referred to; some of these were attributable to antibiotics but some were not. The justification for using "poor" blood samples has been presented.

Pheniplate (Ames Co.) is a commercially produced kit for Guthrie tests which has proved convenient and reliable in our hands. It is not yet clear how the cost of Pheniplate would compare with our usual procedure.

The presence of a possible growth-promoting factor in this test, other than phenylalanine, in fresh serum, plasma and probably blood, is of some interest. According to Guthrie and Susi,[12] in addition to phenylalanine, phenyllactic and phenylpyruvic acids can reverse the inhibitory effects of β-2-thienylalanine on this strain of *B. subtilis*. It is unlikely that these substances can explain our findings since, under normal conditions, there are only negligible quantities of either of them in the blood.[25] Originally, Guthrie[9] stated that proline could also reverse the inhibition of β-2-thienylalanine but he has since denied this.[12] Guthrie[9] has also identified a factor (the TA factor) in the urine of normal newborn babies and some patients with phenylketonuria which will reverse the effects of β-2-thienylalanine in this test. We do not know whether this factor could account for our findings.

It is hard to know whether the Guthrie test ever gives "false negative" results, i.e. results which indicate a normal blood phenylalanine level when in fact the level is raised. There was no suggestion of this possibility from the observations on patients known to have high blood phenylalanine levels or from the blood standards. It seems likely that a "false negative" result would occur if the blood sample contained antibiotics or other factors giving rise to clear zones as well as an increased phenylalanine concentration.

In addition to criticism of the test itself, objections have been raised to the use of Guthrie tests as a screening procedure for phenylketonuria on all babies in the early newborn period. It is argued that, except in the very rare situation where the mother also has the untreated disease,[26] the newborn baby with phenylketonuria has normal phenylalanine levels. By the second or third week of life, in the absence of specific treatment, the level of phenylalanine in the blood rises to 30 or 40 times the normal.[27] It is assumed that the rate at which the blood phenylalanine level rises depends on the intake of protein in the diet. Theoretically, if the newborn baby takes little milk in the first two or three days, it is possible for the child to have phenylketonuria and yet maintain normal or near normal blood phenylalanine levels up to the usual time of discharge from hospital on the third to sixth day of life. In other words, the Guthrie test may be normal at this age, the case may be missed, and both parents and physician may enjoy a false sense of security.[14] A very similar argument is valid with regard to urine tests with ferric chloride or Phenistix in the early newborn period.[7]

Observations on the blood phenylalanine level in the first week of life in subjects with phenylketonuria are few, but the indications are that in most cases the blood phenylalanine level rises high enough in the first two or three days of life to be detected by screening procedures.[28] In practice several cases of phenylketonuria have now been diagnosed by Guthrie tests at this age. In the PKU Screening Program,[23] 20 of the 22 babies with phenylketonuria diagnosed in the first 238,161 tests were detected by blood samples taken on the second to sixth days of life. Furthermore, 163,712 follow-up Guthrie tests on urine collected a few weeks after birth failed to detect any new cases of phenylketonuria, which suggests that, in fact, none had been missed by the Guthrie[23] tests on the blood in the early newborn period.

A further objection to the performance of Guthrie tests on all newborn babies stems from some recent observations of Hsia *et al.*[28] and La Du *et al.*[29] These workers have found that a proportion of babies, especially premature babies, develop a transient rise in the blood phenylalanine level in the neonatal period. The explanation for this phenomenon is not clear[30] but it is feared that such babies may be regarded as cases of phenylketonuria and given unnecessary dietary treatment. This seems to be a theoretical possibility, although the serum phenylalanine levels reported by Hsia and La Du were considerably lower than those usually found in phenylketonuria. It is not proposed that phenylketonuria be diagnosed by Guthrie tests alone,[12] but that the plasma or serum phenylalanine levels should be formally estimated by other procedures if the Guthrie test is positive. Furthermore, if there were doubts and a low phenylalanine diet were started, the diagnosis of phenylketonuria could be confirmed or refuted a few weeks later by

phenylalanine load tests or reversal to a milk diet. Less harm would be done by giving a low phenylalanine diet to a normal baby for a few weeks than by failure to treat a case of phenylketonuria.

A final objection to the use of Guthrie tests on all newborn babies may be mentioned. It is argued that screening procedures in the newborn period should also test "for other disorders which are amenable to prevention, detection and correction and are more significant to the health of the population" and that concentration of effort on the Guthrie test could "preclude attention to other problems and even delay research on PKU itself".[19] Few would deny that it is desirable to develop screening procedures for other disorders "amenable to prevention, detection and correction" in the newborn period, but this is no argument against a screening procedure for phenylketonuria. The overwhelming arguments for the Guthrie test are that it is simple, comparatively cheap and detects cases of phenylketonuria at an age when treatment is most effective.

SUMMARY

Experience with over 6000 Guthrie tests has been reviewed. Observations with whole blood standards indicated a linear relation between the logarithm of the concentration of phenylalanine in the blood and the diameter of the bacterial growth zone on the Guthrie test. The maximal sensitivity of the test was in the range from 1 to 10 mg. of phenylalanine/100 ml. of blood.

Certain technical aspects of the Guthrie test (type of filter paper, size of blood spot on the paper, cost) were investigated. Evidence of the presence in fresh blood, plasma or serum of a bacterial growth-promoting factor in this test, other than phenylalanine, is presented.

The Guthrie test was shown to distinguish clearly between normal subjects and patients with untreated phenylketonuria.

Examples of the use of the Guthrie test in screening populations for phenylketonuria are described; these included a mental hospital, a penitentiary and 2400 tests on newborn babies.

The Guthrie test is discussed in the light of objections which have been raised to it on technical grounds and as a screening procedure for phenylketonuria in the newborn period.

It is concluded that the Guthrie test is sufficiently simple, cheap and reliable in the detection of phenylketonuria to warrant its serious consideration as a screening procedure for all babies in the newborn period.

We wish to thank the large number of people who co-operated in the collection of blood samples. These included the medical superintendents of the Ontario Hospitals or Hospital Schools at Orillia, Smiths Falls, Cedar Springs, Aurora, Port Arthur and Kingston, Ontario; the physicians and nursing staff of the newborn nurseries at Kingston General Hospital and Dr. William Amodeo and his staff at Kingston Penitentiary. We would also like to thank Professor N. A. Hinton, Queen's University, for help with setting up the Guthrie test, and the Ames Company, Elkhart, Indiana, for supplies of Pheniplate.

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PAGES OUT OF THE PAST: FROM THE JOURNAL OF FIFTY YEARS AGO

THE INCORRECT DIAGNOSIS

We all know the man who has made an incorrect diagnosis, but who, before the operation or post mortem is over, has nearly convinced himself that he did make the correct diagnosis and before night is quite sure of it. For him no good has come from the lesson . . . In this connexion is an excellent saying, "It is easy to be wise after the event, but very difficult to be wiser," which can be illustrated by an example. A patient dies in whom you have made a diagnosis of typhoid fever, and at autopsy miliary tuberculosis is found. You are *wise* after the event but the laboratory *Diener* or a first year student is just as wise as you. To be *wiser*, or in other words to lessen the chance of your making the same mistake again, is quite another matter. You will certainly be no wiser if you have persuaded yourself that after all you did think it was miliary tuberculosis. For one's own training it is better to make an incorrect diagnosis than none at all—if you call yourself to account afterwards.—Thomas McCrae, *Canad. Med. Ass. J.*, 4: 586, 1914.