An Improved Screening Procedure for Blood Phenylalanine

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THIS PAPER describes a modification of the method of Hill and associates (*1*) for the screening of blood phenylalanine. The procedure is both simpler and more sensitive. It is based on the observation that the steaming of dried blood on filter paper fixes the proteins irreversibly on the paper, whereas the other blood solutes can be recovered by subsequent elution. This procedure takes the place of dialysis and permits a fivefold saving of material, since in our experience the Technicon dialyzer yields at most only 20 percent of dialyzable solutes. Another improvement is the use of a smaller flow cell, which eliminates carryover and makes it possible to run the specimens without a water wash in between.

Materials and Methods

Manifold and sampler. The manifold is shown in figure 1. The double crook, described by Gray and associates (*2*), is easily made and fitted to sampler I. If sampler II is available, the "sample air" line becomes the sample line and the "air-water" line pumps only air.

In the work reported here, the usual speed was 60 specimens per hour. With sampler II and a modified pump operating at nearly twice the speed of the original model (a 15-tooth sprocket replacing the 8-tooth sprocket on the motor shaft), it is possible to run 120 specimens per hour.

Flow cell. The modified flow cell consists of a section of ordinary soft glass tubing, about 3.5 mm. in internal diameter and 50 mm. in length. It has about one-half the inside diameter and one-fourth the capacity of the cell provided with the Technicon fluorometric unit. It is used with slits No. 4. A few layers of adhesive tape wrapped around the top and bottom of the tube insure its correct position, facing the middle of both slits. The range selector of the fluorometer is set at 30*X* and the full-scale recorder at 10. The cell outlet on the outside of the fluorometer should drip freely into an open container to avoid back pressure. With the smaller flow cell, the danger of carryover is practically eliminated. For example, if normal blood disks follow a 12-mg. control, the first normal peak remains distinct and is raised above the following normal peaks by only about 20 percent.

Reagents. The reagents, essentially those of Hill and associates (*1*), are made with ordinary distilled water—dipeptide, 80 mg. of L-leucyl-L-alanine in 100 ml. of water; ninhydrin.H_2O, 5.3 gm. per liter of water; and succinate buffer, 0.04 M solution, which is prepared daily from a stock solution that is molar in succinic acid and whose pH has been adjusted to 5.8 with NaOH. This stock solution is stable for months. The three reagents are stored in the refrigerator.

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The copper reagent consists of 16 gm. of NA_2CO_3, 0.65 gm. of $KNaC_4H_4O_6.4H_2O$, and 0.6 gm. of $CuSO_4.5H_2O$. The three salts are dissolved separately, mixed in the given order, and made up to 1 liter.

Standards. The standards consist of 0.05, 0.10, 0.15, and 0.20 mg. of phenylalanine in 100 ml. of water. Add chloroform as a preservative. These standards correspond to 3, 6, 9, and 12 mg. per 100 ml. of the unknown specimens. Add 0.5 ml. of Brij (*A*) per liter of dilute buffer or distilled water used in the procedure.

Controls. Add 20λ of a 1 percent phenylalanine solution in saline to 10 ml. of normal blood. Deposit this blood on filter paper S and S 903 (*B*) in the form of even drops of about 0.025 ml. Allow to dry at room temperature. The final phenylalanine concentration of this material (about 4 mg. per 100 ml.) is determined on a trichloracetic acid filtrate by the method of McCaman and Robins (*3*) or the procedure we shall outline.

Steam treatment of specimens. Specimens and controls are autoclaved together for 3 minutes at 121° C. To avoid water condensation, care should be taken that a vacuum follows the steam before the autoclave is opened. It has since been found that steaming at atmospheric pressure for 15 seconds instead of autoclaving is as effective in fixing blood proteins and permits a better recovery of blood solutes.

Procedure. The sample cups are filled with 0.6 ml. of distilled water with the aid of an automatic syringe. This is the minimal volume required by the tube arrangement in the manifold. It was chosen in preference to 1 ml. to minimize the waste of material. Each cup receives a quarter-inch disk punched from the middle of the steamed blood spot. The disks should be fully immersed. After 15 to 30 minutes they are stirred and flicked off with a fine metal hook. They may also be speared on ordinary pins, with which they are stirred, and discarded. The cups are placed in succession in the sampler. Water cups (to locate the baseline), control disks, and if desired, sets of standards are inserted at regular intervals. Figure 2 illustrates the beginning of a routine run. Standards equivalent to 3, 6, 9, and 12 mg. per 100 ml. are on the right. *C* is a 4 mg. control, *W* a water wash. A borderline specimen, *G*, whose content was 6–8 mg. per 100 ml. by the Guthrie test, stands out conspicuously.

Validity of Method

We wish to discuss several points relative to the validity of the method. For greater accuracy, the results reported were obtained from carefully measured drops (usually 10λ) deposited on the filter paper, and then steamed and cut out.

Effect of steaming. Hot steam is indispensable for the complete fixation of blood proteins on paper. Dry hot air is ineffective. Even after a 24-hour elution in 1 ml. of water, a

Figure 1. Manifold for phenylalanine determination

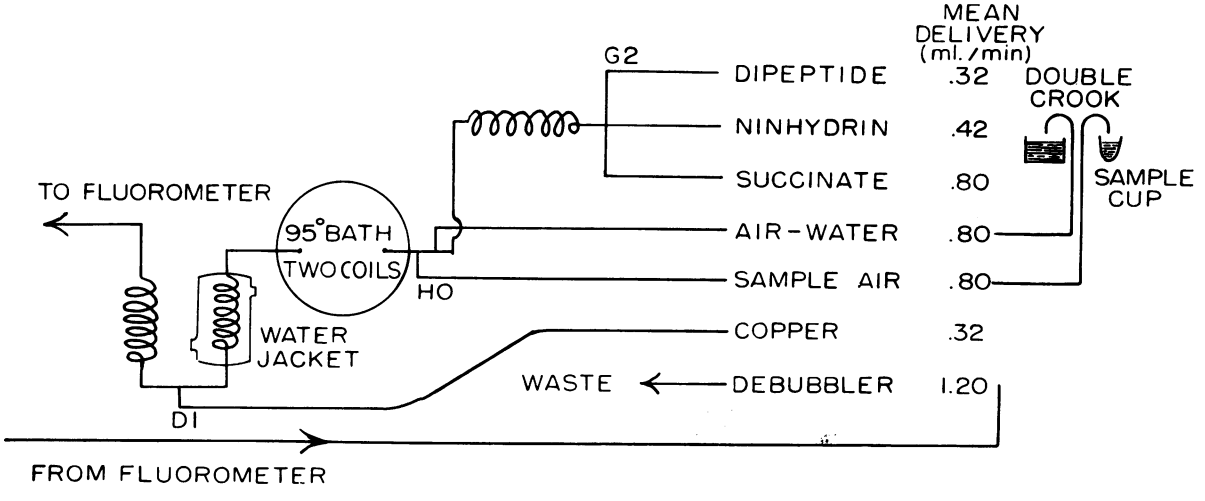
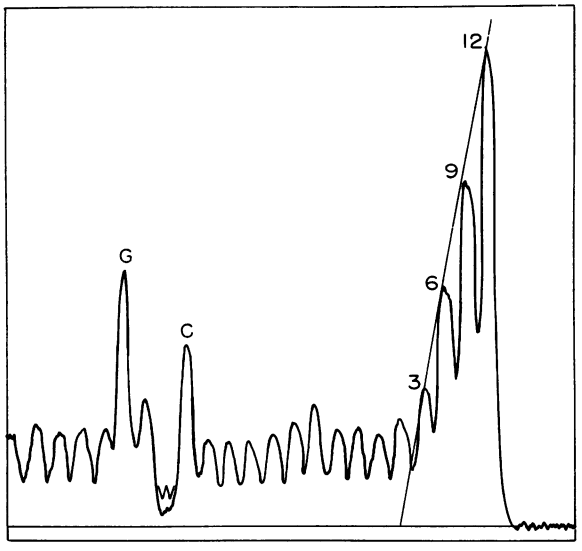


Figure 2. Routine run for blood phenylalanine at 60 specimens per hour



quarter-inch steamed blood disk fails to release any protein detectable by trichloracetic acid. Autoclaving for 3 minutes at 121° C. is a simple procedure and permits the handling of large numbers of filter paper cards in one operation. It is already used in the Guthrie test (*4*) to prevent the spread of blood "pigments" into the agar. We have since found that steaming at atmospheric pressure is just as effective as autoclaving and requires only 15 seconds. This can be done with a simple apparatus to be described elsewhere. It permits a better recovery of solutes such as phenylalanine, urea, and uric acid, and is indispensable with glucose, which is not recovered in adequate amounts after autoclaving at 121° C.

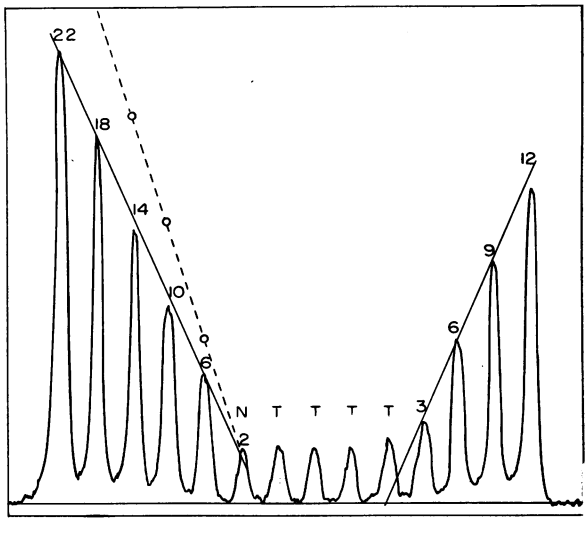
Whether steaming is done at 100° C. or 121° C., a remarkable fact is that the amount of solute recovered is constant and reproducible and represents a constant percentage of the solute, regardless of its concentration. It is also noteworthy that the loss of solute becomes proportionately less as the steaming time is extended. This suggests that the solute becomes bound to the paper or to the denatured proteins in the process but is not actually destroyed.

With phenylalanine, 3 minutes of autoclaving gave a recovery ratio of about 0.85 for water standards, 0.85 for phenylalanine added to serum, and 0.75 for phenylalanine added to whole blood. Figure 3 illustrates the point. *N* is a normal blood and *T* its trichloracetic acid filtrate (obtained by the method outlined below). The straight line on the right is given by standards, the solid straight line on the left by controls (obtained by adding various amounts of phenylalanine to the normal blood). The broken straight line gives the theoretical values expected in the absence of loss. It is clear that the percentage of recovery, estimated here at about 0.76, is practically the same for all the controls, from 6 mg. per 100 ml. to 22 mg. per 100 ml.

Although little variation was observed between different batches of autoclaved controls, it would be advisable to autoclave specimens and controls at the same time. We should like to discourage the use of pre-autoclaved commercial controls, which in our hands have not proved reliable.

Blood content of disks. If an estimation of concentration is desired in reference to measured amounts of standards rather than to control disks, the following information may be of interest. Blood drops varying in size from 5 to 50λ cover an area on the filter paper (S and S 903) that is closely proportional to their volume, namely, 2.6 mm.² per lambda. A quarter-inch disk (32 mm.²) punched out of a large drop would then contain $32 \div 2.6 = 12\lambda$ of blood. Actually, the amount of phenylalanine eluted from punched-out disks of blood or serum con-

Figure 3. Recovery of added phenylalanine



taining added phenylalanine was practically identical with that from 10λ drops, whose area is smaller. The reason for this is the frontal migration of solutes in porus flow (*5*, *6*), which causes them to accumulate at the periphery of the drop and to lower their concentration at the center. It happens that this decrease in concentration is closely balanced by the larger area, so that punched-out disks carry the contents of 10λ of blood within the limits of experimental error.

Nonphenylalanine fluorescence. All the normal blood tested by the present method gave values close to 2 mg. per 100 ml., indicating that fluorescence produced by substances other than phenylalanine was inconsequential. For a closer study of this point, we proceeded as follows: 100λ aliquots of a normal blood and of its plasma were deposited on the filter paper, autoclaved, cut out, and eluted in 1.2 ml. of water. A trichloracetic acid filtrate of the same blood was prepared by the addition of 1 volume of 0.6 *N* trichloracetic acid to 1 volume of blood. Two hundred lambda aliquots of the trichloracetic acid filtrate were then added to 1 ml. of dilute NaOH solution (the molarity of which was such that it practically neutralized the acid, so as not to overtax the capacity of the buffer in the analytical procedure).

The whole blood eluates, the serum eluates, and the filtrates were run in the AutoAnalyzer (*C*) by the present procedure and gave the following concentrations: whole blood 2.5 mg. per 100 ml., serum 2.0 mg. per 100 ml., and serum trichloracetic acid filtrate 2.2 mg. per 100 ml. It is obvious that in the autoclaving and elution technique, normal whole blood yielded a small amount of a nonphenylalanine fluorescent substance which offset the phenylalanine loss due to autoclaving. If it is assumed that the normal whole blood had the same phenylalanine concentration as that given for its serum by the trichloracetic acid filtrate, 2.2 mg. per 100 ml., a 25 percent loss in autoclaving would have reduced this concentration to about 1.7 mg.

Since the value for whole blood was 2.5 mg., the red cells must have released some heat-stable substances equal in fluorescence to about 0.8 mg. of phenylalanine per 100 ml. Since the purpose of the screening method is the detection of abnormal concentrations equal to at least twice the normal values, and not the accurate determination of normal concentrations, this source of error may be disregarded.

Results

The results so far are best illustrated by a few examples. In a series of 47 newborns considered normal by the Guthrie test, the mean height of the peaks in millimeters was 33.9 ± 3.6 (mean deviation) and that of five 4-mg. controls 61 ± 2.8 mm., which gave a concentration of 2.2 ± 0.2 mg. per 100 ml. for the normal newborn. This figure, which is partially the result of two compensating systematic errors, happens to agree with values from other sources (*7*). In a group of 24 specimens, the concentration of which ranged from 2 to 13 mg. per 100 ml., the correlation of the AutoAnalyzer results with those obtained by the manual method of McCaman and Robins (*3*) was good. Agreement was less satisfactory with the results from the Guthrie test.

When these same specimens were run without the dipeptide reagent, the peaks were 10 to 18 mm. in height as against 70 mm. for the 4-mg. controls with dipeptide. This confirmed our conclusion that nonphenylalanine fluorescence was equivalent to less than 1 mg. of phenylalanine per 100 ml. It may be added that false positive results, presumably due to accidental outside contamination, were infrequent. More extensive data are now being gathered for a definitive assessment of this method.

Summary

An improved method is described for the automated screening of blood phenylalanine. When dry blood spots on filter paper are exposed to hot steam, the proteins are irreversibly bound to the paper, whereas the other solutes can be subsequently released by water elution. This step takes the place of dialysis and permits bypassing the dialyzer in the Technicon automated methods, resulting in a fivefold increase in sensitivity. Dry blood disks containing added phenylalanine and subjected to autoclaving for 3 minutes at 121° C. gave a phenylalanine recovery of about 75 percent. Under controlled conditions, this figure was constant

and independent of the concentration. The fluorescence of nonphenylalanine substance was equivalent to only 0.8 mg. of phenylalanine per 100 ml.

With a smaller fluorometric cell, the method could be run at 60 specimens per hour without carryover. A modified pump allowed the speed to be increased to 120 specimens per hour. The method, which requires only a one-quarter-inch paper disk of dried blood, is considered accurate enough for screening purposes and sensitive enough to detect borderline cases.

REFERENCES

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(*7*) Wong, P. W. K., O'Flynn, M. E., and Inouye, T.: Micromethods for measuring phenylalanine and tyrosine in serum. Clin Chem 10: 1098–1104 (1964).

EQUIPMENT REFERENCES

(*A*) Brij, Technicon Chemical Co., Ardsley, N.Y.

(*B*) S and S 903 filter paper, Schleicher and Schuell, Keene, N.H.

(*C*) AutoAnalyzer, Technicon Instruments Corp., Ardsley, N.Y.

$2 Million in Grants for Antismoking Projects

Forty grants and contracts totaling almost $2 million were awarded by the Public Health Service during fiscal year 1966 for antismoking projects. They will involve parents and children, college students, health workers, hospital patients, educational television broadcasters, and communities.

The largest single award is a $182,614 contract to San Fernando Valley State College, Northridge, Calif. The college's department of health science will study the immediate, rather than the long-range, effects of smoking on college students. Laboratory findings involving analysis of expired air, serum lipids, clotting time, and work performance will be used to develop new methods and materials for antismoking education.

The San Diego County Medical Society and the Research Foundation of the State University of New York received grants totaling $209,345 for 5-year countywide educational programs to test methods by which organized community action can change cigarette smoking habits. The programs are to be carried out in San Diego County, Calif., and Onondaga County, N.Y., through local interagency councils on smoking and health.

Five contracts, totaling $517,337, were awarded for national surveys on smoking attitudes, beliefs and behavior among college students, and health workers and adults in the general population. The surveys are to be conducted by the American College Health Association, Coral Gables, Fla., National Opinion Research Center, Chicago, National Analysts, Inc., Philadelphia, and Opinion Research Corp., Princeton, N.J.

The District of Columbia, the University of Oklahoma, and the State health departments of Colorado, Florida, Idaho, Maine, Michigan, New York, Ohio, Pennsylvania, Vermont, and Washington will share a $213,739 grant to support statewide interagency programs on smoking and health.