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Specific Detection of Phenylalanine Following Chromatographic Separation from Serum and Urine of Phenylketonuric Patients. (29076)

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Previous studies from our laboratory have shown that a specific reaction for phenylalanine in complex biological mixtures is produced when the ninhydrin-stained chromatograms are subsequently treated with dilute sodium bicarbonate(1). It was also later shown that the same reaction can be used for a quantitative method that permits determination of phenylalanine on paper chromatograms in the presence of other amino acids even under conditions of poor separation and resolution(2). In this communication is shown how this reaction may be applied to serum and urine of phenylketonuric (PKU) patients following paper chromatographic separation of the amino acids. The paper chromatographic procedure used by Berry(3) was also studied.

Procedure. Chromatography. Two chromatographic procedures were applied to whole blood, serum and urine samples from PKU patients, (1) descending one-dimensional paper chromatography and (2) horizontal one-dimensional circular paper chromatography. These samples were compared with pooled lots of normal whole blood, normal serum and normal urine. All PKU urine samples were first morning voids, and blood samples containing EDTA as anticoagulant were taken shortly afterwards. Volumes of whole blood and serum ranging from 0.1 to 5 ml and urine specimens in 5 ml amounts were treated as follows:

One volume of absolute alcohol was added to blood or serum specimens and mixed well with a vortex mixer. After standing for 15 minutes at 0° to 4°C they were centrifuged at 2000 rpm for 20 minutes. The precipitated proteins were then washed twice with approximately 50% alcohol (v/v); all the supernatants were pooled and then dried over concentrated sulphuric acid *in vacuo*. These were then reconstituted into 50 or 100 μl of distilled water and appropriate volumes applied to paper in order to detect concentrations above 3 mg % of phenylalanine. Five milliliter aliquots of the urine samples were dried directly over concentrated sulphuric acid *in vacuo* without the alcohol treatment. The dried urine specimens were reconstituted to 0.2 or 0.3 ml with distilled water and suitable microliter volumes spotted on the filter paper.

Descending one-dimensional chromatography was carried out with the redeveloping technique as described previously(4,5,6). In addition, circular one-dimensional horizontal paper chromatography was employed for

shorter separation times. The solvent combination recommended for use in both systems is either: 1) 1-butanol, acetic acid and water or 2) 1-butanol, ethanol and water prepared as previously described(4,5,6). The circular chromatograms were developed horizontally between 2 large desiccator tops supported on a desiccator bottom. The solvent was introduced by means of a filter paper wick to a small hole in the center of the filter paper. The filter papers used were Whatman No. 4 and S & S 597. After solvent development the chromatograms were dried at 80° to 100°C for 2 to 3 minutes and dipped into a solution of 0.25% ninhydrin in acetone. They were then dried at the above temperature for 2 to 4 minutes to allow full ninhydrin color development and subsequently dipped into a 1% sodium bicarbonate solution. It is recommended that for quantitative studies the ninhydrin reagent be 0.4% ninhydrin in water saturated 1-butanol as previously described(2). The phenylalanine region on the chromatograms immediately appeared as a deep blue spot which was clearly visible even in the presence of the purple colors of the other amino acids. This blue color did not fade on standing or drying provided the conditions remained alkaline even though the color intensity of the other amino acids decreased steadily. The blue phenylalanine area can be quantitated by one of 3 techniques previously reported (2).

Results and discussion. The ninhydrin-bicarbonate reaction for phenylalanine was applied to separated amino acid mixtures from 10 serum samples. Some of the results of these experiments are shown in Fig. 1. This Figure shows two descending one-dimensional chromatograms of PKU serum samples and of a pooled control of 37 normal sera marked CK, which contained less than 3 mg % of phenylalanine. The chromatogram on the left shows the separated amino acids developed with ninhydrin. The chromatogram on the right is a duplicate stained with ninhydrin and subsequently treated with sodium bicarbonate and shows the specific selectivity of the phenylalanine reaction. Number 4 on both of these chromatograms was a non-PKU serum sample and gave similar results to the serum control, CK.

A further application of this reaction for phenylalanine to urine specimens is shown in Fig. 2, which shows results of 7 different PKU urine samples most of which were taken from the same patients as were the sera seen in Fig. 1. Fig. 2 shows 2 one-dimensional paper chromatograms. The one on the left is the ninhydrin stained chromatogram. The one on the right is the duplicate ninhydrin chromatogram treated with sodium bicarbonate. Urine samples from patients 1, 2, 4 and 5 had a much higher concentration of phenylalanine compared with the control CK, which contained less than 2 mg % phenylalanine. Samples 3, 6, and 7 were higher compared to the control but the elevation was not as marked. All urines shown on these chromatograms were positive to the Phenistix test.* On visual inspection of the series under study, differences in phenylalanine content were more marked with urine specimens than with serum.

A simpler chromatographic procedure employing the ninhydrin-bicarbonate reaction for phenylalanine was investigated. The circular chromatographic technique was selected due to the simple equipment required and the shorter separation time. The choice of 2 desiccator tops allows use of equipment commonly found in most small clinical laboratories. A major concern in setting up such a technique was the ability to detect phenylalanine in the minimum volumes of whole blood or serum which are available from infants. Fig. 3 shows a photograph of a circular chromatogram where whole blood samples were used from 3 PKU patients, labelled 1, 2 and 5, and compared with a pooled whole blood control marked CK. This figure shows on the lower half of the chromatogram the ninhydrin control (NIN.CK.) and on the upper half the specific phenylalanine (S.P. REACTION) reaction. The specificity and selectivity of the phenylala-

* Phenistix-reagent strips manufactured by Ames Co., Inc., Elkhart, Ind.

nine reaction can be seen. The amount of whole blood used was 0.5 ml from which the pooled alcohol extracts were taken to dryness and reconstituted to 0.1 ml. Six microliters of each reconstituted sample were spotted on the filter paper. Under these conditions any serum or whole blood containing more than 5 mg/100 ml of phenylalanine can be observed readily; by altering the concentration of the sample and the volume applied to filter paper, as little as one or two mg/100 ml of phenylalanine may be detected.

The use of the Berry method(3) appears to be limited in that the phenylalanine reaction described by that author is not specific. The ninhydrin reagent used by Berry gives a color for phenylalanine that too closely resembles that produced by the other amino acids. On the other hand, the reaction we have described here and previously is associated with a shift in the absorption maximum from 560 to 600 mμ as well as an increase in color stability even under conditions of poor resolution and separation(1, 2). The Berry solvent of 1-butanol, ethanol and water (70, 20, 20) does not separate the leucines cleanly from the phenylalanine and in our experience there is a tendency for the phenylalanine to streak. When the sol-

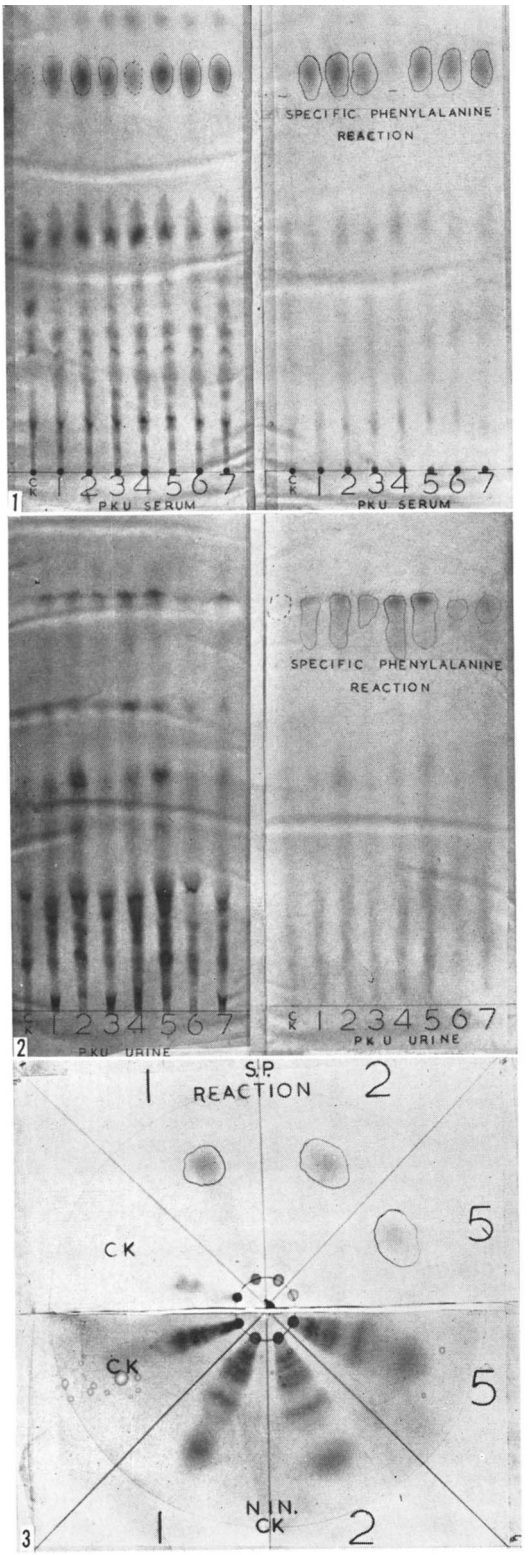

FIG. 1. A photograph of 2 identical descending paper chromatograms showing separated amino acids from normal and several different PKU sera. Chromatogram on the left has been stained with ninhydrin. Chromatogram on the right has also been stained with ninhydrin, with subsequent sodium bicarbonate treatment thus rendering a specific color reaction for phenylalanine.

FIG. 2. Separated amino acids from a pooled normal urine sample and from several PKU urines are shown. The specific reaction for phenylalanine has been applied to chromatogram on the right. Chromatogram on the left is the ninhydrin control. The following urine samples in Fig. 2 correspond to serum samples indicated in Fig. 1: No. 2 urine and No. 6 serum are from the same patient. No. 3 urine and No. 2 serum, No. 4 urine and No. 5 serum, No. 5 urine and No. 7 serum, No. 6 urine and No. 3 serum correspond as do No. 7 urine and No. 1 serum.

FIG. 3. A horizontal circular paper chromatogram of amino acids from a pooled whole blood sample and from 3 different whole blood PKU samples. Lower half of the photograph shows the ninhydrin control. In upper half the specific phenylalanine reaction has been applied to the pooled whole blood control marked CK and to whole blood samples from PKU patients designated 1, 2, and 5.

vents described here and previously(4,5,6) are used, the leucine and isoleucine appear as a separate spot above the phenylalanine area (Fig. 1, 2). In addition, the specific phenylalanine reaction described here eliminates contaminating amino acids as they are washed away or extracted, leaving only the phenylalanine for quantitative measurements (Fig. 1, 2, 3).

Van de Heuvel and Wadman(7) described a paper chromatographic method for determining phenylalanine in serum in which the chromatographic process was performed at 37°C. They removed salt prior to chromatography and used a ninhydrin reagent for staining the amino acids. In our method no desalting is required and the bicarbonate treatment following ninhydrin is selective for phenylalanine.

Recently the paper chromatographic technique of Hudson, Dickinson and Ireland(8) for detection of phenylketonuria has come to our attention. They report that with the solvent system used it is possible to separate phenylalanine completely from valine and the leucines.

Summary. A specific reaction for phenylalanine has been applied after chromatographic separation of amino acids from urine, serum and blood specimens of phenylketonuric patients. This reaction is demonstrated by means of photographs of the actual chromatograms obtained.

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Flow Rate and Composition of Thoracic Duct Lymph With and Without Cisterna Chyle Ligation. (29077)

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It was suggested(1) that one method by which anti-inflammatory agents may act to reduce induced pleural edema may be by increasing lymphatic drainage. In attempting to determine the role of lymphatic drainage it was found that little information concerning the normal flow rate and composition from this area in rats was available(2). In view of the above it was thought that whether or not lymphatic drainage is associated with the reduction of pleural edema, some information on normal rats would be of value.

* Recipient of N.I.H. Fellowship Award.

The thoracic duct is known to be the final collecting pathway in many animals(3), receiving lymph from the lower extremities, peritoneal cavity and contents, left upper extremity and the left side of the head and neck. The right lymphatic duct drains the right extremity and the right side of the head and neck. Although the thoracic cage is drained mainly *via* the right lymph channel (4), we have shown that T-1824 dye appears in the thoracic duct within 20-30 seconds after injection into the left pleural space.

It was also of interest to determine contri-