TABLE II. Fractions of Concentrated Parotid Saliva Separated by Boundary Electrophoresis.*

Component	A	B	C	D	E-1†	E-2†	Mean ± S.E.
			Mobility × 10^{-5} cm^2 $sec.^{-1}$ $volt^{-1}$				
I	+1.90	+2.24	+2.11	+2.28	+2.14	+2.15	+2.14 ± .08
II	+ .85	+ .60	+ .86				+ .77 ± .08
III	+ .17	+ .24	+ .57	+ .50	+ .39	+ .04	+ .32 ± .08
IV	− .74	− .60	− .83	− .81	− .71	− .93	− .77 ± .08
V	−2.11	−1.94	−2.22	−1.99	−2.22	−2.02	−2.08 ± .05
VI	−3.02	−2.84	−3.07	−2.91	−3.31	−2.95	−3.02 ± .07
VII	−4.25	−4.05	−4.20	−4.21	−4.49	−3.86	−4.18 ± .09
			Relative concentration (%)				
I	6.6	13.2	8.0	7.4	8.0	5.6	8.1 ± .93
II, III, IV	50.8	52.8	50.0	53.3	52.0	51.4	51.7 ± .51
V	21.3	20.8	32.0	20.5	22.7	23.6	23.5 ± 1.77
VI	16.4	7.5	6.0	14.7	8.0	9.7	10.4 ± 1.72
VII	4.9	5.7	4.0	4.1	9.3	9.7	6.3 ± 1.05

* Taken from descending boundary. † Same individual run 3 wk apart.

cm^2 $sec.^{-1}$ $volt^{-1}$.

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Paper Chromatographic Method for Estimation of Phenylalanine.* (23124)

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In phenylketonuria serum phenylalanine levels are high(1). Removal of phenylalanine from the diet results in a marked decrease in blood phenylalanine to near normal levels(2,3). The methods for determination of phenylalanine are largely based on modifications of the Kapeller-Adler procedure(4) in which phenylalanine is oxidized to benzoic acid and subsequently nitrated to the 3,4-dinitro derivative(3,5). The enzymatic decarboxylation of phenylalanine to phenylethylamine and determination of the resulting base as described by Udenfriend and Cooper has also served as a method for obtaining phenylalanine values in serum(6). When children are to be treated with a phenylalanine-low diet, there is need for a simple and rapid method for determination of serum phenylalanine. We have used a paper chromatographic method requiring a very small volume of blood in order to obtain a clear picture of the individual response to a low phenylalanine diet.

Methods. Phenylalanine in blood serum. A small sample of blood such as can be obtained from skin puncture is sufficient for the estimation of the phenylalanine content of serum. Protein is removed by adding 2 ml

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of 95% ethyl alcohol to 0.5 ml of serum. The precipitated protein is removed by centrifugation or suction filtration. The filtrate, a 5-fold dilution of the original sample, is used directly for chromatography. Aqueous solutions of dl-phenylalanine containing 0.2, 0.3, and 0.4 mg/ml are prepared for use as standards. Sheets of Whatman No. 1 filter paper, 7 in. x 11 in., are marked along a line 2 cm from the long edge of the paper at intervals of 2 cm. Along this line the standard solutions are applied in increasing amounts on positions 1, 3, 5. Positions 11, 9, 7, are duplicates of 1, 3, 5, respectively. The solutions are applied with 5 μl capillary pipettes obtainable from Microchemical Specialties Co., Berkeley, Calif. The ethanol filtrate of the unknown serum is applied in 5 μl increments until volumes of 50, 75, and 100 μl have been added to spots 2, 4, and 6. Spots 8, 10, and 12 are duplicates of 2, 4, and 6. These increasing amounts are applied to the paper by pipetting a 5 μl volume on a spot several times, allowing the spot to dry each time, until the total volume has been added to the spot. This time-consuming method of application cannot be avoided, since the spot diameter must be kept small and should not exceed 12 mm. The sheets should be prepared in duplicate. If only a very small volume of blood is available 0.2 ml of serum and 0.8 ml of ethanol give sufficient filtrate to prepare a single sheet. When the sample and standard spots have dried, the sheets are rolled into a cylinder, and the short edges are stapled together with great care that the edges do not touch after stapling. Excessive handling of the sheets must be avoided to prevent contamination from ninhydrin-reacting components of sweat. These cylinders are placed upright in a solvent mixture, prepared by mixing 70 ml n-butyl alcohol (technical grade), 20 ml 95% ethyl alcohol, and 20 ml water, in a Pyrex jar of 10 to 18 in. height, approximately 8 in. in diameter. Airtight closure is provided by a glass plate to cover the jar. The depth of solvent should be such that the bottom edge of the cylinder is covered to within ½ in. of the line along which the spots were applied. The solvent mixture may be used for several chromatograms prepared at different times provided the container is always kept tightly closed. If the solvent composition is altered by evaporation, phenylalanine will not be separated from other serum constituents. Chromatographic equipment can readily be improvised. As an alternative to Pyrex jars, stone crocks are perfectly suitable as chromatogram chambers. Since the bottoms are usually uneven, the solvent should be placed in a Pyrex pie plate obtained from a hardware or variety store, or in a Petri dish. The sheets should remain in the solvent mixture about 6 hours. The sheets are then removed and dried in air at room temperature at least two hours before further treatment. The dried sheets are sprayed with a reagent prepared by adding 200 mg ninhydrin to 85 ml butyl alcohol, 10 ml water, 5 ml 95% ethanol. The sheets are heated in an oven at 90°C for 10 minutes.

Results. Phenylalanine appears as a blue spot at Rf .42. (Rf is the ratio of the distance the phenylalanine has traveled to the total distance the solvent has traveled.) Other amino acids are characteristically purple in color and migrate to different positions. Phenylalanine content of the serum can be estimated by visual comparison with the standard spots. A more precise determination can be made with a densitometer, if available. Density measurements (1 – log % transmission) when plotted *vs.* micrograms of phenylalanine give a straight line in the range 0.5 – 2.5 μg of phenylalanine.

When the serum phenylalanine concentration is below 5 mg %, spots containing 0.5 μg of phenylalanine standard may replace the 2 μg phenylalanine standard to extend the concentration range downward to 2.5 mg %, corresponding to the concentration found in normal serum. The concentration range can be extended upward by using a smaller volume of diluted serum. For example, 15, 25 and 50 μl of serum filtrate should be used in examining blood from untreated phenylketonurics. As mentioned earlier, greater accuracy is possible if densitometric measurements can be made, but for clinical guidance of children with phenylpyruvic oligophrenia treated with a phenylalanine-free diet the changes in serum phenylalanine content are

TABLE I. Serum Phenylalanine Levels in a Phenylketonuric Child.

	Date	Phenylalanine (mg %)
Regular diet	4/ 3/56	34
	5	45
	7	24
	9	47
	11	24
Low phenylalanine diet (begun 4/12/56)	4/14/56	18
	16	15
	18	11
	19	9.5
	24	4.3
	31	4.9
	5/ 2	5.3

so marked that the more exact measurements are not needed. Table I gives values for serum phenylalanine in a phenylketonuric child several days before and after institution of treatment with a phenylalanine-low diet.

In our experience phenylalanine in serum from phenylketonuric children on a regular diet may vary from concentrations of 20 mg% to 50 mg%. When phenylalanine has been removed from their diet, the serum levels gradually decrease to a minimum value between 2 and 4 mg%. The blood levels generally found among the phenylketonuric children on diets low in phenylalanine are usually between 4 and 8 mg%.

Data listed in Table II show recoveries of phenylalanine added to serum. The determinations were made as described above, using a Model 525 Photovolt Densitometer. The range of recovery was from 80% to 106%, with an average recovery of 95%.

Phenylalanine in urine. When urinary excretion of phenylalanine is high relative to other urinary amino acids the method described for serum phenylalanine can be applied to urine, using duplicate spots of 5 μl of ¼ dilution on spots 2 and 8, 5 μl of ½ dilution on spots 4 and 10, and 5 μl spots of undiluted urine on positions 6 and 12. Urinary levels from 0.10 mg/ml to 1.6 mg/ml may be determined.

TABLE II. Recovery of Phenylalanine Added to Serum.

Sample No.	Phenylalanine Added, μg	Phenylalanine Found, μg	Phenylalanine Recovered, μg	Phenylalanine %
1	0	69		
	400	454	385	96
	200	228	159	80
2	0	0		
	200	204	204	102
3	0	22		
	50	75	53	106
4	0	95		
	50	138	43	86
5	0	71		
	100	172	101	101

Phenylalanine in cerebrospinal fluid. Phenylalanine levels may be determined on untreated cerebrospinal fluid using the technic described for serum. Neither deproteinization nor desalting is required. Duplicate spots of 20, 50, and 100 μl are used. The concentration range covered using these volumes is from 0.5 mg% to 10 mg%. The average value for untreated phenylpyruvic oligophrenic children is 8 mg%. Phenylalanine concentration in the spinal fluid falls below 0.5 mg% after a period on phenylalanine-free diet. Glutamine is generally the only other ninhydrin-reacting substance visible in 100 μl of spinal fluid.

Summary. For paper chromatographic estimation of phenylalanine, 0.5 ml samples of blood serum are deproteinized with 4 volumes of 95% ethyl alcohol and the filtrate used for preparation of the chromatogram. The method is especially useful in study of phenylketonuric infants, and in following the changes in serum phenylalanine when a low-phenylalanine diet is used for their treatment. The recovery range of phenylalanine added to serum was from 80% to 106% with an average recovery of 95%.

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