parathormone itself (*17*) and resembles similarly induced desensitizations in the cases of epinephrine and prostaglandin E_2 (*18*). This observation, and the fact that the various changes induced by the two agents were not additive at maximal concentrations of each, suggest that the same bone cells were the targets for both hormones and that generally the same metabolic pathways were used.

Our results demonstrate that 1,25-$(OH)_2D_3$ affects the metabolism of those cells of bone that may represent, respectively, the bone-resorbing (CT) and bone-forming (PT) cells of the tissue (*19*). If the changes in vitro that we observed are, at the concentration of both agents tested, representative of metabolic events that would occur in bone in vivo, then our results suggest that 1,25-$(OH)_2D_3$ induces bone resorption both by stimulating osteoclastic and by inhibiting osteoblastic activities. Moreover, these results would argue against one hormonal agent being a prerequisite for the other—a possibility often considered in the literature (*1*).

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Phenylketonuria: A New Method for the Simultaneous Determination of Plasma Phenylalanine and Tyrosine

Abstract. *This quantitative spectrophotometric method is based on the conversion of phenylalanine and tyrosine by phenylalanine ammonia-lyase to* trans-*cinnamic acid and* trans-*coumaric acid, respectively. Neither deproteinization nor prior incubation of the sample is required, and the entire procedure can be performed in 20 minutes. The method is sensitive to 1-micromolar concentrations of the two compounds, and only 20 microliters of plasma or serum is required to determine both phenylalanine and tyrosine simultaneously. These amino acids were determined between molar ratios (phenylalanine to tyrosine) of 0.1 to 40 in the serum or plasma of healthy individuals and plasma of phenylketonuric patients.*

Classic phenylketonuria, which is transmitted by an autosomal recessive gene, is a result of a defect in liver phenylalanine hydroxylase (*1*). This deficiency causes an excess of phenylalanine to accumulate in the blood and spinal fluid. Individuals with this disease usually develop significant irreversible brain damage and behavioral derangement unless they are placed on a diet low in phenylalanine within 1 to 3 months of age (*2*). The prompt use of dietary treatment is dependent upon an effective screening test. For this purpose, a semiquantitative assay based on the reversal by phenylalanine of β-2-thienylalanine inhibition of the growth of *Bacillus subtilis* was developed by Guthrie (*3*) and has been applied by health departments in most of the states in this country.

Table 1. Phenylalanine (Phe) and tyrosine (Tyr) concentrations in the serum of healthy individuals determined by enzymatic spectrophotometry and automated amino acid analysis. Serums were prepared from 20 healthy nonfasting individuals with a mean age of 24.7 ± 3.5 years [± standard deviation (S.D.)]. The Phe and Tyr determinations were performed by our enzyme method described in the text. For automated amino acid analysis, serums were deproteinized with four volumes of 3.75 percent sulfosalicylic acid in 0.3*N* lithium citrate buffer (*p*H 2.2), and the mixture was centrifuged. Amino acid concentrations were expressed as milligrams per 100 ml of serum. Values obtained by the enzyme method were the mean of triplicate determinations, and those obtained by the amino acid analyzer were the average of duplicate determinations.

Sample No.	Phenylalanine ammonia-lyase		Amino acid analyzer	
	Phe	Tyr	Phe	Tyr
1	0.99	0.89	1.00	0.51
2	0.93	0.73	0.86	0.45
3	1.25	1.03	1.08	0.79
4	1.11	0.97	1.07	0.58
5	1.48	0.89	1.46	0.89
6	0.93	0.68	0.85	0.39
7	0.76	1.05	0.99	0.86
8	0.90	1.22	1.21	1.15
9	0.93	1.35	1.14	1.23
10	1.61	0.95	1.15	0.84
11	1.15	0.77	0.74	0.60
12	1.24	0.76	1.22	0.80
13	1.87	1.04	1.29	0.88
14	1.30	0.72	0.84	0.75
15	1.27	0.90	1.11	0.82
16	1.44	1.10	1.22	1.18
17	1.17	0.74	1.11	0.71
18	1.23	0.91	1.29	0.74
19	0.96	1.33	1.28	1.04
20	1.08	1.08	0.60	0.56
Mean ± S.D.	1.18 ± 0.27*	0.96 ± 0.20‡	1.08 ± 0.21*	0.79 ± 0.24‡

*$P < .20$. ‡$.02 < P < .05$.

Table 2. Phenylalanine and tyrosine concentrations in the plasma of phenylketonurics. Fresh plasma was prepared for enzyme assay and automated amino acid analysis. Spectrofluorometric assays of the same blood specimens were performed by the Texas Health Department, Austin. Amino acid concentrations were expressed as milligrams per 100 ml of plasma.

Patient No.	Time sample taken after a diet (months)	Phenylalanine ammonia-lyase		Amino acid analyzer		Spectrofluorometry	
		Phe	Tyr	Phe	Tyr	Phe	Tyr
1*	1	28.01	0.51	23.05	0.58	25.0	2.0
1	2	21.59	0.71	20.39	0.54	19.0	1.9
1	3	22.47	0.98	22.75	0.62	22.7	1.8
2†	1	9.59	0.97	9.41	1.13	10.7	
3‡	1	25.14	0.81			31.1	2.2
4§	1	26.49	0.60	26.72	0.68	33.1	1.4
5‖	1	21.85	0.98	21.90	0.99		
6¶	1a	58.36	0.65				
6	1b	16.29	1.00				
6	1c	7.39	3.01				
6	1d	2.33	0.95	3.69	1.40	4.4	1.1
6	1e	4.30	1.92				

*Eleven-year-old male on modified diet for 6 months. †Twenty-month-old female under dietary treatment for 19 months. ‡Eight-year-old female on regular diet for 3 months. §Seven-year-old male on regular diet for 1 year. ‖Eight-year-old male on regular diet for 1.5 years. ¶A 28-day-old female with an initial plasma phenylalanine level of 58 mg/100 ml (see sample 1a) and placed under dietary control immediately.

When a presumptive positive (usually > 6 mg of phenylalanine per 100 ml of blood) phenylketonuric is detected, confirmation is required by quantitative methods such as automated amino acid analysis or spectrofluorometry (*2*). Furthermore, since variant forms of hyperphenylalaninemia occur in which both phenylalanine and tyrosine levels fluctuate significantly for several months after birth, quantitative analyses of both amino acids are required for proper management of these diseases.

We have developed a simple, rapid, and sensitive method to determine quantitatively the concentrations of both phenylalanine and tyrosine in serum or plasma (*4*) of healthy individuals and plasma of patients with phenylketonuria. Our method is based on the use of yeast phenylalanine ammonia-lyase (E.C. 4.3.1.5), an enzyme that catalyzes the deamination of phenylalanine to *trans*-cinnamic acid and tyrosine to *trans*-coumaric acid (*5*). The amount of tyrosine in the sample is determined directly by measuring the rate of increase of absorbance (ΔA) of *trans*-coumaric acid at 315 nm (*6*). Since both *trans*-cinnamic acid and *trans*-coumaric acid absorb light at 290 nm, phenylalanine is determined by difference at this wavelength (*7*).

For these experiments, the yeast *Rhodotorula glutinis* was either purchased (from P-L Biochemicals) or cultured in this laboratory (*8*). Highly purified phenylalanine ammonia-lyase with a specific activity of 2.8 units per milligram of protein and demonstrating a single major band on polyacrylamide gels was prepared as described in detail previously (*8*). One unit of enzyme is defined as that amount of protein that catalyzes the appearance of 1 μmole of cinnamic acid per minute at 30°C. This preparation, stored at −60°C at a concentration of 30 mg of protein per milliliter of 0.01*M* potassium phosphate buffer (*p*H 7.0), demonstrated no loss of activity in 6 months. Stock solutions (approximately 1.0 mg of protein per milliliter of 0.01*M* potassium phosphate buffer, *p*H 7.0) were prepared weekly and stored at −60°C. Dilutions were made daily with 0.1*M* tris-HCl buffer (*p*H 8.75) to give 7 to 10 μg of protein (approximately 0.02 unit) per 1.0 ml of assay mixture for measurement of standards or unknowns.

The assay was standardized to determine phenylalanine and tyrosine (analytical grade, Sigma) at molar ratios (phenylalanine to tyrosine) of 0.1 to 40. The reaction mixture contained 1 to 5 μM of one amino acid plus the other amino acid at the specified concentration in 0.1*M* tris buffer (*p*H 8.75) and 0.02 unit of phenylalanine ammonia-lyase at 30°C. Blood (approximately 80 μl) was collected from donors in heparinized capillary tubes by heel or fingertip puncture for the determination of phenylalanine and tyrosine by our enzymatic method. When larger volumes were required for purposes of comparison with automated amino acid analysis or spectrofluorometry (*9*), blood was obtained by venipuncture (*10*). Plasma was obtained by centrifugation of the blood in an Adams microhematocrit centrifuge (Clay-Adams). Serums were prepared by defibrination of the blood with 5 percent (by volume) of 0.2*M* $CaCl_2$ and centrifugation (portable refrigerated centrifuge; International Equipment). The determinations of phenylalanine and tyrosine were performed with a spectrophotometer (Gilford model 250) and recorder (Gilford model 6050) and with an amino acid analyzer (Beckman model 121M).

In order to determine whether the rates of increase in absorbance at 290 and 315 nm are linear over the range of phenylalanine to tyrosine ratios expected in serums or plasma of healthy individuals or patients with phenylketonuria, we performed standard measurements on solutions of these two amino

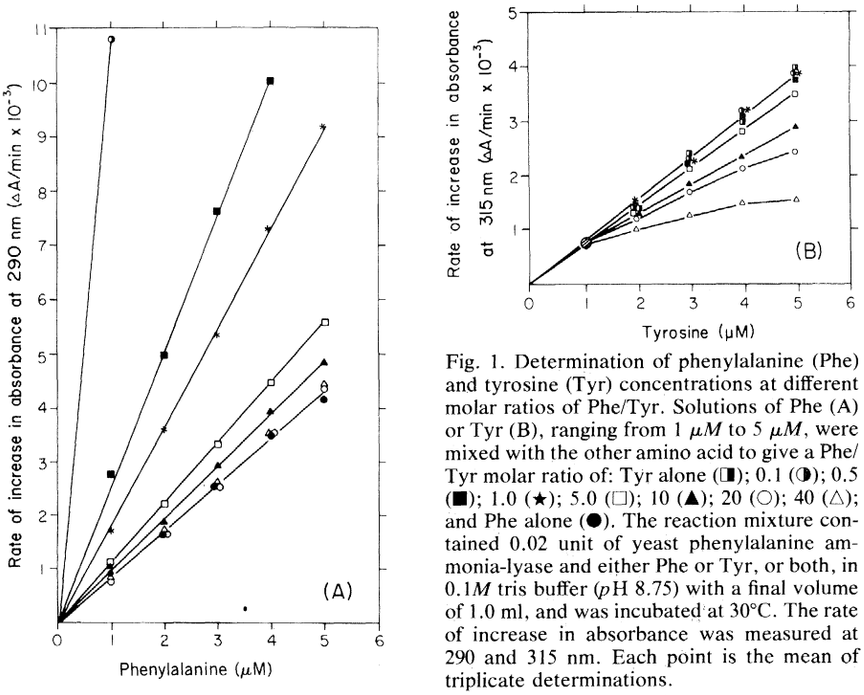

Fig. 1. Determination of phenylalanine (Phe) and tyrosine (Tyr) concentrations at different molar ratios of Phe/Tyr. Solutions of Phe (A) or Tyr (B), ranging from 1 μM to 5 μM, were mixed with the other amino acid to give a Phe/Tyr molar ratio of: Tyr alone (◧); 0.1 (◑); 0.5 (■); 1.0 (★); 5.0 (□); 10 (▲); 20 (○); 40 (△); and Phe alone (●). The reaction mixture contained 0.02 unit of yeast phenylalanine ammonia-lyase and either Phe or Tyr, or both, in 0.1*M* tris buffer (*p*H 8.75) with a final volume of 1.0 ml, and was incubated at 30°C. The rate of increase in absorbance was measured at 290 and 315 nm. Each point is the mean of triplicate determinations.

acids with ratios between 0.1 and 40. The rate of increase in absorbance at 290 nm is directly proportional to phenylalanine concentrations at the molar ratios studied (Fig. 1A). Solutions with high ratios of phenylalanine to tyrosine (10 to 40), however, do not exhibit a linear relationship in the rate of increase in absorbance at 315 nm with tyrosine concentrations from 2 to 5 μM (Fig. 1B). Thus, plasma or serum tyrosine was determined at approximately 1 μM levels.

Table 1 demonstrates that analyses by our method and by the amino acid analyzer are comparable. Both phenylalanine and tyrosine values were slightly higher when measured by the phenylalanine ammonia-lyase assay, but they were not statistically significant within 20 donors (*11*). Phenylalanine ammonia-lyase has no catalytic activity on any of the common amino acids except phenylalanine and tyrosine, nor on the D-isomers or structurally related derivatives (*12*). Table 2 shows a comparative study of six patients with phenylketonuria. Plasma phenylalanine levels are comparable by all three methods, but values for tyrosine were considerably higher by the spectrofluorometric procedure for reasons unknown.

The results of this study demonstrate that phenylalanine ammonia-lyase can be used for the quantitative determination of plasma or serum phenylalanine and tyrosine. Although this method can be easily applied for screening of phenylketonuria, its use in the management of this disease is appropriate because it is rapid, sensitive, and requires only a small portion of blood. Patient No. 6, originally undetected by the Guthrie test, was diagnosed immediately by our enzyme method, and plasma concentrations of these two amino acids were monitored subsequently until the patient exhibited a stable pattern of plasma phenylalanine and tyrosine through dietary manipulation.

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7. The ratio of absorbance of *trans*-coumaric acid at 290 nm/315 nm is 1.48. Therefore, phenylalanine is determined by measuring the rate of increase of absorbance of *trans*-cinnamic acid at 290 nm according to the following formula: $\Delta A_{290} - (1.48)\Delta A_{315}$.
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Hepatic Binding Protein: The Protective Role of Its Sialic Acid Residues

Abstract. *Removal of sialic acid from a specific hepatic binding protein virtually abolishes its capacity to bind certain asialoglycoproteins. The loss of this capacity is the result of competition for the binding sites by galactosyl residues, of hepatic binding protein, that become terminal after desialylation.*

Removal of the terminal sialic acid residues of the carbohydrate chains of many mammalian serum glycoproteins exposes galactosyl residues as terminal. Intravenous injection of such a desialylated protein seems to be followed by a sequence of reactions that rapidly transfers the protein from plasma to hepatocytes. There appears to be a transient binding of the asialoglycoprotein to a component of the hepatic plasma membrane that is itself a glycoprotein with several terminal sialyl residues (*1*). A complex of a plasma asialoglycoprotein and the membrane glycoprotein can be formed in vitro, as well, but only if the terminal sialyl residues of the latter are not removed (*2*). Our study was directed toward understanding the functional significance of sialyl residues of the hepatic plasma membrane glycoprotein.

The relative affinities of the hepatic binding protein (HBP) for a number of sugars and glycoproteins were estimated from measurements of the capacity of each substance to inhibit the binding of [^{125}I]asialoorosomucoid (Table 1). The assumption is made that the lower the concentration required for 50 percent inhibition, the more tightly the inhibitor is bound to HBP.

The inhibition of binding by HBP of

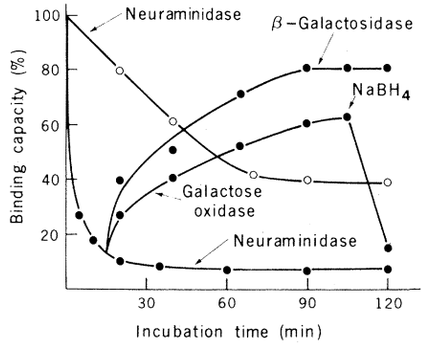

Fig. 1. Effect of enzymatic treatment on the binding capacity of HBP. Hepatic binding protein (2 mg/ml) and neuraminidase (*Clostridium perfringens*; Sigma) (100 milliunit/ml) were incubated at 22°C in 0.05*M* Na acetate buffer (*p*H 6.2) and 0.15*M* NaCl. After 15 minutes of incubation, two portions of the mixture were removed and either β-galactosidase (*Diplococcus pneumoniae*; provided by G. Ashwell) or galactose oxidase (*Polyporus circinatus*; Sigma) was added to a final concentration, respectively, of 100 milliunit/ml or 40 unit/ml. After 90 minutes of incubation with galactose oxidase, 5 μg of $NaBH_4$ were added. The samples of 5 μl that were taken for the determination of the binding capacity of HBP for [^{125}I]asialoorosomucoid (•) and [^{125}I]DOSM (○) were each diluted 40-fold with buffer, as a consequence of which there was no necessity for removal of enzymes present. Binding of [^{125}I]DOSM to HBP, after incubation for 10 minutes as described for asialoorosomucoid (Table 1), was estimated by precipitation of the complex with an equal volume of a saturated solution of ammonium sulfate, adjusted to *p*H 7.8, and filtration on a Whatman GF/A glass disk to remove unbound [^{125}I]DOSM. Desialylated ovine submaxillary mucin was labeled by conjugating it with ^{125}I containing *N*-hydroxysuccinimide ester of 3-(4-hydroxphenyl)propionic acid, by the method of Bolton and Hunter (*8*). The unreacted ester was removed by exhaustive dialysis against water. The labeled protein had specific activities ranging from 0.1 to 0.2 μc per microgram.