SC 2198

Studies on phenylketonuria

II. The excretion of *N*-acetyl-L-phenylalanine in phenylketonuria

In the course of studies with phenylalanine-loaded rats, some results of which have been reported[1], chromatographic analyses of urines on anion-exchange resins suggested the presence of small but measurable amounts of an N-conjugated amino acid with elution characteristics like phenylacetylglutamine[2]. However, the single ninhydrin-positive spot detectable on paper upon chromatography of the products resulting from mild acid hydrolysis had an R_F corresponding to that of phenylalanine. Further studies with added synthetic unlabelled and [^{14}C]*N*-acetyl-L-phenylalanine, utilizing paper-strip and anion-exchange chromatography before and after hydrolysis by acid or carboxypeptidase[3], and application of the snake-venom oxidase procedure of LADU AND MICHAEL[4] for L-phenylalanine determination substantiated the identity of the excreted compound.

For the determination of acetylphenylalanine in the urine of phenylketonurics, 3–5 ml samples were applied to 0.9 × 30-cm columns of Dowex-1 X2 (acetate) which were eluted as described previously[1]. The effluent fractions comprising the mixed phenylacetylglutamine and acetylphenylalanine peaks (usually in 10 tubes of 4 ml each) were made 2 N with respect to HCl and heated in a boiling-water bath for 3 h. The contents of the tubes and washings were evaporated batchwise under reduced pressure, finally to the point of dryness, in a small flask on the rotary evaporator. The residue was treated twice by rotation in contact with 7 ml of *n*-butanol to extract the phenylalanine. The butanol extracts were evaporated to dryness, the residue mixed with 4 ml of water, again brought to dryness, and finally dissolved in 2.0 ml of 0.1 M sodium phosphate buffer (pH 6.5). The smallest aliquot required for a satisfactory assay (0.05–0.20 ml) was used in the LADU AND MICHAEL procedure. Recoveries of *N*-acetyl-L-phenylalanine added to control urines were 95 and 104 % for 250- and 100-μg samples, respectively.

The acetylphenylalanine content of first morning-urine samples from 18 insti-

TABLE I

ACETYL-L-PHENYLALANINE CONCENTRATION IN THE URINE OF PHENYLKETONURICS

Subject No.	*N-Acetyl-L-phenylalanine (mg/mg creatinine)*	*Phenylpyruvic acid (mg/mg creatinine)*
1	0.095	2.3
2	0.047	1.7
3	0.048	2.3
4	0.082	1.9
5	0.042	1.6
6	0.041	2.3
7	0.025	2.0
8	0.100	3.3
9	0.053	2.5
10	0.055	1.6
11	0.018	1.2
12	0.144	5.0
13	0.025	2.2
14	0.016	2.0
15	0.047	1.8
16	0.037	2.1
17	0.060	1.4
18	0.032	2.0

tutionalized phenylketonurics of varying chronological and mental ages on unrestricted diet is shown in Table I. The values range from 0.016–0.14 mg/mg creatinine with a mean of 0.054 mg/mg creatinine. Determinations performed on 24-h urine samples indicate a total daily excretion of the order of 30–100 mg. Employment of larger sample volumes (7.5–10 ml) of urine from 27 healthy humans in an otherwise similar procedure yielded a mean value of 0.002 mg/mg creatinine and an extreme of 0.003 mg/mg creatinine. The urine of phenylketonurics on restricted phenylalanine intake also contained minimal, if any detectable acetylphenylalanine.

The effect of a single orally administered load dose of L-phenylalanine (2 mmoles per kg) on the urinary excretion of acetylphenylalanine by a phenylketonuric (homozygote with respect to inheritance of the condition), a parent of a phenylketonuric (heterozygote), and a normal control is shown in Fig. 1. Excretion of the acetyl derivative, like that of other phenylalanine metabolites, rises sharply following the ingestion of a massive dose of the amino acid by a phenylketonuric. Peak values approaching 1.0 mg/mg creatinine are reached near the 4th h. Marked variation was encountered in the case of heterozygotes. Of four investigated, the response of one was similar to that of a phenylketonuric, while two displayed reactions indistinguishable from that of individuals considered of normal genotype, and one showed a moderate increase (middle curve).

It seems likely that the observed anomaly arises from an accumulation of acetylphenylalanine exceeding the renal reabsorptive capacity. Work with several mammalian species suggests a low reabsorption of the compound. Following the ingestion of 1.0 g in divided doses of 125 mg at hourly intervals, 14% was present in the first 24-h urine collection of the healthy 48 kg human control. Concerning a mechanism for the accumulation of acetyl-L-phenylalanine in phenylketonurics, evidence for an acetylation of L-phenylalanine by rat-liver slices was obtained by BLOCH AND BOREK[5]

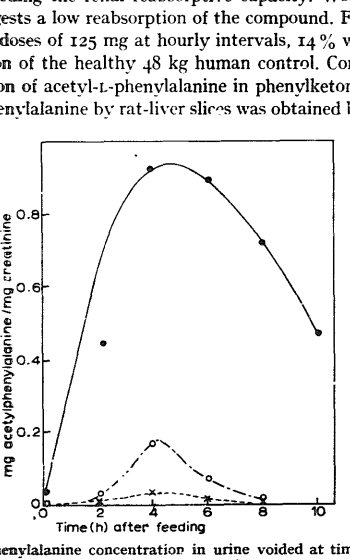

Fig. 1. *N*-Acetyl-L-phenylalanine concentration in urine voided at time intervals following the ingestion of a single oral dose of L-phenylalanine (2 mmoles kg). ●—●, phenylketonuric; ○–·–○, heterozygote with respect to phenylketonuria; ×---×, normal control.

in early isotope studies. In current investigations related to the present report, a net synthesis of acetyl-L-phenylalanine by slices of liver, kidney, or brain of the rat was easily demonstrated. Since a primary metabolic pathway for excess acetylphenylalanine—deacetylation by appropriate acylase systems—would be of little value to the phenylketonuric, its excretion might be anticipated.

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On the titration behavior of dimethylaminonaphthalene–protein conjugates

It has been suggested that the unusual displacement of the pK_a of certain groups conjugated to proteins is due to masking of these groups by an ice-like structure surrounding the protein[1]. For example, the pK_a of the dimethylamino group of 1-dimethylaminonaphthalene-5-sulfonyl chloride conjugates of glycine in water is 3.99, whereas the same group attached to bovine serum albumin exhibits a pK_a of 1.67 (ref. 2). Interpretation of these results in terms of frozen-water layers in the vicinity of the protein, however, has been questioned[3].

The present communication reports the titration behavior of two DNS–protein conjugates: bovine pancreatic RNAase and egg-white lysozyme.

RNAase A was prepared by chromatography on Amberlite IRC-50 of Sigma Chromatographic Grade RNAase by the procedure of HIRS, MOORE AND STEIN[4]. Lysozyme was obtained from Worthington Biochemical Corp. (Lot No. 583). Both proteins were coupled to DNS by reaction for 24 h at 4° in 0.1 M $NaHCO_3$ (pH 8.2). RNAase was freed from unreacted dye by 3 precipitations with acetone at 0°, lysozyme, by passage over G-25 Sephadex (Pharmacia, Uppsala) in 0.08 M acetic acid, followed by CG-50 type II chromatography in 0.3 M phosphate buffer (pH 7.18)[5]. The latter procedure revealed a single peak absorbing at 280 mμ. Both proteins were labelled to the extent of 1.0 mole dye/mole protein as calculated from the molar absorbancies of RNAase[6], lysozyme[7], and DNS[8]. Both conjugates retained full enzymic activity when assayed using RNA and *Micrococcus lysodeikticus* cell walls, respectively. Re-

Abbreviation: DNS, 1-dimethylamino-5-naphthalene-sulfonyl chloride.