rates of thoracic duct lymph flow in unanaesthetized male rats maintained under the experimental conditions of fasting and feeding. with free access to either water or 1% NaCl solution.

2. Thoracic duct lymph flow is at a low level in the fasted animal with free access to water. The rate of flow is increased by feeding or access to 1% NaCl solution. The rate of flow in the *fed* animal with free access to 1% NaCl is increased appreciably over that of the controls, and out of proportion to any hitherto employed method for *voluntarily* increasing lymph flow in the otherwise normal animal. In some instances, the lymph flow of the NaCl treated animal exceeded the body weight of the animal in a 24-hour period.

3. Alternation of periods of access to water and saline solution produced corresponding low and high rates of lymph flow in the fed animals.

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Specificity of Paper Partition Chromatography for Analysis of Free Amino Acids in Unhydrolyzed Urine.* (17495)

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The paper partition chromatographic technique devised by Consden, Gordon, and Martin(1) may be more specific for detecting free amino acids in urine than microbiological methods. The number of amino acids found by microbiological assay in unhydrolyzed urine excreted by normal human subjects is greater than the number detected by paper partition chromatography.

Paper partition chromatography has been used to investigate the amino acids excreted in the urine by normal subjects and selected cancer patients. Aliquots of a 24-hour urine collection or of pooled samples obtained over a 5 or 6 day period were standardized by Kjeldahl analysis to contain either 10 mg of total nitrogen or by the Van Slyke ninhydrin method(13,14) to contain 0.5 mg of α-amino nitrogen per milliliter. All samples were stored at 3°C under toluene. Aliquots requiring concentration were lyophilized and re-diluted to the desired volume. Other aliquots were hydrolyzed by boiling with 50% hydrochloric acid for 24 hours. After removal of the acid under reduced pressure, the hydrolysate was also standardized to contain 10 mg of total nitrogen per milliliter.

Standardization of the urine permits comparison of urine specimens from period to period and from patient to patient. The concentration selected yields chromatograms of satisfactory color intensity and insures detection of most of the amino acids present in the specimen. Concentration of normal urines to contain more than 40 mg of total nitrogen per milliliter prevents adequate separation since salts and other substances interfere. Removal of salt by common ion effect precipitation, impregnation of the filter paper and saturation of the solvents with salt has been unsatisfactory.

A two-dimensional chromatogram on Whatman No. 1 filter paper was prepared from each of the samples(1). One hundred microliters of the standardized solution were applied to the paper, and 25 microliters of 30% hydrogen peroxide were superimposed on the spot to effect quantitative oxidation of cystine and methionine to cysteic acid and

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methionine sulfone, respectively, which appear as discrete spots on the developed chromatogram. Without such conversion, cystine is destroyed and methionine is obscured by leucine, isoleucine, tryptophane, and phenylalanine. Phenol and a mixture of equal parts of γ-collidine and 2,4-lutidine were the solvents regularly employed.

Each spot on the chromatogram was outlined in pencil as soon as possible after color had been developed with 0.1% triketohydrindene hydrate (ninhydrin) in n-butanol to insure that interpretation was complete before the color faded. The amino acids and peptides were identified after calculation of the R_F values, by comparison with a control sheet to which standardized solutions of the amino acids had been applied, or by reference to a map of the spots similar to the one prepared by Dent.(3)

Other ninhydrin-reacting substances than α-amino acids(7) have seldom been found in urine in sufficient concentration to cause confusion or interference. The identity of the spots which comprise the chromatogram is frequently verified to insure that known spots are not masking the presence of other substances. Glycine, for example, may completely obscure asparagine, and β-alanine may conceal glutamine. Visual identification of certain spots may be difficult because of their close proximity and identical color as in the case of leucine, isoleucine, phenylalanine, and tryptophane.

Chromatograms of standardized unhydrolyzed urines from a number of normal subjects generally reveal the presence of alanine, cystine, glutamine, glycine, histidine, and serine. Three-fold concentration of an aliquot has revealed only β-alanine, leucine, threonine, tyrosine, and valine in addition. Although glutamic acid may be present, it has been demonstrated in this laboratory that it represents, in part at least, the amide which has undergone hydrolysis during storage or when exposed to the solvents during the run. Glutathione is also seen, as well as "over-glycine", often called taurine in the literature.(3) Doubt exists that "over-glycine" is in fact taurine, however. The infra-red absorption spectrum obtained from a sample of "over-glycine" washed from undeveloped chromatograms after localization differs from that of a standardized taurine solution.(5)

Dent, utilizing the same technique, has reported that normal urines usually contain glycine and alanine.(2) Glutamic acid and valine occasionally appear, and α-amino-n-butyric acid, citrulline, histidine, leucine, serine, and tyrosine may be found. His specimens, however, were not standardized to constant nitrogen concentration and usually contained less α-amino nitrogen than the samples analyzed in this investigation.

Arginine, cystine, glycine, histidine, leucine, methionine, phenylalanine, serine, threonine, tryptophane, tyrosine, and valine are found regularly by microbiological assay in an allegedly uncombined state in the urine of normal human subjects.(4,11,12,15) Proline, lysine, and isoleucine are sometimes present, too. Aspartic acid is detected infrequently. Free glutamic acid is found only when precautions have been inadequate for preservation of the specimens.(4) The amino acids detected in normal, unhydrolyzed urines by both methods are compared in Table I.

Determination of the amino acids in hydrolyzed urines by both microbiological and chromatographic methods yields comparable results. Whether measured microbiologically or chromatographically, hydrolysis results in an increase in the number and amount of amino acids over those found in the free state.(4,11,12,15,16)

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TABLE I.
Free Amino Acids in Unhydrolyzed Urine.

Amino acid	Methods: Paper partition chromatography, 10 mg total N per ml	Methods: Paper partition chromatography, 34 mg total N per ml	Methods: Microbiological assay (4, 11, 12, 15)
—alanine	+	+	0
,,	—	+	0
Arginine	—	—	+
Cystine	+	+	+
Glutamine	+	+	—
Glycine	+	+	+
Histidine	+	+	+
Isoleucine	—	*	+
Leucine	—	+	+
Lysine	—	—	+
Methionine	—	—	+
Phenylalanine	—	*	+
Proline	—	—	+
Serine	+	+	+
Threonine	—	+	+
Tryptophane	—	*	+
Tyrosine	—	+	+
Valine	—	+	+
Total No.	6	11	15

* May be obscured by leucine.
\+ Reported as usually present.
— Not reported as usually present.
0 No information available.

Certain of the amino acids are excreted mainly in the combined form. Ninety-nine percent of aspartic acid, 90% of glutamic acid, 80% of proline, 77% of valine, and 71% of isoleucine, for example, are known to be excreted in the urine in peptide combination or as conjugates.(15) More lysine, threonine, leucine, and arginine have also been found to be excreted in the bound form than in the free state.(12) Harvey and Horwitt(6) concluded that not all of the free amino acid determinations by microbiological assay yield valid results because of the inhibiting effect of urea and other substances in urine.

As the sensitivities of paper partition chromatography and microbiological assay are comparable,(3,8,9) the discrepancy in results suggests that the microbiological procedure may detect other than free amino acids in urine. Peptides or similar substances excreted in urine may not yield a color after reaction with ninhydrin and hence are not detectable on chromatograms, but their component amino acids apparently are available to the micro-organisms. The growth-promoting action of certain peptides has been demonstrated with some strains of micro-organisms,(10) and utilization by other strains may yet be demonstrated.

Summary The number of free amino acids found in unhydrolyzed urine by paper partition chromatography is less than that indicated by microbiological assay. No discrepancy is apparent between the methods when hydrolyzed urines are analyzed. As the sensitivities of the two methods are comparable, it is suggested that microbiological techniques are not as specific as the chromatographic procedure for the detection of urinary amino acids in the uncombined state.

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