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Alanine: Identification as Compound Interfering with Chromatographic Assays of Formiminoglutamic Acid in Urine* (33895)

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The estimation of urinary formiminoglutamic acid (FIGLU) has become widely used as an index of folic acid deficiency in man. FIGLU is a catabolic product of histidine metabolism which requires a folic acid coenzyme for its normal degradation to glutamic acid. When folic acid coenzymes are in short supply, FIGLU accumulates. Its level in urine, following a standardized histidine oral loading procedure, generally parallels the degree of folic acid deficiency (1).

Among the methods that have been employed for the detection and assay of urinary FIGLU are chromatograms on paper (2) and more recently on thin-layer cellulose (3). Paper chromatograms have not been popular because they lack sufficient sensitivity to assay FIGLU in the range of concentrations necessary for an accurate diagnosis of folate deficiency. The thin-layer technique is of considerable interest, however, because it is sufficiently sensitive for this purpose. However, in both paper and thin-layer chromatography, a ninhydrin reactive material migrates with the same R_f value as FIGLU, and is in sufficient cocentration so that its color interferes seriously with the estimation of the FIGLU present in the urine.

Since FIGLU does not have a free alpha-amino group which can react with ninhydrin, chromatograms for FIGLU are exposed to alkali prior to ninhydrin staining. Alkali removes the formimino moiety converting FIGLU to glutamate, which is ninhydrin reactive (Fig. 1, Channel 2a, 2b). To estimate FIGLU, the color intensity at the FIGLU position is compared with a known FIGLU marker. In *chromatograms of urine*, however, there is invariably considerable ninhydrin staining in the FIGLU position prior to alkali treatment (Fig. 1, Channel 4a). As a result, the color intensity of the FIGLU position *before* alkali treatment, must be subtracted from that *after* alkali treatment and compared with appropriate markers in order to estimate FIGLU concentration. This introduces significant error, even if the material is eluted and the color intensities, before and after alkali, are read in a spectrophotometer. In this report, results of studies to isolate and identify this interfering urinary compound are presented and its significance for the chromatographic detection of urinary FIGLU in clinical folic acid deficiency states is discussed.

Experimental Methods. To identify the unknown ninhydrin-positive compound(s) in urine interfering with FIGLU estimation the following plan was employed:

1. The urinary constituents were initially separated by one-dimensional paper chromatography and the material at the FIGLU locus was eluted.

2. This material was then subjected to two-dimensional chromatography and the compounds present were identified chromatographically by comparison of their position with those of known reference standards.

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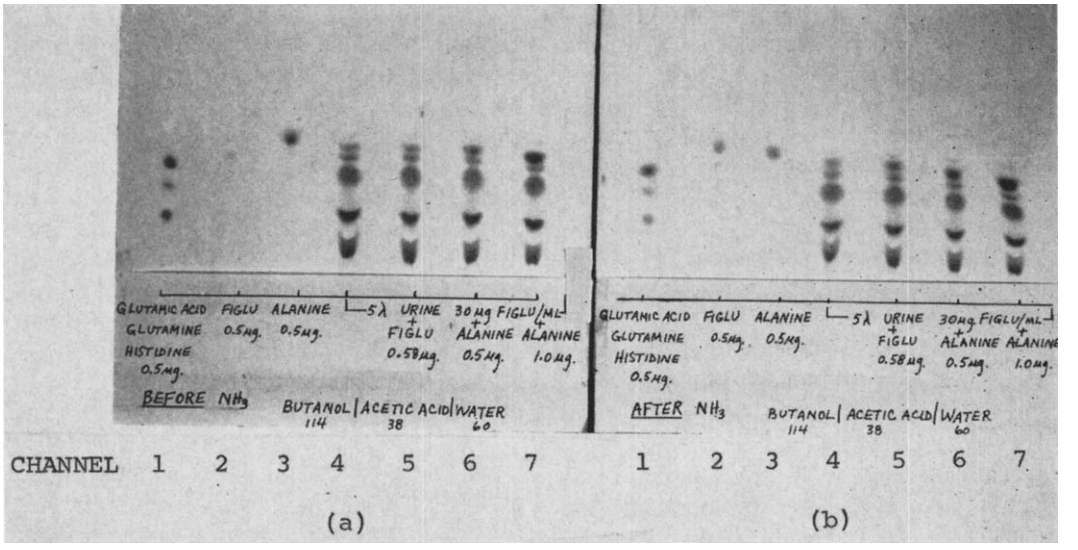

FIG. 1. Thin-layer chromatograms (cellulose), in duplicate, of a FIGLU-containing urine run alone and with crystalline FIGLU and alanine overlays. Crystalline FIGLU, alanine and a mixture of glutamic acid, glutamine, and histidine are included as markers (Channels 2, 3, and 1, respectively). Ninhydrin stained.

FIGLU appears as ninhydrin stained area only after alkali (ammonia vapor) treatment (compare channel 2b with 2a). (The lightly stained area below the FIGLU spot represents glutamic acid incompletely removed during the purification of the FIGLU). Alanine is ninhydrin stained before and after alkali treatment (3a, b). In FIGLU-containing urine, however, note the ninhydrin staining material in the FIGLU position (uppermost stained areas) both before and after alkali treatment (4a, b). Urine with crystalline FIGLU overlay shows darker spot after alkali exposure (5b compared to 5a). Channel 6a shows an immediate darkening effect in the FIGLU position when a similar amount of alanine is overlaid; channel 7, the effect of an overlay with twice that amount of alanine. Channels 5, 6, and 7 also illustrate that FIGLU and alanine in the urine of a folic acid deficient subject have the same R_f values with this developing system.

3. The identity of the unknown compound found in step 2 was confirmed chromatographically by its R_f value in three different developing systems and by its specific growth promoting properties in a microbiological assay.

Separation of the ninhydrin reactive materials at the FIGLU locus. One-dimensional paper chromatography. A urine from a folic acid deficient subject containing 100 μg of FIGLU/ml was spotted at 10 positions along the base line of a sheet of Whatman no. 1 paper, 25 μl/spot. The sheet was developed by descending chromatography using an *n*-butanol, acetic acid, water solvent system (114:38:60). This slightly acidic system gave the best separation of the FIGLU area material from other urinary constituents in paper or thin-layer chromatography. Acidic developing systems are necessary because FIGLU decomposes at neutral and alkaline pH. After an overnight run, the sheet was air dried and the right and left edges were cut off. These were treated with ammonia vapor and sprayed with ninhydrin to identify the FIGLU position. The unstained areas on the remaining portion of the chromatogram corresponding to FIGLU were cut out. These were eluted with 1 *N* ammonium hydroxide and the eluates were combined and evaporated to dryness on a water bath.

Two-dimensional paper chromatography. For further separation of the substances in the above eluate, a portion of the dry residue was dissolved in a small amount of water and reapplied on Whatman no. 1 paper for two-dimensional chromatography. Five μg of glycine were also applied as a marker amino

acid. The sheet was first developed with a pyridine, acetone, 3 *N* ammonium hydroxide solvent system (50:35:25). For the second dimension chromatogram, an isopropanol, formic acid, water solvent system was utilized (8:1:1). Ascending technique was used. The sheet was then dried and sprayed with ninhydrin. For initial identification of the unknown, reference chromatograms were run under identical conditions with amino acids known to occur in appreciable amounts in urine: asparagine, alanine, serine, threonine, glutamic acid, and glutamine.

Only four stained areas appeared. By comparison with chromatograms of synthetic amino acids these were identified as owing to glutamic acid, glutamine, glycine, and alanine. Since the area of glutamic acid and glutamine in the chromatogram of step 1 was not cut out, the glutamic acid and glutamine here must have arisen from the conversion of FIGLU in the residue by the ammonium hydroxide used for elution (1). Since the only other compound which could not be accounted for, appeared in the position in which alanine is expected, the presumptive evidence was that the unknown ninhydrin reactive material in the original chromatogram of urine at the FIGLU position prior to ammonia vapor treatment was alanine.

Confirmation of the unknown as alanine. Corroborative evidence that the unknown material in the FIGLU area residue of the test urine was in fact alanine, was obtained as follows:

Chromatographic evidence. The R_f values of synthetic alanine and FIGLU were compared in paper and thin-layer chromatograms, using the three solvent developing systems above. They were found to be identical with all three systems. In overlay chromatograms, in which urine, urine plus alanine, and urine plus FIGLU, were run simultaneously, the alanine regularly appeared in the area which coincided with the unknown ninhydrin stainable material of the native urine prior to ammonia treatment. After ammonia treatment, synthetic FIGLU always appeared at the same R_f position as the unknown and the overlaid alanine (Fig. 1, Channels 4, 5, 6, 7).

Microbiological evidence. An alanine microbiological assay [Sauberlich and Baumann (4)] was set up, employing as the test microorganism, *Pediococcus cerevesiae* (ATCC no. 8081), which requires alanine for growth. A dose–response curve was determined using synthetic alanine at five concentrations. Employing five dilutions of the residue eluted from the FIGLU area of the chromatogram in step 1, a similar assay was run in duplicate. Since increasing concentration of this residue yielded proportionately increased growth in a manner which paralleled the growth observed with increasing concentrations of synthetic alanine, the material in the residue satisfied the growth requirements of this microorganism as if it were alanine, confirming microbiologically, the presence of alanine in the residue.

It would thus appear established that the ninhydrin reactive material in urine which interferes with the chromatographic estimation of urinary FIGLU by paper and thin-layer techniques, is indeed, the amino acid alanine.

Discussion. In normal human urine, alanine is among the amino acids endogenously excreted to the greatest extent. Stein (5) found the daily excretion to average 46 mg (range 21–71); Mabry and Todd (6) 45 mg. We found the daily urinary alanine output in normal subjects, assayed microbiologically, to average 72 mg (range 37–105). In patients with megaloblastic anemia, the urinary alanine concentration may be even greater owing to the generalized aminoaciduria often associated with this condition (7, 8, 9).

The endogenous daily excretion of FIGLU in the normal human, averages less than 2 mg (range 0–6) (1). In clinical medicine, it has been found advantageous, in order to diagnose folic acid deficiency, to measure the 24-hr urinary output of FIGLU after a 15-g L-histidine $\cdot$ HCl $\cdot$ H_2O metabolic loading procedure (10). Outputs above 35 mg/day are diagnostic of the deficiency state; a large portion of such patients having excretions in the range of 35–100 mg/day.

It is in the latter range of diagnostic sig-

nificance that neither thin-layer nor paper chromatograms can be used to estimate confidently the concentration of urinary FIGLU because of the interfering color effect of endogenous alanine, even though the thin-layer technique is sufficiently sensitive to detect as little as 0.1 μg of synthetic FIGLU when 25 μl of a solution containing 4 μg/ml are applied. In addition alanine, μg for μg, stains more intensely with ninhydrin than alkali-treated FIGLU. It has thus not been possible to subtract the color intensity of alanine sufficiently accurately from that of FIGLU, to obtain a meaningful result, unless the FIGLU concentration exceeds that of alanine by at least 3–4-fold. Because of the limitations of these chromatographic assay methods, the enzymatic assay for urinary FIGLU which has the specificity and sensitivity (1.0–4.5 μg of FIGLU/ml) to quantitate accurately the level of urinary FIGLU over the entire range of clinical significance should be used for this purpose.

Summary. The assay of urinary formiminoglutamic acid (FIGLU) by paper or thin-layer chromatography for the diagnosis of folic acid deficiency has been difficult to quantitate because of the inability to separate FIGLU from a ninhydrin reactive compound in urine. This ninhydrin-positive interfering compound has been identified by chromatographic and microbiological means as the previously unsuspected amino acid alanine. Since the endogenous urinary excretion of alanine may equal or exceed that of FIGLU in normal and folic acid deficient subjects, the usefulness of paper and thin-layer chromatographic techniques is limited to qualitative estimation of FIGLU in urines with FIGLU concentrations of 150 μg/ml or more.

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