tomas. While adenosine deaminase data in our study did not correlate with observations of Reid and Lewin(4), DeLamirande and Allard‡ reported data which, in agreement with present findings, showed increased adenosine deaminase activity in precancerous livers from 3′-Me-DAB feeding.

Comparison of enzyme activities in liver and hepatomas led Potter(8) to formulate an enzyme deletion hypothesis in which it was suggested that loss of catabolic enzymes in tumor tissues functioned to preserve metabolites for synthetic pathways associated with cell multiplication. Activities of several purine-catabolizing enzymes, such as 5′-nucleotidases, 5′-nucleoside phosphorylases, guanase and adenase, were reported(3) considerably lower in Novikoff hepatomas compared with normal liver; while xanthine oxidase and uricase appeared to be absent. Decreases of these purine-catabolizing enzymes could be interpreted as functioning to preserve purines for nucleic acid synthesis. Since adenylic acid deaminase and adenosine deaminase may function in conversion of adenine compounds to guanine compounds for incorporation into nucleic acids, their increased enzymatic potential during azo-dye carcinogenesis may be correlated with the metabolite preservation concept of Potter(8). Moreover, it is interesting to note that studies with other fast growing tissues such as chick embryos(9) and regenerating rat livers(10) also showed increased adenosine deaminase activity.

*Summary.* Adenosine and adenylic acid deaminase activities were determined in precancerous livers and in primary hepatomas induced by feeding 3′-methyl-4-dimethylaminoazobenzene. The deaminase activities in precancerous livers were increased about 100% after 30 days of dye feeding compared with control livers. Activities in primary hepatomas and in the liver adjacent to primary hepatomas were essentially the same as that of precancerous livers, indicating that no additional changes occurred upon formation of neoplastic tissues.

‡ This report was presented at Am. Assn. for Cancer Research, Atlantic City, N. J., 1959.

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## Determination of Apparent Hippuric Acid and Free Glycine in Urine by Microbiological Assay.* (25158)

MERRILL N. CAMIEN, AUDREE V. FOWLER AND MAX S. DUNN
*Chemical Laboratory, University of California, Los Angeles*

Values for "bound" or "combined" amino acids in urine have commonly been reported as the difference between amino acid levels found by microbiological assay of this material before and after subjecting it to acid hydrolysis (1-4). It has generally been recognized that such values may lack quantitative significance,† but that they may not be meaningful

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† Qualifying quotation marks are almost always used with the terms "free" and "bound" in this sense.

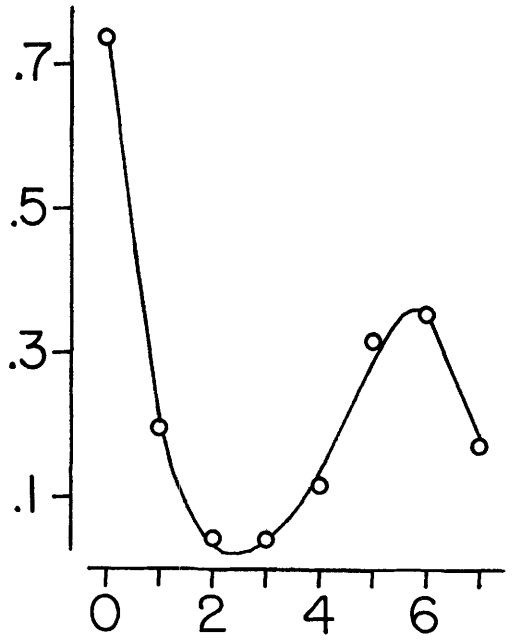


FIG. 1. A 7-transfer countercurrent distribution of glycine and hippuric acid with *n*-butanol and 5% hydrochloric acid. Cell numbers are indicated on horizontal scale. Fractions of total glycine (left hand peak) and hippuric acid (right hand peak)/cell are indicated on vertical scale. Plotted values were calculated from titrations of residual glycine hydrochloride and hippuric acid with sodium hydroxide after evaporating cell contents to dryness. Curve drawn through the points is the theoretical one (derived mathematically) for 2 components with partition ratios of 0.04 (left hand peak) and 3.7 (right hand peak), respectively.

has not been so widely appreciated. Microbiological assay of glycine in urine before and after hydrolysis, for example, yields values (5-7) which give no indication whatever of the relative amounts of free and bound glycine. The reason is that hippuric acid, a major bound form of urinary glycine, effects a microbiological response, qualitatively and quantitatively similar to that of free glycine (8). The present report concerns separation of hippuric acid from free glycine in urine before subjecting it to microbiological analysis. This modification of the usual glycine assay procedure was developed as a means of furthering current investigations of glycine and benzoic acid metabolism in the authors' laboratory.

*Methods.* Countercurrent extraction procedure and solvent system were those described by Craig *et al.*(9). The partition ratios of glycine (0.04) and hippuric acid (3.7) in this system (*n*-butanol, 5% hydrochloric acid) permitted quantitative separation of these metabolites with a 7-transfer distribution (Fig. 1). Eight cells of conventional countercurrent extraction train(9) were employed for this trial separation (Fig. 1), although 4 of 8 cells were eliminated in subsequent separations by employing "single withdrawal" procedure(9). Centrifuge tubes (narrow neck with rubber stoppers) were used as extraction cells to distribute the urine samples‡ because this permitted rapid separation of emulsified solvents by centrifugation. In these cases the lower phase was taken as the mobile phase because it proved most convenient of the 2 phases to transfer by pipet from one tube to the next. Fresh lower phase was added to cell 0 (first tube in the series) after the first through third transfer. Lower phase was withdrawn from cell 3 (last tube in the series) after fourth through seventh transfer.§

‡ Pooled urine with and without measured additions of glycine and hippuric acid (for recovery tests) was acidified to contain 5% hydrochloric acid and saturated with *n*-butanol before subjecting it to the described countercurrent extraction.

§ Tubes were shaken well and centrifuged (if required to separate the phases) before each transfer. Initially, 15 ml of fresh upper phase solvent (*n*-butanol saturated with 5% hydrochloric acid) was placed in each of the 4 cells, and 15 ml of urine sample was placed in cell 0. The first transfer was accomplished by pipetting lower phase from cell 0 into cell 1 and adding 15 ml of fresh lower phase solvent (5% hydrochloric acid saturated with *n*-butanol) to cell 0. The second transfer was accomplished by pipetting lower phase from cell 1 into cell 2 and from cell 0 into cell 1 and adding 15 ml of fresh lower phase solvent to cell 0. The third transfer was accomplished by pipetting lower phase from cell 2 into cell 3, from cell 1 into cell 2, and from cell 0 into cell 1 and adding 15 ml of fresh lower phase solvent to cell 0. The fourth transfer was accomplished by pipetting lower phase from cell 3 into a collecting flask (to contain the "free-glycine fraction"), from cell 2 into cell 3, from cell 1 into cell 2, and from cell 0 into cell 1. This procedure was repeated until 7 transfers were completed and a total of 4 lower phase portions from cell 3 were collected in the collecting flask. Fresh lower phase was not added to cell 0 after fourth

TABLE I. Glycine in Urine Fractions.*

| Addition to sample, μM/ml | Fraction assayed† | Glycine found,‡ μM/ml | Glycine recovered§ μM/ml | % |
|---|---|---|---|---|
| None | F.G. | 3.07 (2.93-3.14) | | |
| | H.A. | 4.62 (4.60-4.65) | | |
| Glycine 4.28 | F.G. | 7.16 (7.00-7.28) | 4.09 | 95.6 |
| | H.A. | 4.68 (4.57-4.77) | .06 | 1.4 |
| Hippuric acid 3.55 | F.G. | 3.06 (2.83-3.40) | .0 | .0 |
| | H.A. | 8.04 (7.86-8.28) | 3.42 | 96.3 |

* Equal volumes of urine from 5 apparently normal male volunteers were pooled. Aliquots of combined material were supplemented as indicated (column 1 of Table) and subjected to fractionation and assay procedures given in text.

† F.G. = "free glycine fraction;" H.A. = "hippuric acid fraction."

‡ Each value is avg (range in parentheses) of 4 independent assay results.

§ Includes glycine added as hippuric acid.

The combined lower phase solutions withdrawn from cell 3 were taken as the "free glycine fraction," and the combined upper phase solutions remaining in the 4 cells were taken as the "hippuric acid fraction." Both fractions were freed of butanol by distillation, and the aqueous residues were subjected to acid hydrolysis and microbiological assay for glycine essentially as described previously(5). Results are compiled in Table I.

*Discussion.* The experimental data (Fig. 1, Table I) suggest that the abbreviated countercurrent extraction procedure described here is capable of effecting a quantitative separation of free glycine from hippuric acid in urine as well as in artificial mixtures of these metabolites. The apparent ratio of hippuric acid to free glycine in normal urine is approximately 1.5 : 1 according to these data. It is evident that the apparent ratio could deviate considerably from the true ratio if either fraction of urine sample should contain some bound form of glycine other than hippuric acid. It seems likely, however, that despite this potential source of error, determination of apparent free glycine and apparent hippuric acid in this manner can provide useful information for metabolic, clinical and nutritional studies, particularly those directly concerning glycine and benzoic acid.

Classical data for hippuric acid in normal urine yield the average value of 3.3 μM/ml,‖ with "normal" concentrations ranging to several times the average under the influence of dietary factors(10). The present value of 4.6 μM/ml (Table I) is therefore in reasonable accord with the classical data. It is of interest that total glycine (including hippuric acid glycine) ranged from 3.1 to 11 μM/ml (median 6.8 μM/ml) in fourteen 24-hour urine samples from 6 normal subjects on free-choice diets and from 11 to 17 μM/ml (median 15 μM/ml) in eight 24-hour urine samples from one of the same individuals maintained solely on army "K-ration" according to earlier data from this laboratory(5).

Seemingly reliable literature data for free glycine in urine appears limited to those obtained by chromatographic procedures, and the values reported on this basis for normal urine(11-13) vary from 0.3 to 1.8 μM/ml (range of 13 values, median 1.3 μM/ml). The discrepancy between these values and the present value of 3.1 μM/ml (Table I) suggests that "apparent free glycine" determined by the present method may include considerable amounts of combined glycine (other than hippuric acid). It is of interest that one author(11) has reported the presence of 2 glycine-containing peptides at concentrations totalling approximately 0.3 μM/ml in normal urine. It is possible, on the other hand, that "normal" values for free glycine in urine may actually range to levels considerably higher than those reported.

The solvent system and simple stage by stage extraction described here have proved satisfactory for separation of hippuric acid from free glycine in urine. Other solvent systems and extraction procedures might nevertheless be advantageous. Preliminary tests

and subsequent transfers since such additions would not be carried beyond cell 3 by these transfers and would therefore be superfluous.

‖ Calculated from 0.7 g hippuric acid and 1200 ml water, reported as average 24 hour normal urine values by Hawk *et al.*,(10) (and in most other physiological chemistry text books).

of ether *vs.* dilute hydrochloric acid and ethyl acetate *vs.* dilute hydrochloric acid, for example, yielded partition ratios of 0.3 and 2, respectively, for hippuric acid, but less than 2 x $10^{-7}$ and 4 x $10^{-4}$, respectively, for free glycine. These data suggest that quantitative separation of hippuric acid from glycine could be effected by either carefully controlled continuous liquid-liquid extraction with either of these solvent systems¶ or appropriate stage by stage extraction from a single cell with ethyl acetate versus dilute hydrochloric acid system.

*Summary.* Apparent free glycine and apparent hippuric acid in urine have been determined by microbiological analysis after fractionating it by a 4-cell, 7-transfer countercurrent distribution between *n*-butanol and 5% hydrochloric acid. The effectiveness of this procedure was demonstrated by separating hippuric acid from free glycine in known mixtures and by recovering known amounts of these metabolites added to urine before subjecting it to the fractionation procedure. The apparent concentrations of free glycine and hippuric acid in normal pooled urine were 3.1 and 4.6 $\mu$M/ml, respectively.

¶ Earlier investigators employed continuous liquid-liquid extraction with ethyl acetate(14) and ether (15,16) in connection with chemical determinations of urinary hippuric acid.

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## A Spectrophotometric Technic for Measuring Erythrocyte Chimerism in Cattle.* (25159)

ARTHUR P. MANGE AND W. H. STONE (Introduced by M. R. Irwin)
*Dept. of Genetics, University of Wisconsin, Madison*

The vascular anastomosis known to occur frequently between pairs of cattle dizygotic twins(1) permits an interchange of blood-forming tissues resulting in chimerism of the erythrocytes(2). The 2 kinds of erythrocytes within each member of such twin pairs can be demonstrated by a technic known as differential hemolysis(3,4). Generally, cells of an individual sensitized by an appropriate antibody (reagent) are completely hemolyzed by addition of complement (normal rabbit serum) (5). However, cells of chimeras often are only partially hemolyzed by this treatment. Such partial reactions indicate presence of 2 distinct cell populations, those with and without the antigenic specificity against which the reagent reacts. Complete antigenic constitutions of the 2 cell types can usually be determined by testing residual cells (unhemolysed)

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