uniformly encountered appear to be related to an alteration in hepatic handling of hydrocortisone in patients treated with ethinyl estradiol.

Hydrocortisone is normally metabolized in large part by a series of steps involving (1) removal from the circulation by the liver, (2) enzymatic reduction to its dihydro and then (3) tetrahydro derivatives, (4) conjugation with glucuronic acid and, finally, (5) reintroduction of the conjugated derivatives into the circulation for excretion by the kidneys. The delay in clearance of infused hydrocortisone from plasma found in this study could reflect alterations in steps 1-4 of this mechanism. The data at hand do not permit more exact localization of the effect of ethinyl estradiol in the liver. Studies are in progress in this laboratory to define more precisely the physiological alteration in hydrocortisone metabolism induced by ethinyl estradiol.

Summary. 1. Ethinyl estradiol in doses of 0.1 and 0.5 mg daily by mouth caused marked elevations in plasma 17-hydroxycorticosteroid levels in 18 post-menopausal and castrate females and in 2 patients whose hypopituitarism was associated with only moderate decreases in adrenocortical secretory capacity. 2. No increases in plasma 17-OHCS levels during ethinyl estradiol therapy were found in a male Addisonian and in a patient with a pituitary tumor and marked secondary adrenocortical insufficiency. 3. Elevations in plasma 17-OHCS were accompanied by augmented responsiveness to a standard ACTH infusion in all 10 of the post-menopausal females studied and in 1 of 2 patients with hypopituitarism. 4. A marked delay in rate of plasma clearance of infused hydrocortisone was present in all 4 of the ethinyl estradiol treated patients so tested. 5. The data suggest that ethinyl estradiol causes an alteration in hydrocortisone metabolism.

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Determination of α-Amino Acid Nitrogen in Urine. (23370)

CHARLES SOBEL, RICHARD J. HENRY, NEIL CHIAMORI AND MILTON SEGALOVE
Bio-Science Laboratories, Los Angeles 64, California

The gasometric-ninhydrin method(1,2) is generally conceded to be the most specific technic available for determination of α-amino acid nitrogen in urine, but is rather tedious for routine analyses. The gasometric-nitrous acid(2,3) and the formol titration methods (2,4) are less specific. Folin's photometric technic(5) employing sodium β-naphthoquinone-4-sulfonate reacts with uric acid and ammonia present in urine(6,7). Cagan and coworkers(8) boiled off ammonia and used charcoal to remove uric acid, but did not investigate the specificity of their technic. Pope and Stevens(9) introduced a method wherein copper is bound to the amino group and the bound copper quantitated by iodometric titra-

tion, a technic applied to urine by Albanese and Irby(10). Photometric determination of the copper with sodium diethyldithiocarbamate has been introduced(11,12,13) but not applied to analysis of urine. It was believed worthwhile to investigate the specificity of the technic of Cagan, *et al.* and a modified photometric copper technic. Preliminary isolation of the amino acids on Dowex 50 x 8, a resin used by Stein(14) in chromatographic separation of urinary amino acids, enhanced the specificity.

Methods. Isolation by ion exchange column. Dowex 50 x 8, 200-400 mesh, analytical reagent grade, hydrogen form, is suspended in water and poured into a small column (10 mm internal diameter), in the bottom of which has been placed a glass wool plug. The column is packed by gravity to a depth of about 50 mm and then a glass wool plug placed on top of the resin. Ten ml of the urine is acidified to a pH of 1 to 2 (pH paper) with 1 N HCl and then passed through the column (the liquid level is never allowed to pass the upper surface of the upper glass wool plug). The column is washed with two 1 ml aliquots and one 3 ml aliquot of 0.01 N HCl, then with distilled water until the eluate is approximately pH 5 by pH paper. Add 1 ml of 1 N NaOH and, after the level has reached the top of the upper plug, add 10 ml of 1 N NaOH. When the NaOH front has descended to about the middle of the resin bed (detectable as a color change in the resin from a light brown in the hydrogen form to a darker brown in the sodium form), *i.e.*, after about 3 ml of eluate has appeared, begin collecting eluate in a graduated centrifuge tube to a total volume of 10 ml.

Copper method. Reagents. 1. Cupric chloride, 2.8% $CuCl_2 \cdot 2H_2O$, filtered. 2. Sodium phosphate, 6.85% $Na_3PO_4 \cdot 12H_2O$, filtered; discard if turbidity develops. 3. Borax solution, 1.9% $Na_3BO_3 \cdot 10H_2O$, filtered. 4. Copper phosphate suspension. To 40 ml of the sodium phosphate solution add, slowly with constant shaking, 20 ml of the cupric chloride solution. Centrifuge and discard the supernate. Stir up the precipitate in about 15 ml of the borax solution, using a glass rod to break up the clumps. Add an additional 45 ml of the borax solution, mix, centrifuge and discard the supernate. Repeat this washing procedure. Finally, suspend the precipitate in 100 ml of the borax solution containing 3 g NaCl. This suspension is usable for several weeks if kept in the refrigerator. This is the reagent as proposed by Schroeder(15) except for alteration in the sodium chloride content. The 5% concentration of NaCl used by Schroeder led to clumping of the suspension in a few days, rendering it unusable. 5. Sodium diethyldithiocarbamate, prepared freshly by dissolving 40 mg of reagent in 2 ml of distilled water and filtering. 6. Standard. Transfer 0.268 g glycine, 0.525 g glutamic acid and 1 g sodium benzoate to a 500 ml volumetric flask. Add about 200 ml water and shake to dissolve the solids. Add 4 ml conc. HCl, dilute to mark and mix. One ml contains 0.2 mg α-amino acid nitrogen.

Procedure. For greatest accuracy, or if the urine contains abnormally large amounts of ammonia, aerate the column eluate for one-half hour to remove the ammonia. Transfer 2.5 ml of column eluate to a 35 ml glass-stoppered centrifuge tube. Add 2.5 ml water, a drop of thymolphthalein indicator and add 6 N HCl drop by drop until indicator is decolorized, then add 1 N NaOH drop by drop until indicator just turns blue. A reagent blank is prepared by transferring 5 ml water to a similar tube, adding a drop of indicator followed by 0.1 N NaOH dropwise to the color change. A standard is prepared with 2.0 ml of the standard amino acid solution, 3.0 ml water, a drop of indicator and adding 1 N NaOH dropwise to the color change. To each tube, add 5.0 ml copper phosphate suspension, stopper and shake for 5 minutes. Centrifuge and filter the supernate through Whatman No. 1 filter paper. Dilute 1.0 ml of each filtrate to 25 ml with water. Transfer 2.0 ml of each diluted filtrate to a test tube. Add 0.1 ml of the diethyldithiocarbamate reagent, 3.0 ml isopropyl alcohol and mix. Read the absorbances of the unknowns (A_x) and standard (A_s) against the reagent blank at 435 mμ. The color is stable for at least 2 hours and obeys Beers law, employing either a Beckman Model B spectrophotometer or a

Klett-Summerson photometer with a number 42 filter.

Calculation: $\frac{A_x}{A_s} \times 16 =$ mg α-amino acid N per 100 ml urine.

β-Naphthoquinone - 4 - sulfonate method. The method of Cagan *et al.* was applied to the column eluate, including removal of ammonia by boiling but deleting the charcoal treatment since uric acid has already been removed by the ion exchange procedure.

Gasometric ninhydrin method. The technic of Van Slyke *et al.*(1,2) was employed except that, to effect a complete transfer of the CO_2, it was found necessary to wash back and forth 12 times from the reaction tubes used to the Van Slyke chamber.

Results. Stoichiometry of the 3 methods. Taurine, glycine, histidine and 3-methyl histidine account for about 70% of the free amino acids normally excreted in the urine (14). Cystine, lysine, arginine and phenylalanine are also of interest because of increased excretion in certain metabolic diseases. Table I compares the apparent α-amino acid N yielded by these amino acids, employing the 3 methods applied directly to pure standard solutions. The relative values observed for the copper phosphate method do not agree exactly with similar studies by others(9,11). Examination of the relative values leads to the prediction of lack of exact correlation between the 3 methods.

TABLE I. Relative Values Obtained by the 3 Methods on Pure Standards.

	Relative values*		
	Gasometric ninhydrin	Copper phosphate	Naphtho-quinone
Glycine	.95	.93	.81
Histidine	1	1.28	.99
l-Methylhistidine	1	1.24	1.07
Phenylalanine	1	.95	1.10
Lysine	1.05	.89	1.33
Arginine	1	.91	.93
Taurine	.0	.0	1.06
Cystine	1.9	†	1.64

* Gasometric ninhydrin method—mols of CO_2 recovered per mol of amino acid. Copper phosphate and naphthoquinone methods—values are relative to a mixed glycine-glutamic acid reference standard, all in equimolar concentration.

† Low and variable.

Recovery of amino acids from ion exchange column. Recoveries of pure standards of glycine, alanine, glutamic acid, histidine, l-methylhistidine, lysine, phenylalanine, arginine and cystine ranged from 94 to 104% as measured by the gasometric, copper phosphate and naphthoquinone methods. Recovery of amino acid nitrogen by column treatment of urines as determined by the gasometric technic ranged from 81 to 97% with a mean of 87% (10 urines, Table II). Recovery of taurine from the column, however, was less than 5%. This loss by column treatment would not be detectable by the gasometric technic in any case since taurine is not measured by this method.

Column vs. batch operation with resin. Removal of amino acids from urine by batch adsorption gave up to 50% less recovery than column treatment even when 2 successive batch treatments were employed.

Removal of uric acid and protein by column treatment. Uric acid gives color in the naphthoquinone procedure and is removed by charcoal adsorption in the method of Cagan *et al.*(8). They observed no loss of amino acid in the cases of glycine, glutamic acid and casein hydrolysate as long as the amount of charcoal (Norit A) employed did not exceed 100 mg per 100 ml of solution. Awapara and Sato(16) used charcoal (Darco G-60) adsorption as a purification step prior to chromatography of urinary amino acids. They added chloroform to prevent adsorption of tyrosine, phenylalanine and tryptophan and claimed that no loss of amino acids occurred. Following the technic of Cagan and coworkers, and employing 50 mg Norit A, losses of 20 to 60% were observed with arginine, cystine, glycine, glutamic acid, histidine, lysine, proline and serine added to urine. Losses amounting to 70 to 95% were observed with tyrosine and tryptophan. Use of Norit A washed with ether-acetic acid and then ether still gave large losses of tyrosine and tryptophan but gave considerably better recoveries of the other amino acids (losses of 0 to 20%). Using the technic of Awapara and Sato and Darco G-60, losses of 15 to 62% were observed with tyrosine, tryptophan, arginine,

TABLE II. Comparison of the 3 Methods, with and without Ion Exchange Treatment.

	mg α-amino acid N per 100 ml urine					
	Gasometric method		Copper method		Naphthoquinone method	
Nature of donor	Without resin	With resin	Without resin	With resin	Without resin	With resin
Normal adults	18.4	15.0	20.6	13.1	19.5	13.3
	8.2	6.8	12.6	7.0	13.7	6.5
	5.2	4.7	5.5	2.6	7.2	6.4
	16.2	13.2	25.0	14.7	27.1	19.5
	18.5	15.5	27.6	22.7	27.8	19.0
	10.5	9.9	12.8	8.8	14.7	9.9
	13.4	12.5	27.9	14.8	22.7	17.5
	10.3	10.0	16.3	10.6	16.8	12.8
	19.6	15.9	34.7	21.3	24.7	18.4
		7.3		7.0		8.7
		20.2		24.6		21.1
		12.2		18.6		12.9
		7.6		8.3		11.0
		10.6		10.2		13.0
		15.1		16.2		20.3
		9.7		14.4		12.3
		5.7		2.6		5.4
	14.4			13.6		14.7
	12.1			15.2		15.9
	18.7			27.0		18.1
	20.0			21.8		20.0
Child	4.0		2.0	2.8	6.5	2.9
"		8.3	19.0	10.2	11.8	6.7
Cirrhosis	2.9			1.8		5.1
"	6.3		21.5	13.5	18.7	14.5
Wilson's disease	15.0			14.9		13.4
" "	27.8			39.3		27.7
" "	17.5		20.2	18.8	24.0	17.1
" "	25.0		29.2	30.8	25.3	25.3
" "	25.3	23.1	44.5	36.0	35.0	27.0

glutamic acid, histidine, glycine, lysine, proline and serine, either as pure solutions or internal standards added to urine.

It was found that approximately 95% of uric acid was removed by the ion exchange column procedure when the initial uric acid concentration was as high as 85 mg %. From a study of the color produced by uric acid in the naphthoquinone reaction, it was concluded that a final concentration of 5 mg uric acid per 100 ml of column eluate would introduce an error of about 1% at a level of 20 mg α-amino acid N per 100 ml urine.

Experiments also showed that serum proteins added to urine to a concentration of 1% were effectively removed by the ion exchange column procedure.

Effect of heating. Only about one-third of the amino acids excreted are present in the urine in the free form, the remainder being conjugated(14). It is commonly presumed that the 3 technics employed in this study determine the unconjugated α-amino acid N, preliminary acid hydrolysis being required if the total is desired. In the gasometric-ninhydrin method, the reaction is carried out at pH 2.5 and 100°C for 8 minutes(2) and, in the β-naphthoquinone method, the ammonia is removed from the urine by boiling at a pH of about 9.5 for 15 minutes. Several urine samples were analyzed, employing these conditions, and also doubling reaction time with ninhydrin and removing the ammonia by vacuum distillation at 50°C for the β-naphthoquinone method. No differences in results were observed, indicating no hydrolysis of the conjugated amino acids present.

Interference by ammonia in the copper phosphate method. Albanese and Irby(10) claimed that urea, uric acid, creatine, creatinine and ammonia do not interfere in the copper phosphate method. In view of the fact that ammonia complexes copper in

alkaline solution this seemed implausible. This was reinvestigated and it was found that, by the technic proposed, a urine concentration of 0.12% NH_3 gave a color in the copper phosphate method equivalent to 1.7 mg α-amino acid N per 100 ml. Since the normal output of ammonia is about 0.7 g per day(17), the positive error in a urine with normal ammonia content is about 1 mg % if the ammonia is not removed. If the ammonia is high as a result of disease or degradation of urea on standing, the ammonia should be removed. It was found that an ammonia concentration of 1% in the original urine was effectively removed by the aeration technic suggested.

Comparison of results obtained by the 3 methods. Table II lists the results obtained in a comparison of the 3 methods, with and without preliminary ion exchange treatment. Each value given in the table is the mean of duplicate determinations. In the case of the naphthoquinone method applied to urine not treated by ion exchange, uric acid was removed by charcoal adsorption by the procedure of Cagan *et al.* It is seen that results obtained by the copper phosphate and naphthoquinone methods without ion exchange treatment ranged up to double those obtained by the gasometric ninhydrin method. Thus, in spite of loss of amino acids in the charcoal treatment for removal of uric acid, the naphthoquinone method without the ion exchange isolation yielded falsely high results in several instances. Preliminary isolation of the amino acids by ion exchange significantly improved correlation of results given by the 3 methods.

Reproducibility. The standard deviations calculated from the duplicate determinations were 0.51, 0.68 and 0.81 mg % for the gasometric, copper phosphate and naphthoquinone methods, respectively.

Stability of urine samples. Four normal urine samples held at room temperature were analyzed for α-amino acid N daily for 4 days with no change indicated by any of the 3 methods. After 1 week standing, 3 of the samples appeared unchanged, whereas the fourth urine showed an increase of about 50%. By all 3 methods, the same 4 urines were found to be stable for 5 weeks at refrigerator temperature.

Discussion. In the spectrophotometric technics of Woiwod(12) and Beauchene and coworkers(13) the color complex produced with diethyldithiocarbamate, which is relatively water-insoluble, is extracted into amyl or isoamyl alcohol. In the present technic, this extraction is obviated by the addition of isopropyl alcohol to the aqueous solution.

By preliminary isolation of the urinary amino acids by ion exchange resin, taurine is lost because it is not adsorbed by the technic used. This is of concern only in the case of the β-naphthoquinone method since the other 2 methods do not determine taurine. This would tend to bring the 3 methods into better agreement. Normally, about 12 to 20% of the total free amino acid N is present as taurine(14,18). It is obvious, however, that the 3 methods under consideration will not give exactly the same values. Nevertheless, these results can be of value for clinical purposes.

From the results obtained in this study, the choice between the copper phosphate and naphthoquinone methods applied to urines after preliminary isolation of the amino acids by ion exchange appears to be one primarily of convenience. It is the authors' opinion that for the occasional sample the naphthoquinone method is somewhat more convenient but for extended series of samples the copper phosphate method is somewhat simpler.

Summary. A modification of the photometric copper phosphate method for determination of α-amino acid nitrogen in urine has been presented. A comparison of results obtained in urines by the copper phosphate, naphthoquinone and gasometric-ninhydrin methods indicates that the specificity of the copper phosphate and naphthoquinone methods is materially improved by preliminary isolation of the amino acids on an ion exchange column.

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Histology and Regenerative Capacity of Liver Following Multiple Partial Hepatectomies.* (23371)

DWIGHT J. INGLE AND BURTON L. BAKER
Ben May Laboratory for Cancer Research, University of Chicago, Chicago, Ill., and Department of Anatomy, University of Michigan Medical School, Ann Arbor

Repeated partial hepatectomies were performed in the rat in order to study in the remaining portion of the liver (a) maintenance of regenerative capacity, (b) histological alterations, and (c) possible appearance of neoplasia as a result of prolonged stimulus to regeneration. The regenerative capacity was not exhausted by as many as 12 partial hepatectomies and neoplasia was not seen.

Procedure. The surgery was carried out at the University of Chicago and the histological studies were done at the University of Michigan. The procedure for repeated partial hepatectomy in the rat has been described(1). Thirty male rats having initial weights of 240-260 g were given Archer Dog Pellets to eat *ad libitum* during the experiments. Twelve of the rats were subjected to sham operations which consisted of a laparotomy once each month. Eighteen rats underwent repeated partial hepatectomies, once each month. The procedure was to remove all of the liver but the median lobe at the first operation, a part of the median lobe was excised at the second operation and thereafter liver was aspirated by negative pressure applied to a pipette inserted in a slit in the now thickened capsule of liver. The post-operative care of the rats has been described(1).

At the termination of the experiment, the rats were killed by exsanguination under ether anesthesia. The liver was fixed in ice-cold Carnoy's fluid and left in the refrigerator overnight. On the next day the Carnoy's fluid was replaced with absolute alcohol and the livers shipped to Ann Arbor. A large piece of the liver was prepared for microscopic study. Sections were stained with periodic acid-leucofuchsin(PAS) and counter-stained with methylene blue in a citric acid-phosphate buffer (pH 5.6).

Results. All of the sham-operated rats survived for the duration of the experiment. All of the hepatectomized rats survived 8 operations. During the following 4 months 5 rats died as the result of self-inflicted wounds after the incision of the last operation had healed. Two rats died from post-operative hemorrhage due to breaking of adhesions. There were no deaths due to failure to control hemor-

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