tissue per hour of incubation.

Clearly the convention of expressing aldosterone output in terms of tissue weight minimizes the secretory capacity of the decapsulated portion since the non-productive medulla is included in the weight of this part of the gland. It is believed that this consideration does not invalidate the conclusion that aldosterone is produced primarily by the capsular portion and by the zona glomerulosa. This conclusion is based on the following reasons: an arbitrary correction factor which assumes the weight of the medulla to be 50% of the decapsulated gland still yields a significant difference when the corrected output of the decapsulated glands is compared to that of the capsules ($P<.001$). Calculation of aldosterone output in terms of total weight of tissue incubated shows a significantly greater output by the capsules ($P<.001$). Finally as pointed out previously, the aldosterone production of the intact glands (Table I) is strictly comparable to that of the sum of the two portions of the glands incubated separately ($P>.3$), emphasizing the difference in output in favor of the capsule containing the zona glomerulosa. It is therefore concluded that the present findings strongly suggest that aldosterone is produced principally by the cells of the zona glomerulosa.

Conclusion. Rat adrenal glands incubated *in vitro* release aldosterone at the rate of 0.66 μg/100 mg of tissue per hour. Decapsulated rat adrenal glands secrete only negligible amounts of the hormone. Comparison of aldosterone production between decapsulated rat adrenals and their capsules shows that most of the hormone is secreted by the capsular portion which contains the cells of the zona glomerulosa. These findings are strong evidence that the principal site of aldosterone secretion in the rat adrenal is the zona glomerulosa.

We are deeply indebted to Mr. M. Vulpe, Department of Neuro Anatomy, Montreal Neurological Institute, for the histological study and for his valuable advice.

1. Giroud, C. J. P., Saffran, M., Schally, A. V., to be published.
2. Deane, H. W., Shaw, J. H., Greep, R. O., *Endocrinol.*, 1948, v43, 133.
3. Saffran, M., Schally, A. V., *ibid.*, 1955, v56, 523.
4. Bush, I., *Biochem. J.*, 1951, v50, 370.
5. Venning, E. H., Dyrenfurth, I., Giroud, C. J. P., *J. Clin. Endocrinol. & Metab.*, in press.

Received April 9, 1956. P.S.E.B.M., 1956, v92.

Evidence for Occurrence of 10-Formyltetrahydrofolic Acid in Human Urine. (22417)

ALBERTA M. ALBRECHT AND HARRY P. BROQUIST.
Nutrition and Physiology Section, American Cyanamid Co., Lederle Laboratories, Pearl River, N. Y.

The term "citrovorum factor activity" is commonly used to denote the growth-promoting effect of certain substances for *Pediococcus cerevisiae,* formerly known as *Leuconostoc citrovorum*(1,2). Citrovorum factor (CF) activity is shown by 5-formyl-5, 6, 7, 8-tetrahydropteroylglutamic acid (5CHOTHFA) (3,4), by crude preparations of tetrahydropteroylglutamic acid (THFA)(5), and by massive concentrations of pteroylglutamic acid (FA)(1).

The urinary excretion of CF activity in adult males was shown to increase markedly following the oral administration of FA(6,7), thus indicating that FA was converted *in vivo* to CF. However, these studies were based upon microbiological assays in which the samples were autoclaved and hence gave no indication as to whether heat-labile substances with CF activity were present in the urine. Recent studies of the mechanism of CF formation in certain enzyme systems have demonstrated the existence of such heat-labile substances(8,9) thus prompting the con-

TABLE I. Citrovorum Factor (CF) Activity in Urine following Oral Administration of Folic Acid. Values are expressed in terms of DL-5CHOTHFA, 4 samples.

Dose administered		CF excretion in 12 hr		Heat labile CF
Folic acid, mg	Ascorbic acid, g	Heated sample, γ	Unheated sample, γ	% of total CF
10	—	17	36	53
50	—	219	420	48
50	1.0	59	277	78
50	1.0	35	180	81

sideration that human urine might contain such forms of CF. This communication presents evidence from microbiological studies and paper chromatography that a heat-labile form of CF, termed HLCF, exists in human urine. The relationship of HLCF to 10-formyltetrahydropteroylglutamic acid (10CHOTHFA), anhydroleucovorin (AHL) and 5CHOTHFA is discussed.

Methods. CF assays were carried out with *Pediococcus cerevisiae* (*Leuconostoc citrovorum*, ATCC #8081) following the technic of Sauberlich(1). Leucovorin (synthetic DL-5CHOTHFA) was used as the reference standard; results are expressed in terms of the DL anhydrous free acid. Microbiological assays were carried out in 11 x 102 mm test tubes in a final volume of 2 ml. Test samples were either autoclaved with the medium for six minutes, or by contrast were added aseptically in a volume of 0.1 to 0.2 ml to previously autoclaved and cooled medium. For paper chromatography 0.05 ml samples of urine were spotted on one-half inch strips of Whatman #1 paper and developed with 0.1 M sodium acetate, pH 5.4, for 2 hours at 25°C with ascending technic. Bioautography of paper chromatograms was accomplished by placing strips on agar plates of medium which were appropriate for growth of either *L. citrovorum* or *Streptococcus faecalis* (ATCC #8043) and were seeded with an inoculum of the respective organism. Location and size of zones were recorded after the plates were incubated for 16 hours at 37°C. Urine samples were obtained from adults who had taken an oral dose of folic acid and ascorbic acid before retiring. Urine was collected for the next twelve hours, divided into a series of aliquots and stored at −10°C. Aliquots for assay were withdrawn, thawed, and used immediately.

Results. The total amount of CF excreted in 12 hours following the oral administration of FA singly or with ascorbic acid is shown in Table I. Significantly higher excretion values of CF were obtained when the samples were assayed aseptically indicating a possibility that more than one form of CF might be present in the samples. Further evidence for this possibility was obtained by paper chromatography. In many experiments difficulty was encountered in obtaining rigidly reproducible R_f values for the CF forms in urine; this was believed due to the presence of salts in urine and the effects of heating urine. More weight was given to the positions of the growth zones relative to each other rather than to constant R_f values.

Chromatograms of freshly thawed urine showed 2 zones of growth for *L. citrovorum* (Table II). No growth zone appeared at R_f 0.31 if the samples were first subjected to 30 minutes autoclaving. This located the HLCF area on the chromatogram. The zone at R_f 0.7 (± 0.10) coincided with the growth response zone obtained with 5CHOTHFA. The data of Table I indicated that 81% of the CF content of sample #4 was HLCF, yet a qualitative inspection of the bioautograph of the sample (Table II) showed the major form of CF to be 5CHOTHFA as judged by the size of the zone. It is possible that there was some conversion of HLCF to 5CHOTHFA during the course of the experiment, although

TABLE II. Paper Chromatography of Urine and Folic Acid Derivatives. System: 0.1 M acetate (pH 5.4); bioautography with *L. citrovorum*.

Sample	Treatment	pH	R_f and zone size	
Urine	None	6.5	.31 (2+)	.67 (5+)
"	*	6.5	—	.70 (7+)
"	†	2.3	.28 (2+)	.70 (2+)
Leucovorin	†	6.5	—	.76
"	†	2.2	.36	—
Anhydroleucovorin	†	6.5	—	.77 (hazy)
	†	2.2	.43	—

* Autoclaved 30 min. at 120°C.
† Stood 2 hr at 25°C.

it seems more likely that much HLCF was destroyed during the experimental manipulations whereas 5CHOTHFA was stable.

Table II also shows that when urine was incubated at pH 2.3 and subsequently chromatographed, the growth area corresponding to HLCF remained and the 5CHOTHFA zone simultaneously diminished in size. The interpretation of this experiment was complicated by the fact that 5CHOTHFA is converted to AHL under these conditions and could not be distinguished from HLCF in this chromatographic system (Table II). The possibility was considered that HLCF might be AHL; however, it was found that HLCF could be consistently detected in whole urine standing at pH 6.5, whereas, when AHL was held under these conditions, only a slight growth zone could be demonstrated and this zone corresponded to leucovorin (Table II). The finding that AHL at pH 2.2 should support growth of *L. citrovorum* with a clearly discernible zone at 0.43 was unexpected in view of the low microbiological activity of AHL in conventional tube assays(10). Evidently AHL was decomposed during the assay procedure in the earlier investigation(10), presumably during autoclaving. This prompted a study of the effect of pH and reducing conditions on the microbiological activity of AHL for *L. citrovorum*. Table III illustrates that AHL at pH 2.3 had about 13% of the CF activity of leucovorin for *L. citrovorum* and the activity was independent of ascorbate. However, when AHL was incubated at pH 6.5, ascorbate was necessary to maintain biological activity, whether AHL was dissolved in buffer or normal urine. The possibility that HLCF was AHL or a closely related compound stabilized by the ascorbate in the urine is compatible with the findings of Table III.

TABLE III. Lability of Anhydroleucovorin to Air and pH. 100 γ anhydroleucovorin dissolved in 1 ml 0.1 M PO_4 buffer (pH 2.35). Initial activity, 13.5 μg/ml in terms of DL-5CHOTHFA.

Conditions during next 2 hr at 27°C			CF activity as 5CHOTHFA, μg
Solvent		0.1% ascorbate	
.1 M PO_4	pH 6.5	Absent	.8
Idem	"	Present	6.4
"	pH 2.35	Absent	12.0
"	"	Present	13.3
Normal urine*	pH 6.5	Absent	2.3
Idem	"	Present	13.0

* From an adult male not dosed with FA and ascorbate.

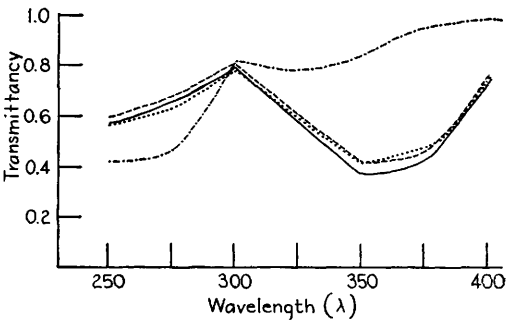

FIG. 1. Ultraviolet absorption spectrum of AHL (0.1 M phosphate buffer) solutions diluted to 10 γ/ml in 0.01 N HCl just prior to reading in a model DU Beckman spectrophotometer. Curves: ———, AHL in pH 2.3 buffer, 2 min. at 25°C; •••••, AHL in pH 2.3 buffer after 2 hr at 25°C; – – – –, AHL in pH 6.5 buffer, 2 min. at 25°C; •–•–•–, AHL in pH 6.5 buffer after 2 hr at 25°C.

Spectrophotometric analyses of solutions of AHL in 0.1 M phosphate buffer at pH 2.2 and 6.5 were made at zero and 2 hours. Fig. 1 shows that a loss in absorption between 350 and 360 mμ occurred at pH 6.5 after 2 hours, showing a chemical change, but no spectral change was observed at pH 2.2. An observable chemical change thus accompanied the loss of microbiological activity of AHL at pH 6.5.

An attempt was made to purify HLCF by large scale paper chromatography to permit a more detailed study of its chemical relationship to other FA derivatives. HLCF and FA had approximately identical R_f values in the solvent system employed. The high concentration of FA in the urine used as a source of HLCF however, was not a problem in these studies because the location of HLCF on chromatograms was determined by bioautography with *L. citrovorum* which was insensitive to the amount of FA in the aliquot. Aliquots of 0.05 ml of urine containing HLCF were spotted along a line 1" from the lower edge of a Whatman #1 paper sheet (18½ x 11½). The chromatographic technic used was similar to that described under *Methods*.

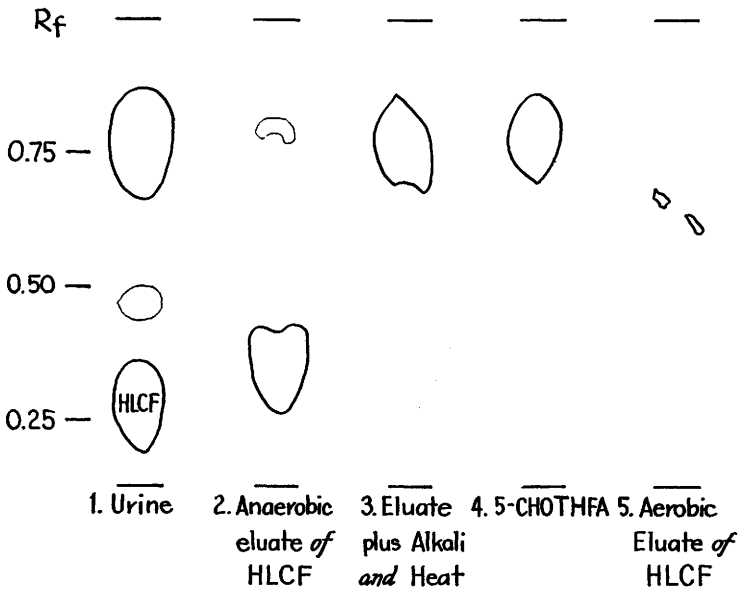

FIG. 2. Evidence for conversion of HLCF to 5CHOTHFA. Chromatography system; 0.1 M sodium acetate, pH 5.4; bioautography with *L. citrovorum*. 1. Urine; 2. Anaerobic eluate of HLCF; 3. Anaerobic eluate adjusted to 0.1 N alkali and heated at 100°C; 4. Leucovorin; 5. Aerobic eluate of HLCF.

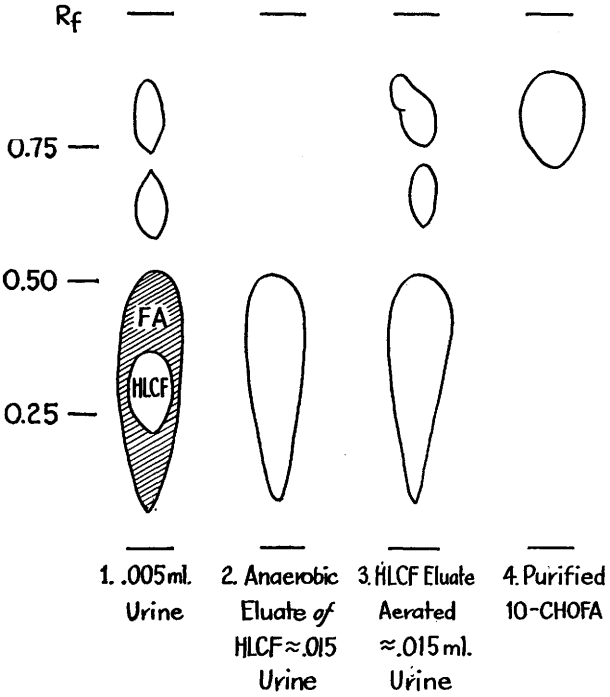

FIG. 3. Evidence for conversion of HLCF to 10CHOFA. Chromatography system; 0.1 M sodium acetate, pH 5.4; bioautography with *S. faecalis*. 1. 0.005 ml urine; 2. Anaerobic eluate of HLCF = 0.015 ml urine; 3. Aerated eluate of HLCF = 0.015 ml urine; 4. purified 10-CHOFA.

The HLCF area on the chromatogram, predetermined by bioautography of a ½" vertical section was cut out and cut into smaller squares which were stacked in a small pyrex funnel. An appropriate buffer was then applied to the paper with a pipette. The volumes of eluant were about twice the original volume of urine.

When the HLCF area was eluted with 0.1 M phosphate buffer, pH 6.5, and aerated for one hour, HLCF activity could not be demonstrated (strip #5, Fig. 2), but it could be shown that such "aerobic" eluates contained a substance (R_f 0.78-0.80) active for growth of *Streptococcus faecalis* (Strip #3, Fig. 3) and this factor could not be distinguished in 3 solvent systems from a sample of 10CHOFA, purified by the method of Silverman (11). This experiment indicated the presence of a formyl group in HLCF.

When the HLCF area was eluted with 0.1 M phosphate pH 6.5 buffer with the addition of 0.1% ascorbate to bring about anaerobic conditions, HLCF could be recovered from paper chromatograms (strip #2, Fig. 2). These observations emphasized the highly reduced state of HLCF. When such an anaerobic eluate was made alkaline (0.1 N) and heated at 100°C under nitrogen for one hour (3), 5CHOTHFA was formed as indicated by Fig. 2, strip 3, leading to the conclusion that HLCF is a formyl derivative of tetrahydrofolic acid.

Discussion. The chemical changes pertinent to the present investigation are shown in abbreviated form in Fig. 4. The chemical studies of May *et al.*(4) showed that AHL presumably exists only in acid solution, and that when such a solution is made neutral or alkaline the imidazoline ring is ruptured and 10CHOTHFA is formed provided that air is excluded from the solution. Moreover, 10CHOTHFA presumably does not exist in acid solutions for under these conditions it is transformed into AHL. The following lines of evidence support the conclusion that HLCF present in human urine under the conditions described herein was 10CHOTHFA: (a) HLCF was stable at a neutral pH in the presence of ascorbic acid, (b) under aerobic conditions HLCF was converted to 10CHOFA, and (c) under anaerobic conditions when HLCF was made alkaline and heated 5CHOTHFA was readily formed. These properties of HLCF correspond to those of 10CHOTHFA which are shown in Fig. 4. Similar evidence has been obtained to establish the identity of 10CHOTHFA formed from THFA by enzyme systems from pigeon liver and pig heart(12,13). The present study demonstrates that 10CHOTHFA is formed in man.

Nichol and coworkers(8) recently described the enzymatic formation of a labile precursor of CF, termed "CFX", from FA by several enzyme systems. "CFX" was converted non-enzymatically to CF by heating and it was labile to oxygen; in these respects "CFX" resembles HLCF. However HLCF possesses microbiological activity for *L. citrovorum* prior to heating. The fact that a form of CF having an R_f indistinguishable from 5CHOTHFA exists in fresh unheated human urine implies that 5CHOTHFA may arise by

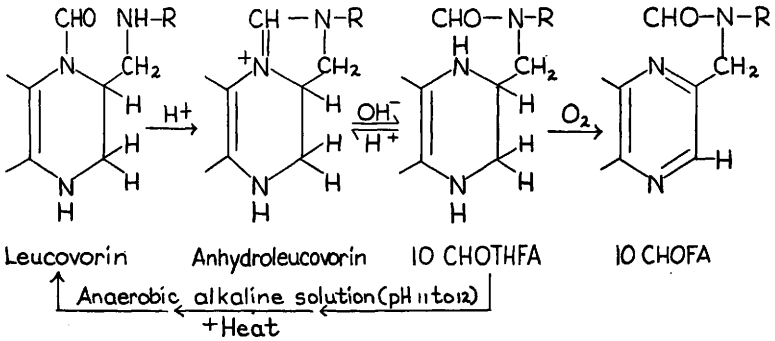

FIG. 4. Chemical transformations involving leucovorin; R = p-aminobenzoylglutamic acid. Pyrimidine ring is omitted to save space.

physiological processes as well as by chemical transformation from 10CHOTHFA-like compounds. Studies by Kisliuk and Sakami(14) and Blakley(15) have established the existence of a form of CF in which the one carbon functional group is at the formaldehyde level of oxidation (5 or 10 hydroxymethyl-THFA, or the corresponding 5-10 bridge compound). It seems unlikely that HLCF is a hydroxymethyl derivative since such a compound would have to undergo an oxidative step before chemical conversion to 5-CHOTHFA.

While this work was in progress we were informed(16) that Silverman has also demonstrated the existence of a labile derivative of CF in human urine and has reached conclusions similar to ours regarding its chemical nature.

Summary. Microbiological studies with *Leuconostoc citrovorum,* ATCC 8081 (*Pediococcus cerevisiae*) showed that a heat labile form of citrovorum factor (HLCF) occurs in the urine of normal adults after an oral dose of folic and ascorbic acids. Anhydroleucovorin (AHL) resembled HLCF in that AHL was active for growth of *L. citrovorum* (13% as active as leucovorin) when acid solutions were assayed aseptically, but neutral solutions of AHL were low in microbiological activity unless ascorbic acid was present. HLCF was purified by large scale paper chromatography which permitted a study of its chemical properties. The chemical relationships between 5CHOTHFA, AHL, and 10-CHOTHFA were discussed with regard to the chemical properties that were demonstrated for HLCF from which it was concluded that HLCF is probably 10CHOTHFA.

The authors wish to thank Dr. Thomas H. Jukes for his interest and encouragement in this investigation and for his helpful suggestions and criticisms in the preparation of this manuscript.

1. Sauberlich, H. E., and Baumann, C. A., *J. Biol. Chem.,* 1948, v176, 165.

2. Felton, E. A., and Niven, C. F., *J. Bacteriol.,* 1953, v65, 482.

3. Roth, B., Hultquist, M. E., Fahrenbach, M. J., Cosulich, D. B., Broquist, H. P., Brockman, J. A., Jr., Smith, J. M., Jr., Parker, R. P., Stokstad, E. L. R., and Jukes, T. H., *J. A. C. S.,* 1952, v74, 3247.

4. May, M., Bardos, T. J., Barger, F. L., Lansford, M., Ravel, J. M., Sutherland, G. L., and Shive, Wm., *ibid.,* 1951, v73, 3067.

5. Broquist, H. P., Fahrenbach, M. J., Brockman, J. A., Jr., Stokstad, E. L. R., and Jukes, T. H., *ibid.,* 1951, v73, 3535.

6. Anker, R. M., Boehne, J. W., and Welch, A. D., *Fed. Proc.,* 1950, v9, 351.

7. Broquist, H. P., Stokstad, E. L. R., and Jukes, T. H., *J. Lab. Clin. Med.,* 1951, v38, 95

8. Nichol, C. A., Anton, A. H., and Zakrzewski, S. F., *Science,* 1955, v121, 275.

9. Deodhar, S., Sakami, W., and Stevens, A. L., *Fed. Proc.,* 1955, v14, 201

10. Cosulich, D. B., Roth, B., Smith, J., Jr., Hultquist, M. E., and Parker, R. P., *J. A. C. S.,* 1952, v74, 3252.

11. Silverman, M., Keresztesy, J. C., and Koval, G. J., *J. Biol. Chem.,* 1954, v211, 53.

12. Jaenicke, L., *Biochimica et Biophysica Acta,* 1955, v17, 588.

13. Greenberg, J. Robert, Jaenicke, L., Silverman, M., *ibid.,* 1955, v17, 589.

14. Kisliuk, R. L., and Sakami, W., *J. Biol. Chem.,* 1955, v214, 47.

15. Blakley, R. L., *Biochem. J.,* 1954, v58, 448.

16. Silverman, M., personal communication.

Received April 13, 1956. P.S.E.B.M., 1956, v92.