their excretion and mental diseases(6) offer a plausible explanation for the results of Hill, *et al.* Although it was possible to assure reasonably similar intake of meals with patients and control subjects, it was difficult to control intake of beverages and refreshments such as coffee, tea, cola beverages, milk shakes, ice cream, sweets, etc., between meals. These foods, unfortunately, represent a major source of chromogenic phenolic substances in urine. Thus, in the driving-stress experiments, even though consumption of meals was controlled, a variable intake between meals could account for appearance of variable total amounts of vanillic and ethylvanillic acids. The varying proportions might be due to the fact that commercial vanilla preparations contain varying mixtures of vanillin and ethylvanillin, and different materials eaten could have been prepared with different flavoring preparations. In the absence of rigorous experimental proof of a new type of metabolic reaction, biological ethylation, it would seem safer to ascribe the appearance of ethylvanillic acid in urine to intake of ethylvanillin.

Summary. Ethylvanillic acid (3-ethoxy-4-hydroxybenzoic acid) occurs occasionally in human urine. Its presence follows ingestion of ethylvanillin, a component of some artificial vanilla flavorings. After ingestion of ethylvanillin or of ethylvanillic acid, free ethylvanillic acid, a glucuronide conjugate, and a glycine conjugate are excreted.

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Multiplication of Single Mammalian Cells in a Nonbicarbonate Medium. (25647)

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It is standard practice to use a CO_2-bicarbonate system to buffer mammalian cell cultures, even in single cell plating technics where one would expect the other constituents of the medium such as amino acids and serum protein to exert an adequate buffering action. The result of this practice is that cultures must be incubated in a high pCO_2 to control pH. If bicarbonate could be omitted from the medium, incubation could be in air instead of the usual 5% CO_2, and plating out of single cells and manipulation of such cultures would be considerably simplified. This paper describes attempts to attain this objective. It was found that L cells did not form colonies consistently under these conditions unless a Krebs cycle intermediate, oxalacetic acid (OAA), was added to the medium.

Methods. Stock cultures were grown in defined medium CMRL-1066(1) with 20% dialyzed horse serum for the L strain(2) and 20% whole sheep serum for HeLa(3). Both strains were clonal lines. Primary cultures of

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trypsinized rhesus monkey kidney were grown in Hanks BSS with 0.5% lactalbumin hydrolysate and 1% bovine serum. For the single cell plating experiments, medium with and without bicarbonate was prepared by first making up sterile 2X CMRL-1066 with the bicarbonate omitted. To half of this medium $NaHCO_3$ solution was added to make a 1X medium containing 2.2 g $NaHCO_3$ per liter. The other half was treated similarly except that an equimolar concentration of NaCl was used to replace the bicarbonate. Both of these solutions were then supplemented with bicarbonate-depleted sera at a concentration of 20%. Whole sera could not be used, since loss of CO_2 caused the pH to rise to about 8. Horse serum used for plating the L strain was depleted of bicarbonate by an aseptic dialysis at 4° against 10 volumes of physiological saline, changed twice daily for 3 days. For HeLa and monkey kidney cells, which did not grow in such exhaustively dialyzed serum, bicarbonate was removed by lowering pH of the serum to 6 with 0.1 N HCl and leaving it overnight in a cotton-stoppered flask to allow the CO_2 so liberated to escape. Before use bicarbonate-depleted sera were checked for pH stability on contact with air. A neutralized 0.25 M solution of oxalacetic acid in bicarbonate-free CMRL-1066 was stored frozen and added directly to the Petri dishes. L and HeLa cells were dispersed with 0.035% trypsin (1:300), while a mixture of 0.25% trypsin and 0.05% Versene was used for primary monkey kidney. Dilutions were made in bicarbonate-free CMRL-1066 and 100 cells were added to each dish of medium. The Petri dishes used were 6 cm in diameter and contained 5 ml medium. Cultures with bicarbonate were incubated at 37 ± 0.5° in a flow of 5% CO_2, while nonbicarbonate cultures were incubated in air. Incubation was continued for 2 weeks. In some experiments, as an index of growth after 6-7 days, the cells in 10 randomly selected colonies were counted and averaged. For colony counts the cultures were stained as described previously(4).

Results. The proportion of cells that formed colonies (efficiency of plating, EOP) varied from 0 to 61% in the bicarbonate-free system (Table I), but with OAA in the medium EOP was consistently high, variation being only what one would expect when plating from cultures grown on glass(4). In addition OAA increased total number of cells which developed after about 1 week of incubation. Those colonies which did develop in bicarbonate-free medium without OAA exhibited cells which were granular and rounded. This necrotic condition was not apparent when OAA was present (Fig. 1). A concentration of 1 to 2 mM OAA was optimum (Table II). Above 3 mM, probably due to spontaneous decarboxylation of the OAA, an appreciable rise in pH occurred. L cells were isolated twice in succession from bicarbonate-free-OAA medium. On replating in the same medium no decrease in EOP or rate of growth was apparent.

TABLE I. Plating Efficiency of L Cells in Bicarbonate and Nonbicarbonate Media Supplemented with Dialyzed Serum. Effect of adding oxalacetic acid to medium in 4 experiments.

Plating efficiency (%)			
Bicarb.*	Bicarb. + OAA†	Nonbicarb.	Nonbicarb. + OAA
43 ± 5‡	73 ± 7	0	83 ± 4
43 ±12	73 ± 9	0	94 ± 3
		1 ± 1 (50)§	43 ± 15 (285)
		61 ± 4 (40)	125 ± 13 (68)

* Cultures in bicarbonate medium were incubated in 5% CO_2, those in nonbicarbonate medium were incubated in air.

† Oxalacetic acid, 2 mM.

‡ Avg plating efficiency for 3 to 5 plates ± S.E.

§ Figures in parentheses are avg No. of cells/colony after 6 to 7 days incubation.

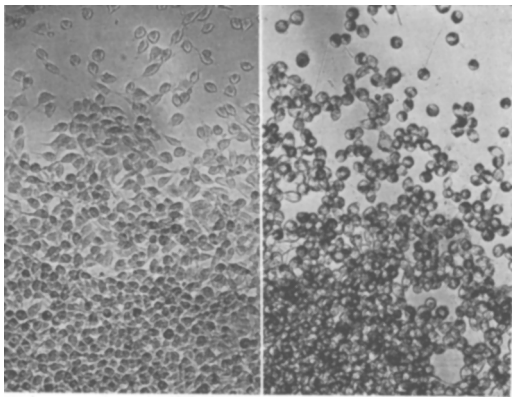

FIG. 1. Marginal areas of L strain colonies that developed in nonbicarbonate medium supplemented with dialyzed serum and equilibrated with air (left) and, under same conditions with 2.5 mM oxalacetic acid (right). Note that abnormal granular condition did not develop when oxalacetic acid was present. (× 90.)

TABLE II. Effect of Varying Concentration of Oxalacetic Acid on Growth of Single L Cells in Nonbicarbonate Medium Supplemented with Dialyzed Serum and Equilibrated with Air.

Conc. of oxalacetic acid (mM)	Plating efficiency (%)	Avg No. of cells/colony after 7 days
.0	0	
.05	0	
.1	0	
.5	35 ± 7*	59
1	53 ± 2	61
2	50 ± 2	69
3	26 ± 6	47

* See corresponding footnote in Table I.

tabolites in these 2 groups of cells. OAA was tested because it substitutes, at least partially, for CO_2 in bacteria, where CO_2 appears to be needed to provide the requisite amount of OAA for the Krebs cycle(8). However, it is well known that CO_2 participates in other areas of metabolism, *e.g.*, in RNA synthesis (9). It would be of interest to study the effect of RNA derivatives, which are not present in CMRL-1066, on ability of monkey kidney cells to multiply in nonbicarbonate media.

In all 3 types of cells, CO_2 and OAA sometimes had an additive effect. A possible reason for this may be that dispersing and diluting tend to arrest the Krebs cycle by washing intermediates from the cells. Under these conditions an intermediate such as OAA may be required in addition to CO_2 to get the cycle started. Neumann and McCoy(10) have shown recently that single cells of Walker carcinosarcoma 256 require Krebs cycle intermediates which are not needed by bulk cultures.

TABLE III. Plating Efficiency of L, HeLa and Monkey Kidney Cells in Nonbicarbonate Medium Supplemented with Serum Selectively Depleted of Bicarbonate by Acid Treatment. Effect of adding oxalacetic acid to the medium.

Cells	Exp. No.	Plating efficiency (%)			
		Bicarb.*	Bicarb. + OAA†	Nonbicarb.	Nonbicarb. + OAA
L	1	59 ±11‡(74)§	21 ±13 (30)	13 ± 2 (21)	92 ± 3 (65)
	2	13 ± 5 (29)		1 ± 1 (17)	66 ± 4 (50)
HeLa	1	82 ± 3 (26)		52 ± 2 (32)	109 ± 8 (38)
	2	17 ±15 (24)	72 ± 4 (39)	0	14 ± 2 (21)
Primary monkey kidney	1	18 ± 2 (4 mm)¶	35 ± 4 (4 mm)	2 ± 1 (.5 mm)	6 ± 1 (1 mm)
	2	8 ± 2 (3 ")	18 ± 2 (")	0	6 ± 0 (")

*†‡§ See corresponding footnotes in Table I. ¶ Avg diameter of colonies to nearest mm.

The usefulness of OAA-medium for plating other types of mammalian cells was studied using HeLa and monkey kidney. Since these cells failed to grow in exhaustively dialyzed serum, bicarbonate was removed from the serum by acidifying as described under 'Methods'. Sheep serum was employed for HeLa and horse serum for monkey kidney. The results (Table III) suggest that HeLa as well as L cells can be plated out satisfactorily without addition of bicarbonate to the medium provided OAA is present. In contrast monkey kidney cells, which failed to multiply in the bicarbonate-free system, responded only slightly to OAA.

Discussion. It is clear from these experiments that even in a medium containing serum as well as a large number of amino acids and other growth factors a lack of bicarbonate and/or CO_2 interferes with cell multiplication. This agrees with the results others have obtained with bulk cultures of several types of cells(5,6,7). The fact that OAA replaced the CO_2-bicarbonate system for L and HeLa but not for monkey kidney, suggests that CO_2 may be involved in synthesis of different me-

Summary. The feasibility was explored of using nonbicarbonate media, equilibrated with air, for plating single mammalian cells. L strain mouse cells, with which most of the experiments were done, multiplied erratically under such conditions and cell morphology was abnormal. Satisfactory plating could be obtained by substituting oxalacetic acid for the CO_2-bicarbonate system.

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Failure of Acute Bilateral Adrenalectomy to Influence Brain Serotonin Levels in the Rat. (25648)

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Recently, De Maio reported differential increases of serotonin concentration in pooled fractions of the base, hemispheres, and medulla oblongata of the rat brain 24 hours after bilateral adrenalectomy or hypophysectomy (1). His report suggests a possible influence of endocrine function on brain serotonin metabolism. It was considered of interest to repeat the studies with the acute, bilaterally adrenalectomized rat using a chemical, fluorimetric procedure instead of a bioassay method. Since the surgical procedures employed by De Maio are well known to activate the pituitary-adrenal cortex system, it was considered important to include a sham operated group of animals in order to distinguish specific from nonspecific effects of ablative surgery. In addition our estimations have been made on the individually treated animals, and the results subjected to standard statistical analysis.

Methods. Two studies were made on the effect of bilateral adrenalectomy on brain serotonin concentration. In the first experiment 15 albino rats (Sprague-Dawley strain), weighing between 150 and 200 g, were divided into 3 groups: control, sham operated, and bilateral adrenalectomized. Ether was used as anesthesia in all of the operations. The animals were sacrificed by decapitation 24 hours after surgery. The brain was immediately removed, weighed, and homogenized in 3 volumes of 0.1 N HCl. Serotonin was assayed according to the indirect method of Weissbach, Waalkes, and Udenfriend, involving extraction with butanol(2).

Results. The control group exhibited a mean concentration of 0.65 μg of serotonin per gram of fresh brain tissue. Mean values found in the sham operated and adrenalectomized groups were 0.61 and 0.67 μg of serotonin per gram of fresh brain tissue, respectively. Values for groups of animals were not significantly different from each other.

The above experiment was repeated with addition of a fourth group injected with 100 mg of iproniazid/kilo of body weight 18 hours prior to sacrifice. As in the first experiment, there was no significant difference in brain serotonin concentrations of control, sham operated, and bilateral adrenalectomized groups. The iproniazid treated group showed a 165% increase in brain serotonin over that of control group ($P = 0.01$). Table I summarizes means, standard deviations, and ranges of the groups tested. The data were analyzed by use of the Mann Whitney U-test(3). P values in Table I refer to comparisons of experimental groups to control group. We have also demonstrated that reserpine treated animals have the expected depletion of brain serotonin using the fluorimetric method(4).

To make direct comparison of our data with that of De Maio's, 10 rat brains were separated and pooled into the 4 fractions described by De Maio. Mean weights for these frac-