presents little obstruction to passage of fluorine from maternal to fetal circulation. The fact that concentrations of fluorine in maternal and cord blood are almost the same was considered by Held(2) to imply that the placenta passively permits fluorine transfer to the fetus. Our finding that fluorine concentration in the placenta is higher than in either maternal or cord blood suggests, however, that the placenta plays a more active role.

Summary. Samples of the blood from pregnant women shortly before delivery, placentas at full term and cord blood, were analyzed for fluorine. All subjects drank from the same water source which contained about 0.55 ppm fluorine. The results suggest that the placenta plays an active role in accumulation of fluorine and its transfer to the fetus.

We are indebted to Miss Pearl Weiskopf for statistical analysis and to Natan Portuguese for technical assistance.

1. Gardner, D. F., Smith, F. A., Hodge, H. C., Overton, D. E., Feltman, R., *Science,* 1952, v115, 208.

2. Held, H. R., *Schweiz. med. Wchnschr.,* 1954, v84, 251.

3. Feltman, R., Kosel, J., *Science,* 1955, v122, 560.

4. Ziegler, E., *Mitt. d. naturwiss. Gesellsch., Winterthur,* 1956, v28, 39.

5. Gedalia, I., Brzezinski, A., Bercovici, B., *J. D. Res.,* 1959, v38, 548.

6. Gedalia, I., Yardeni, J., *ibid.,* 1957, v36, 203.

7. Voegtlin, C., Hodge, H. C., *The Pharmacology and Toxicology of Uranium Compounds,* McGraw-Hill, New York, 1951.

8. Cremer, H. D., Voelker, D., *Biochem. Z.* 1953, v324, 89.

9. Lerman, D., Gedalia, I., Rosenzweig, K., Brzezinski, A., *Proc. 6th Congr. ORCA,* 1959, in press.

10. Smith, F. A., Gardner, D. E., Hodge, H. C., *J. D. Res.,* 1950, v29, 596.

11. Shaw, J. H., *Fluoridation as a Public Health Measure,* A.A.A.S., Washington, 1954, p54.

12. Buttner, G., Muhler, J., *J. Dental Res.,* 1958, v37, 326.

13. Ericsson, Y., Ullberg, S., *Proc. 5th Congr. ORCA,* 1958, p200.

14. Brzezinski, A., Bercovici, B., Gedalia, I., *Obst. and Gynec.,* 1960, v15, 329.

Received November 9, 1960. P.S.E.B.M., 1961, v106.

Carbon Dioxide Requirement and Nucleic Acid Metabolism of HeLa and Conjunctival Cells.* (26267)

R. SHIHMAN CHANG, HELEN LIEPINS AND MERRY MARGOLISH
Department of Microbiology, Harvard School of Public Health, Boston, and the Virus Laboratory, University of California, Berkeley

This is the third of a series of publications from this Department on the physiological role of carbon dioxide in multiplying human cells *in vitro.* The following are some of the justifications for carrying out such studies: a) while many compounds are known to have arisen through CO_2 fixation in the heterotrophic organisms, the physiological significance of many such reactions remains obscure; b) the importance of CO_2 in the process of differentiation has been reported(1); c) there are speculations that CO_2 may act as a physiological regulator of cell multiplication(2), and d) very few reports are based on study with intact human cells.

* Supported by research grants of U.S.P.H.S.

Our first report described CO_2 as an essential for propagation of human cells *in vitro* (3). A subsequent paper demonstrated the role of CO_2 in RNA and protein synthesis as shown by virus formation in human cell culture(4). The present report describes the fixation of $C^{14}O_2$ by human cells and recovery of most of the fixed C^{14} in the nucleic acids and their derivatives. Under the described experimental conditions, the syntheses of nucleic and oxaloacetic acids are the only demonstrable essential CO_2 fixation reactions of these human cells.

Materials and method. Nutrient media. The tris(hydroxymethyl)aminomethane buffered medium was used exclusively, unless

otherwise specified, in all experiments. This medium consisted of 10% dialyzed equine serum in Eagle's basal medium(5) with certain modifications(4). No bicarbonate was added and any absorbed CO_2 was minimized by exposing the medium to an alkaline trap for at least 24 hours before each experiment.

Human cell cultures. The HeLa(6) and the conjunctival cells(7) derived respectively from malignant and normal human tissues were used. Stock cultures were propagated and checked at regular intervals for possible contamination by the pleuropneumonia-like organism as described previously(8).

Cell culture method. In all experiments, the cells were grown directly on glass surface of culture tubes (18 × 150 mm) containing 1 ml of nutrient medium and tightly closed with rubber stoppers. The culture was incubated at 36° C on a roller drum of about 12 revolutions per hour. The gentle agitation of nutrient medium in a closed system presumably facilitated the equilibration of metabolites.

Incorporation of C^{14} substrates. Twenty-four or 48 hours after addition of C^{14} labelled substrates, 2 of a series of replicate cultures were selected at random for cell enumeration. The remaining cultures were rinsed twice with about 20 ml of 0.85% NaCl. The cells were then dissolved in 0.85% NaCl containing 0.02% sodium lauryl sulphate. Cell solutions from 5 to 10 cultures were pooled and then fractionated by the method of Schmidt-Thannhauser-Schneider(10). Fraction 1 (acid solubles), 2 (lipids), 3 (RNA), 4 (DNA) and 5 (proteins) were obtained. C^{14} activities of these fractions were determined. It should be emphasized that any bicarbonate-C^{14} present in fraction 1 is presumably expelled as CO_2 during the processing of this fraction for counting. In certain experiments the C^{14} activities of the purines and pyrimidines were determined following hydrolysis of whole cells with 7 N perchloric acid(11) and chromatographic separation using isopropanol-HCl as solvent(12).

CO_2 depletion technic. This was accomplished through use of an alkaline trap installed in the gaseous phase of the culture system(3).

Results. $C^{14}O_2$ fixation into cell fractions. The results of 5 representative experiments are presented in Table I; more than 90% of C^{14} activity resided in the acid soluble and nucleic acid fractions. Such results were consistently observed in 10 experiments, although there were considerable quantitative variations from experiment to experiment. No consistent difference between conjunctival and HeLa cells nourished in serum of equine or human origin have been observed.

C^{14} activities of purines and pyrimidines. Following hydrolysis and chromatographic separation of the purines and pyrimidines of cells grown in $NaHC^{14}O_3$ containing-medium for 48 hours, C^{14} activity was regularly detected in spots formed by guanine, adenine,

TABLE I. Fixation of $C^{14}O_2$ into Various Fractions of Conjunctival and HeLa Cells.

Exp.*	Duration of exp. (hr)	Multiplication index†	C^{14} activities‡ in the following fractions 1	2	3	4	5
1	24	152/ 96, 193/ 96	151, 165§	64, 16	1630, 1160		126, 120
2	24	278/180, 292/180	280, 231	0, 0	410, 340		114, 165
3	24	222/165, 232/165	370, 330	0, 0	887, 429		281, 156
4	24	112/81	1330	73	322	191	123
	48	187/81	623	26	560	276	78
5	24	115/76	1329	14	335	132	102
	48	162/76	873	15	500	253	105

* The conjunctival cells were used in Exp. 1, 2, 4; HeLa in Exp. 3, 5. Five μC (5 μmoles) of $NaHC^{14}O_3$/10 ml media was used in all experiments. In Exp. 4 and 5, nutrient media were modified by use of double concentrations of amino acids and inclusion of non-essential amino acids at 0.2 mM.

† Denominator and numerator represented No. of cells, in thousands, per culture at 0 and 24 hr (or 0 and 48 hr) respectively.

‡ Expressed as counts/min./million cells; separation into RNA and DNA not attempted in Exp. 1, 2, and 3.

§ Results of replicate sets of cultures.

TABLE II. Multiplication of CO_2-Depleted Cells in Presence of Intermediate Metabolites.

	Exp. 1		Exp. 2		Exp. 3	
	Cell number,† in thousands, per culture on day					
Metabolites added*	7	14	7	14	7	14
None	65, 77‡	19, 18	31, 29	0, 0	17, 26	0, 0
Bicarbonate	649, 643	555, 550	225, 247	217, 184	394, 278	452, 732
Ribosides	314, 297	101, 104	217, 235	250, 232	73, 78	14, 18
Oxaloacetate	80, 95	21, 16	72, 78	8, 7	63, 40	5, 7
Ribosides, oxaloacetate	851, 745	702, 850	488, 405	525	194, 365	170, 398

* Bicarbonate—5 mM, no alkaline trap; ribosides—adenosine guanosine, uridine and cytidine at 10 μg/ml, and oxaloacetate at 5 mM.

† Zero day counts were 61, 42, and 30 in Exp. 1, 2 and 3 respectively.

‡ Results of replicate culture.

cytosine, uracil and thymine. Total activity of the purines was somewhat higher than the pyrimidines. For example, C^{14} activity, expressed as cpm per million cells as 1665, 1143 and 669 for cell hydrolysate, the purines and the pyrimidines respectively in one experiment. In a second experiment, they were 1450, 799 and 584.

CO_2 substitution study. Since the preceding data suggested that most of the CO_2 was utilized for synthesis of the purines and pyrimidines, experiments were designed to determine if these bases were effective substitutes for CO_2 in supporting cell multiplication. A combination of adenine, guanine, cytosine and uracil (with or without thymine) at concentration of 0.1-10 μg per ml media was found ineffective as CO_2 substitute. Adenosine, guanosine, cytidine and uridine at similar concentrations were partially effective; evidence of partial degeneration was invariably present in all experiments. If oxaloacetate was added, in addition, cell multiplication occurred at a rate at least equal to that in the presence of CO_2. Oxaloacetate, without the ribosides, was ineffective. Malate, with or without the ribosides, was also ineffective. Results of 3 representative experiments are presented in Table II. Occasionally, the accumulation of acid metabolites occurred at such rapid rate that great difficulty was encountered in maintaining the pH of the media at 7.2-7.8. Such difficulty may be readily remedied by replacing glucose in the nutrient medium with galactose. Experiment 1 was performed with the glucose medium, and 2 and 3, the galactose medium. The following metabolites were ineffective substitutes for CO_2 in supporting the multiplication of the conjunctival cells: 1) the deoxyribosides at 10 μg per ml; 2) glycine, serine, alanine, aspartic acid, asparagine and glutamic acid at 0.2 mM; 3) oleic acid at 10 μg per ml; 4) creatine at 0.1 mM; 5) uridine at 10 μg per ml, or 6) adenosine at 10 μg per ml.

Discussion. Our present study of the significance of CO_2 fixation may be compared with similar studies of the bacteria, particularly those of Wilson and his associates(13, 14, 15), Griffin and Racker(16) and Tuttle and Scherp(17). When *Brucella abortus* were grown in the presence of $C^{14}O_2$, most of the activity was found associated with glycine and other amino acids. Only 6% of $C^{14}O_2$ fixed was recovered in the nucleic acids of the *Brucella* and almost all this activity was found in the pyrimidines. No substitutes capable of replacing CO_2 have yet been found for *Brucella*. With *Neisseria meningitis,* no substitute for CO_2 has been found, although a combination of guanine, uracil, cytosine, casein hydrolysate and the B vitamins was partially effective. In the case of *Neisseria gonorrheae,* again only a partial substitute for CO_2 may be found in a combination of hypoxanthine, uracil and oxaloacetate. Our results with human cells differed significantly in that most of the $C^{14}O_2$ fixed by the cells was recovered in the nucleic acids and their purine and pyrimidine derivatives, and that a combination of the ribosides and oxaloacetate was found completely effective as a substitute for CO_2 in supporting cell growth. These findings are in general agreement with Geyer and Niemark who showed that cell extract was an effective substitute

for CO_2(9); their results suggested a relationship between CO_2 and biosynthesis in these cells. Of interest was a recent abstract reporting on recovery of C^{14} activity in the purines, pyrimidines and certain amino acids of mouse cells grown in presence of $C^{14}O_2$ (22).

The completeness of CO_2 depletion in our experiments may be questioned. Since our previous report(3) showed that CO_2-depleted cells continued to oxidize glucose-1-C^{14} to $C^{14}O_2$, a very low level of CO_2 must still be present. That different levels of CO_2 depletion may give rise to different results is shown by the several reports using various methods of depletion. For instance, Eagle, replacing the bicarbonate component of his nutrient medium by tris found that a massive culture multiplied adequately in a closed system(18). Swim and Parker found conditions described by Eagle inadequate if an open tissue culture system was used (19); presumably, endogenous CO_2 cannot accumulate. Gwatkin and Siminovitch(20), working with small cell populations in an open system, found oxalcacetate an adequate substitute for CO_2. By use of an alkaline trap in a closed system, a method first used by Harris for chick tissue(21), the ribosides and oxaloacetate were both essential. If a practical method for further depletion of CO_2 is available, it should not be too surprising if some other metabolites may also be found essential.

The syntheses of the purines, pyrimidines and oxaloacetate from CO_2 are some of the many well established CO_2 fixation reactions in lower forms of life. Our data are significant, not in demonstration of the existence of similar reactions in human cells, but more in the following findings: 1) these CO_2 fixation reactions are essential for survival and multiplication of human cells; 2) among a large number of CO_2 fixation reactions, the syntheses of the purines, pyrimidines and oxaloacetate are the only ones demonstrated essential, and 3) synthesis of the nucleic acid precursors is quantitatively the most significant CO_2 fixation reaction.

Summary. Over 90% of C^{14} fixed by HeLa or conjunctival cells growing in a tris-buffered nutrient medium containing 5 microcuries of $NaHC^{14}O_3$ per 10 ml was recovered in the acid soluble and nucleic acid fractions. Most of this activity was found associated with the purines and the pyrimidines of such cells. The ribonucleosides were partially effective as a substitute for CO_2 in supporting cell multiplication. A combination of ribosides and oxaloacetate provided conditions as good as or better than CO_2 for multiplication of CO_2-depleted conjunctival cells. It may be concluded that, under the described experimental conditions, the chief functions of CO_2 in human cells are to provide specific precursors for syntheses of the purines, the pyrimidines and oxaloacetate.

1. Loomis, W. F., *Science,* 1957, v126, 735.
2. ———, *J. Nat. Cancer Inst.,* 1959, v22, 207.
3. Geyer, R. P., Chang, R. S., *Arch. Biophysics and Biochem.,* 1958, v73, 500.
4. Chang, R. S., *J. Exp. Med.,* 1959, v109, 229.
5. Eagle, H., *Science,* 1955, v122, 501.
6. Gey, G. O., Coffman, W. D., Kubicek, M. T., *Cancer Research,* 1952, v12, 264.
7. Chang, R. S., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 440.
8. ———, *J. Exp. Med.,* 1960, v111, 235.
9. Geyer, R. P., Niemark, J. M., PROC. SOC. EXP. BIOL. AND MED., 1958, v99, 599.
10. Glick, D., *Methods of Biochemical Analysis,* 1957, v1, 290.
11. Marshak, A., Vogel, J. H., *J. Biol. Chem.,* 1951, v189, 597.
12. Wyatt, G. R., *Biochem. J.,* 1951, v48, 584.
13. Tepper, B. S., Wilson, J. B., *J. Bact.,* 1958, v76, 24.
14. Newton, J. W., Marr, A. G., Wilson, J. B., *ibid.,* 1954, v67, 233.
15. Newton, J. W., Wilson, J. B., *ibid.,* 1954, v68, 74.
16. Griffin, P. J., Racker, E., *ibid.,* 1956, v71, 717.
17. Turtle, D. M., Scherp, H. W., *ibid.,* 1952, v64, 175.
18. Eagle, H., *Arch. Biochem. Biophys.,* 1956, v61, 356.
19. Swim, H. E., Parker, R. F., *J. Biophys. and Biochem. Cytol.,* 1958, v4, 525.
20. Gwatkin, R. B., Siminovitch, L., PROC. SOC. EXP. BIOL. AND MED., 1960, v103, 718.
21. Harris, M., *J. Exp. Zool.,* 1954, v125, 85.
22. McCoy, T. A., Orr, G., Maxwell, M., *Proc. Am. Assn. Cancer Research,* 1960, v3, 132.

Received November 9, 1960. P.S.E.B.M., 1961, v106.