Response of CO_2-Deficient Human Cells *in vitro* to Normal Cell Extracts.* (24432)

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Human cells of HeLa and conjunctival strains failed to show a net increase in numbers when grown in absence of CO_2(1). Under conditions of the experiments, cells so arrested for as long as 14 days resumed multiplication when CO_2 was again supplied. Grossly, the cells appeared healthy, and active metabolism of radioactive glucose was found. These characteristics, together with resumption of multiplication when CO_2 was provided, indicated that cell viability was high and suggested depletion of factors concerned with multiplication rather than general interference with cell permeability or overall CO_2-fixation reactions. Experiments herein reported show that depleted HeLa and conjunctival cells can resume growth in absence of CO_2 when provided with normal cell extracts. Extracts of depleted cells were ineffective.

Methods. Stocks of HeLa and conjunctival cells derived from human carcinoma and human conjunctiva, respectively,† were maintained in Eagle's medium containing 10% horse serum inactivated by heating at 56°C for ½ hour. Preparation of roller tube cultures, determination of cell count, and method of making tubes CO_2-deficient have been described(1,2). With CO_2-deficient cells, the sole buffer was 1 mM phosphate buffer which sufficed for adequate pH control. Bicarbonate buffer was present in control tubes. Normal cell extract‡ was prepared as follows: stock bottles of cells were grown in normal manner and when a total of approximately 3-4 x 10^6 cells were present, the medium was removed as completely as possible and the following procedure was employed: 10 ml of Eagle's medium (containing no serum, $MgCl_2$ or $CaCl_2$), was added and the cells incubated overnight.§ Any cell debris remaining on glass was scraped off, and contents of bottle transferred to sterile ampule, sealed, and stored at −70°C until used. Prior to being added to roller tubes, the extract was reconstituted by addition of dialyzed horse serum, $CaCl_2$, and $MgCl_2$. Such media were agitated at 35°C in the presence of a KOH ampule(1) for 4 to 5 hours to remove traces of CO_2, then transferred to appropriate roller tube cell cultures with a minimum of exposure to air. When used with HCO_3^--deficient cells, the KOH-containing ampule was always present in the tube. Ampules in these and other CO_2-deficient tubes were changed frequently to assure adequate CO_2-absorption. In some instances, extracts were prepared from stock cells which were CO_2-deficient for 48 hours. Media were changed every other day and all incubations were done at 35°C. All media employed were kept in containers provided with KOH ampules to minimize CO_2 contamination in so far as possible. Routine assay experiments were done as follows: freshly prepared roller tube cultures were allowed to incubate 3 days, then were made CO_2-deficient for 48 hours. Test material was introduced and incubation continued for 7 days, at which time cell counts were done. Appro-

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‡ The term "extract" denotes the product which results from disintegration of cells by the method described and from which particulate material has not been removed.

§ In absence of Mg^{++} and Ca^{++}, these cells rapidly disintegrate(3). This procedure was adopted because manipulation of sterile materials was minimized while still insuring destruction of the cells. On occasion, other methods were used, such as scraping cells into water or medium, followed by lyophylization, and subsequent reconstitution prior to use. These extracts were as active as those made by procedure outlined in text. Furthermore, lyophylization of the usual extract did not impair its effectiveness.

TABLE I. Effect of Cell Extract on Multiplication of Normal and CO_2-Deficient Human Cells *In Vitro*.

Exp. No.	Test cells*	Extract added†	No. cells/tube $\times 10^3$‡
		Conjunctival cells	
1	Normal	None	369 (292-486)
	Deficient	"	199 (160-230)
	"	Normal	647 (596-700)
2	Normal	None	394 (360-422)
	"	Normal	395 (386-400)
	Deficient	None	172 (156-186)
	"	Normal	297 (236-362)
3	Normal	None	284 (232-328)
	Deficient	"	77 (50- 98)
	"	Normal	183 (170-196)
	"	Deficient	32 (22- 42)
		HeLa cells	
4	Normal	None	291 (192-382)
	"	Normal	301 (280-314)
	Deficient	None	76 (62- 82)
	"	Normal	314 (220-410)
5	Normal	None	264 (210-292)
	Deficient	"	26 (20- 36)
	"	Normal	201 (178-242)
6	Normal	None	144 (124-162)
	Deficient	"	25 (20- 34)
	"	Normal	48 (42- 58)
7	Normal	None	228 (200-270)
	Deficient	"	81 (56-128)
	"	Normal	174 (158-200)
	"	Deficient	29 (21- 40)
8	Normal	None	695 (640-768)
	Deficient	"	69 (60- 78)
	"	Normal	301 (260-378)
	"	Deficient	20 (4- 48)
9	Normal	None	178 (161-194)
	"	Normal	191 (172-211)
	Deficient	None	45 (44- 47)
	"	Normal	245 (198-288)

* In Exp. 1 and 2, cells were allowed to grow for 9 and 18 days, respectively, prior to removal of CO_2. Subsequent treatment was as given in the text.

† Conjunctival cell extracts were used in Exp. 1-3, and HeLa cell extracts in Exp. 4-9. Amount of extract was that derived from approximately 3×10^5 cells.

‡ Expressed as avg and range.

priate sets of control tubes were included. The procedure adopted penalized the deficient cells grown with extract because their growth would have been arrested during the 48 hours of depletion, whereas that of non-deficient cells was not. In experiments where this discrepancy was taken into account, the extract-treated deficient cells always showed at least normal multiplication. However, from the standpoint of the immediate problem, the important point was whether or not multiplication occurred. Where radioactive acetate was employed, the procedures have been previously described (4). Additional pertinent data are given in footnotes of the appropriate Table.

Results. Both human HeLa and conjunctiva cells multiplied in the absence of CO_2 when normal cell extracts were present (Table I). No stimulation of multiplication of non-deficient cells was caused by such extracts. From Fig. 1 it is apparent that the amount of extract employed in the usual assay was well within the optimum range. Material in approximately 4 to 5 normal cells was sufficient to cause one deficient cell to resume multiplication. Extracts from CO_2-deficient cells not only failed to stimulate multiplication of deficient cells (Fig. 1), but actually caused reduction in cell count. This suggests that the deficiency may have been made more severe by such extracts.

Since depleted cells respond to added CO_2 (1), care was exercised to eliminate this as a possible contaminant. Use of KOH ampules is highly effective in this regard(1). As determined by use of $NaHC^{14}O_2$, less than 0.02% of normal CO_2 and HCO_3^- is found in the presence of the ampule. Contamination of cell extracts by viable cells is unlikely because extract added to control tubes caused no increase in cell count (Table I), nor have cells

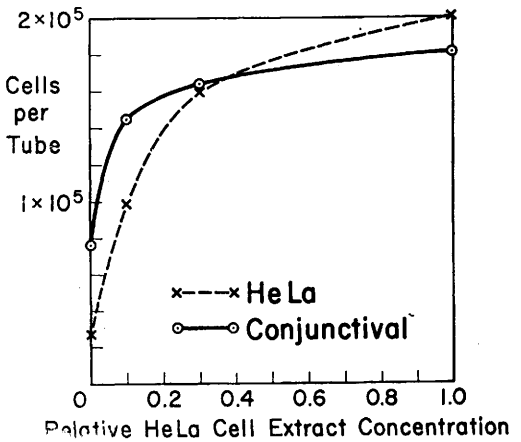

FIG. 1. Effect of concentration of normal HeLa cell extract on the multiplication of CO_2-deficient cells. A cell extract conc. of 1 is equivalent to material contained in approximately 3×10^5 cells made up to a final vol of 1 ml with Eagle's medium. Each point represents avg of 4 results.

TABLE II. Conversion of Acetate-1-C^{14} to $C^{14}O_2$ by Normal and CO_2-Deficient HeLa Cells.*

Kind of cells	Cell count × 10^3	Total conversion to $C^{14}O_2$ (c.p.m. × 10^3)	Relative conversion to $C^{14}O_2$ (c.p.m./10^3 cells)
Normal	177 (161-194)	27 (26-30)	152
CO_2-deficient	45 (43- 49)	12 (9-13)	266

* These results pertain to cells which were 8 days old from time of inoculation. CO_2-deficient cells were without CO_2 for last 5 days of this period. On the seventh day, all tubes received acetate-1-C^{14} to furnish 1.38 × 10^6 counts/min. (specific activity = 15 millicuries/millimole acid). Values are mean of 4 tubes; numbers in parentheses give range.

ever grown out of the reconstituted extracts even after 3 weeks' incubation.

Discussion. Of considerable interest is the fact that whereas within 24 hours after removal of CO_2, net multiplication has ceased (1), metabolism of acetate-1-C^{14} to $C^{14}O_2$ is approximately normal even at the end of 5 days (Table II). Earlier(1), glucose-1-C^{14} conversion to $C^{14}O_2$ by such cells was found to be two-thirds normal after 2 days' depletion. Therefore, inhibition of net multiplication is one of the first effects of CO_2-depletion and is not accompanied necessarily by generally deranged metabolism. This is not to say that other reactions involving incorporation of CO_2 are necessarily normal or adequate in the depleted cells. Interference with nucleic acid synthesis would afford ample cause for inhibition of multiplication. Such a possibility is now under investigation. The importance of CO_2 in living cells has been pointed out by Loomis(5) and Werkman(6). The former has laid special stress on pCO_2 as a major regulatory agent in the control of biological function. He suggests CO_2 is the agent involved in such studies as those of Clowes(7) and Cook and Elvidge(8) on sperm stimulation and those of Puck(9) on a diffusable factor in tissue culture The manner in which CO_2 would function in such cases is not discussed. From the present results, it is apparent that CO_2 can be replaced by cellular material synthesized by normal cells with an adequate CO_2 supply. It is also possible that similar results would be found in other cases where CO_2 has been shown to be essential. Elucidation of the active agent(s) should afford further insight into control of cell multiplication. The earlier finding of Harris(10) that CO_2 was essential for the outgrowth of chick heart fibroblasts suggests that the phenomena observed here are not restricted only to cells which have been maintained in continuous culture for long periods.

Summary. Human cells of HeLa and conjunctival (Chang) strains fail to multiply in absence of CO_2, but net multiplication can be restored by addition of normal cell extract. Such extracts have no effect on multiplication of normal cells. On the other hand, extracts of CO_2-deficient cells have an adverse effect on deficient cells. Although net multiplication was completely arrested in the absence of CO_2, conversion of acetate-1-C^{14} to $C^{14}O_2$ was at least normal, indicating general cell metabolism was still high. Implications of the results to regulation of cell multiplication are discussed.

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