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Partial Replacement of CO_2 in Strain L and HeLa Cultures.* (32341)

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It has been reported that HeLa or conjunctival cells of human origin would not multiply under conditions of CO_2 deficiency (1) but that net multiplication could be restored partially by extracts of normal cells (2) or completely by a combination of ribonucleosides and oxalacetate(3). In the latter study, over 90% of the label fixed from $NaHC^{14}O_3$ by the cells was found in the acid soluble and nucleic acid fractions. It was concluded that, under the experimental conditions used, the chief function of CO_2 was to provide specific precursors for synthesis of purines, pyrimidines, and oxalacetate. Reported here is a study of CO_2 substitution in fibroblast-like mouse cells.

Materials and methods. The cells were a subline of NCTC clone 929 (strain L). The basal medium was Waymouth's MB752/1(4) modified as follows: $MgSO_4 \cdot 7H_2O$(100), Na_2HPO_4(150), nicotinamide(0.5), choline (124), ascorbic acid(8.75), cysteine HCl(45), glutathione(7.5), folic acid(0.5), $FeSO_4$(0.5) (all mg/l) (personal communication, C. J. Waymouth). Horse serum(5%) that had been heated at 52°C for one hour was added; $NaHCO_3$ was omitted. Compounds(all from Nutritional Biochemicals Corp., Cleveland, Ohio) used in the experiments were added to the basal medium which then was filtered through a sterile fritted-glass or membrane filter. Oxalacetate(OA) created a problem when added to media because of a rise in pH due to spontaneous decarboxylation and concomitant loss of an acidic group. This was compounded by the presence of alkaline traps since the CO_2 resulting from the decarboxylation was removed from the medium and did not form H_2CO_3 which ordinarily would offset partially the loss of the carboxyl group from OA. In such cases, the pH was kept in a range suitable for the cells by addition of dilute HCl as frequently as necessary.

After inoculation into 250 ml milk dilution bottles, the cells were allowed to grow in monolayers for 24 hours before establishment of experimental conditions. Deficiency in CO_2 was established by use of an alkaline trap similar to one previously described(1) except that the glass tubing extended completely through the stopper and had its exterior end closed by a sleeve-type serum bottle stopper. Four-tenths ml of 15% NaOH was introduced onto a 1.5 × 2 inch Whatman No. 1 filter paper fan contained in the glass tubing. Media and alkaline traps were changed every 48 hours unless otherwise noted. Cells were scraped from the glass and counted with a Model B Coulter Counter (Coulter Electronics, Hialeah, Fla.). Cultures were periodically tested for PPLO contamination by Madoff's modification of Diene's procedure (5).

Results. Cultures of strain L cells deficient in CO_2 showed no increase in number if the

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TABLE I. Growth of CO_2 Deficient L Cell Cultures in Presence of Ribosides or Ribosides and Oxalacetate.

	Cell count ± S.E./ml × 10^{4}*		
Days	Controls†	CO_2 deficient + R	CO_2 deficient + R + OA
2‡	9.89 ± .63	13.69 ± .73	13.53 ± .41
4	34.89 ± .57	40.79 ± .11	37.53 ±1.35
6	93.59 ±3.85	76.39 ±1.38	75.43 ± .44
8	173.26 ±3.77	134.44 ±2.70	118.71 ±1.79

* N = 4.
† Controls were without added HCO_3^- or alkaline traps.
‡ 0 day count = 5.13 ± .07.

cell count at initiation of the deficiency was 5×10^5 cells/ml or fewer. Deficient cultures progressively larger than that number showed progressively faster growth rates, finally approaching the normal rate at about 2×10^6 cells/ml. It seemed advisable to use cultures substantially below 5×10^5 cells/ml for experiments where potential CO_2 substitutes were added because of the possibility that a partial growth response would appear to be a complete response if the cell density passed this level. An alternative would have been dilution and transfer of supplemented deficient cultures, but this proved impractical because after transfer, diminution in number occurred and cell debris accumulated in all deficient cultures, whether CO_2 substitutes were present or not.

Deficient cultures (5×10^5 cells/ml) remained static for a few days and then began to decrease slowly in number. However, the cells remaining in cultures kept deficient for up to 14 days would multiply after a lag of a few days if the alkaline traps were removed. Time-lapse cinematographic studies showed that the static appearance of CO_2 deficient cultures was not due to some cells slowly multiplying while others disintegrated. Only cells already in mitosis at the start of the deficiency would divide.

Efforts to replace CO_2 in the deficient cultures by supplementation with the ribosides, adenosine, guanosine, cytidine, and uridine (R)(10 μg/ml each) or a combination of R + OA were only partially successful. Table I shows the best growth attained by CO_2 deficient cultures under such conditions in some 20 experiments. Multiplication of the control cells occurred without addition of bicarbonate; however, it should be noted that in spite of the eventual population which the R + OA supplemented CO_2 deficient cells attained, their overall rate of multiplication did not approach that of the controls. In many experiments, the supplemented cultures ceased growing after one or two doublings in number. Table I shows that R alone were more effective in replacing CO_2 than the combination of R + OA. In most experiments, however, there was no significant difference between them. No significant changes in the results were noted when: (a) medium containing R and 1.5 or 5 mM OA was changed every 24 hours, (b) the concentration of R varied from 5 to 15 μg/ml, (c) the experiments were repeated in serum-free media with cells adapted to growing in such media, (d) carbonic anhydrase (20 μg/ml) was added to the media, (e) tris buffer was used in the media(3).

The basal medium used here contained hypoxanthine. When this purine was present, the following replaced CO_2 as effectively as R: a combination of deoxyadenosine, deoxyguanosine, deoxycytidine, and thymidine (10 μg/ml each); a combination of cytosine and uracil(15 μg/ml each); cytidine, uridine, or orotic acid individually(15 μg/ml each). A combination of cytidylic and uridylic acids(15 μg/ml each) was less effective, while thymidine, adenosine, or guanosine(15 μg/ml each) were ineffective. In the absence of hypoxanthine, the pyrimidine compounds above had little or no effect on the growth of CO_2 deficient cultures. Subsequent to the above, it was found that extracts of normal cells(2) would replace CO_2 in deficient cultures as adequately as, but no better than R, even when the equivalent of 3 normal cells to each deficient cell was used. Extracts were equally effective whether the particulate cell material was removed or not. Since adding OA with R did not completely replace CO_2, the following were tried in combination with R: malonate (5mM), pyruvate(5mM), asparagine(5mM), carbamyl phosphate(2mM), carbamyl phosphate and OA(1 and 5mM), formate(10mM). None enhanced the growth response above that from R alone.

Because R and OA completely replaced CO_2

TABLE II. Growth of CO_2 Deficient HeLa Cell Cultures in Presence of Ribosides or Ribosides and Oxalacetate.

Days	Cell count ± S.E./ml × 10^4* Controls†	CO_2 deficient + R	CO_2 deficient + R + OA
2‡	9.90 ± .19	9.15 ± .24	9.99 ± .99
4	19.88 ± .65	19.03 ± .92	18.56 ± .54
6	45.51 ± .82	37.98 ±1.48	37.14 ± .98
8	88.49 ±2.10	64.10 ±2.24	52.12 ±3.14

*, † See corresponding footnotes Table I.
‡ 0 day count = 2.58 ± .09.

in the system used by Chang *et al*(3) for human cells but not in the system used here for mouse cells, it seemed possible this represented a basic difference between at least some human and mouse cells. HeLa cells were obtained from the above authors, grown in essentially the same medium used by them, but with the bottles and alkaline traps as used above for L cells. Table II shows the results of the experiment which resulted in the best growth of 8 such experiments. The results were essentially the same as with L cells, for replacement of CO_2 by R and OA was only partial. The comments above about the difference between R and R + OA apply here also.

Discussion. The failure of these L cells below a certain population density to grow in the presence of an alkaline trap established CO_2 as an essential for their proliferation. Examples of similar findings are those with human cells mentioned above(1); other mouse fibroblasts, human fibroblasts, rabbit fibroblasts(6); fibroblasts from chick heart explants(7); and some bacteria(3). Much of this work has been reviewed(8). The growth of the CO_2 deficient cultures when above 5 × 10^5 cells/ml was likely due to a microenvironment of CO_2 from endogenous production which was not removed rapidly enough by the alkaline traps because of the proximity of the cells to one another. Such a concept is supported by progressively higher growth rates of CO_2 deficient cultures progressively denser than 5 × 10^5 cells/ml.

Whatever caused the cessation of growth in CO_2 deficient cultures seems rather readily reversible in light of the recovery of cells after lengthy deficiencies. Blockage of one or more anabolic steps by the deficiency because of lack of one or more compounds which results from a carboxylation reaction is a possibility. This is not in disagreement with the continuation of division by deficient cells in mitosis since such cells could be expected to have completed the bulk of biosyntheses needed for division. However, the failure of the various additions to the medium in this study to replace CO_2 completely is attested to not only by the slower growth of the supplemented deficient cultures, but also by their intolerance of dilution and transfer.

The maximum amount of CO_2 substitution obtained in this study could be realized by using R, deoxy R, or certain related compounds with no other additions to the medium. The work with and without hypoxanthine indicated that one each of a metabolically intraconvertable purine and pyrimidine is necessary for such a level of CO_2 replacement. The failure of hypoxanthine plus thymidine is in agreement with current knowledge of the metabolic pathways of the latter. The lower growth of CO_2 deficient cultures with hypoxanthine plus uridylic and cytidylic acids was probably due to limited permeability of the cells to those acids.

It would seem that normal cells should contain whatever is needed to replace CO_2 completely, but extracts of such cells were no more efficient than R, thus indicating the extracts were simply another source of R and/or related compounds. However, it is possible the critical compound is present in normal cells in labile or bound form which was unavailable by the time the extracts were used.

The failure of R and OA to completely replace CO_2 in HeLa cells is contrary to the results of Chang *et al*(3). However, those authors pointed out that the deficiency of CO_2 in their system might be incomplete and a system promoting a more severe deficiency might give different results. There is no reason to believe the system used in this study caused absolute depletion of CO_2 either, but consideration of the two systems makes it seem likely the one used here was more efficient. Also, the cultures used here were in bottles while those of the above authors were in roller tubes.

It is clear that a major function of CO_2 in the cells used in this study is the provision of precursors for purines and pyrimidines since they would replace CO_2 partially. However, involvement of CO_2 in some other function which becomes critical to the cells more slowly is apparent. The inability of metabolic compounds, when added to the medium, to replace CO_2 completely in the cells used in this study is similar to results with bacteria(3). It should be noted that these failures to substitute completely for CO_2 do not necessarily mean the critical requirement is not in an anabolic step involving one of the potential substitutes. Some of the compounds tested such as malonate and carbamyl phosphate, that are known to result from metabolic CO_2 fixation, may enter the cells poorly. Even if entry is successful, a compound may not arrive at the proper cellular location, or be in the proper activated form if it does. Supplying such forms as malonyl CoA to intracellular locations by addition to media would often be difficult or impossible. Furthermore, the right combinations of compounds in the right concentration ratios could be critical. The number of possibilities would be extremely large, and therefore impractical to approach. There may also be undiscovered reactions involving CO_2 fixation and the proper substitutes, therefore, are unknown. Efforts to substitute for CO_2 may never be completely successful because cells may have a requirement for CO_2 or bicarbonate *per se*. If the critical fixation reaction were in a degradative pathway essential to the cells, addition of intermediates would only aggrevate the situation. In spite of the negative results with oxalacetate supplementation, carbon dioxide deficiency could cause disturbances in metabolic pathways which produce CO_2, and thereby inhibit the cell. A possible role for CO_2 in metabolic control cannot be ignored. Its role in sexual differentiation of hydra has been demonstrated(9), and possible effects on other biological regulation discussed(10). Finally, CO_2 deficiency might result in a physical phenomenon such as swelling due to ionic changes across the membrane, which is damaging to the cells. In any of these latter cases, efforts to substitute for CO_2 would be futile since substitutes would not exist. In general, knowledge of the nature and composition of CO_2 deficient cells would appear a more fruitful approach to the problem of CO_2 substitution. Such studies are in progress.

Summary. Deficiency of CO_2 stopped the growth of strain L mouse fibroblasts in monolayer cultures. Cells in such cultures would recover after lengthy deficiencies upon replacement of CO_2. Ribonucleosides, deoxyribonucleosides, or extracts of normal cells would substitute partially for CO_2. A comparable level of substitution would occur with a purine and pyrimidine combination as simple as hypoxanthine and orotic acid. Combining other compounds involved in CO_2 metabolism with the ribosides did not result in more complete substitution.

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