The Role of Carbon Dioxide as an Essential Nutrient for Six Permanent Strains of Fibroblasts*

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ABSTRACT

The results of these studies demonstrate that carbon dioxide is required for the growth and maintenance of strains of fibroblasts derived from human tissues, strains FS4-705 and U12-705, from mouse tissue, strain L-705, and from rabbit tissues, strains RM3-56, RS1-56, and RT-56 in a chemically defined medium containing phosphite buffer in place of bicarbonate and supplemented with dialyzed serum and dialyzed embryo extract. Under these conditions, the cells fail to proliferate at a significant rate and begin to degenerate within 5 to 10 days when the flasks are not stoppered. Sufficient carbon dioxide is produced by the cells to promote growth as indicated by the fact that maximal proliferation is obtained in the same phosphite media when stoppered flasks are employed. With the exception of RS1-56, all the remaining strains tested can be propagated serially in open flasks containing phosphite medium prepared with whole serum and embryo extract. The rate of growth under these conditions, however, is only one-half to one-third that obtained in stoppered flasks containing phosphite medium or the conventional bicarbonate medium.

An earlier report from this laboratory described the propagation of several strains of mammalian cells in media containing fixed buffers such as tris(hydroxymethyl)aminomethane and glycylglycine in place of the conventional carbon dioxide-bicarbonate system (1). Studies on the practical application of fixed buffers in tissue culture media were subsequently extended to include other compounds which are compatible with calcium and other cations and which are effective buffers in the pH range 7.0–8.0. In the course of these investigations, it was observed that several strains of fibroblasts proliferate at a reduced rate in certain media containing sodium phosphite (2) in place of bicarbonate when the flasks were closed only by means of loosely fitting metal caps instead of stoppers. These observations suggested that carbon dioxide might be an essential nutrient for fibroblasts propagated *in vitro*. Some support for this speculation was indicated by other investigations which demonstrated that carbon dioxide is beneficial for the outgrowth of cells from explanted rat kidney (3, 4) and essential for explants of chick embryo tissue (5, 6). The results to be presented demonstrate that carbon dioxide is essential for the proliferation of six permanent strains of fibroblasts, derived from human, mouse, and rabbit tissues, when propagated in media containing dialyzed chick embryo extract and dialyzed horse serum.

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Materials and Methods

The following strains of cells were propagated serially as described previously (7–9): human fibroblasts, strains FS4-705 and U12-705 (8), rabbit fibroblasts, strains RM3-56, RS1-56, and RT6-56 (9) and mouse fibroblasts, strain L-705 (10). Medium 705 is composed of solution 703 (11) supplemented with 5 per cent (V/V) chick embryo extract (CEE, 7) and 20 per cent normal horse serum (NHS) and medium 56 contains 5 per cent CEE, 10 per cent NHS, and 85 per cent solution S18 (12). Dialyzed chick embryo extract (DCEE) and dialyzed horse serum (DHS) are prepared by dialysis

TABLE I

Composition of Solution S104

Compound*	Concentration	Compound	Concentration
	mM		mM
Alanine‡	0.3	Serine	0.2
Arginine	0.3	Threonine	0.2
Aspartic acid	0.15	Tryptophan	0.05
Cystine	0.05	Tyrosine	0.1
Histidine	0.05	Valine	0.2
Hydroxyproline	0.15	Choline	0.01
Glycine	0.15	Folic acid	0.005
Glutamine	2.0	Meso-inositol	0.01
Isoleucine	0.2	Nicotinamide	0.005
Leucine	0.2	Pantothenic acid	0.005
Lysine	0.2	Pyridoxal	0.005
Methionine	0.1	Riboflavin	0.001
Phenylalanine	0.1	Thiamin	0.005
Proline	0.15		

* Medium also contains 100 units penicillin G and 12.5 μg. streptomycin sulfate per ml. and the ingredients of a modified Earle's balanced salt solution (11) which contains only 5 mg. per liter phenol red (sodium salt) and lacks sodium bicarbonate.

‡ All amino acids are of the L configuration.

against Earle's saline (13) lacking bicarbonate as described by Haff and Swim (12). The basal media employed contain the following: Basal medium number 1 (BM1) = 5 per cent CEE + 10 per cent NHS + 85 per cent (V/V) S104 described in Table I; BM2 = 10 per cent DCEE + 10 per cent DHS + 80 per cent S104; BM3 = 5 per cent DCEE + 5 per cent DHS + 90 per cent S104. Duplicate cultures are prepared from 5 to 7 day old stock cultures as follows. The cells are harvested as described previously (7–9) and the resulting suspension is diluted with an equal volume of medium and passed through a sintered glass filter (Ace Glass, Inc., Vineland, N. J., porosity B) to remove any clumps of cells that remain. The filtrate is diluted with an equal volume of the appropriate medium (705 or 56) so as to contain 5×10^4 cells per ml. and 4 ml. aliquots are added to S-20[1] flasks which are incubated overnight at 37° C. The medium is removed from groups of 6 to 8 duplicate cultures and the cells which adhere to the glass are washed twice with 2 ml. portions of experimental medium. Four ml. of experimental medium is then added to each flask. These are incubated at 37° C. and the medium is replaced on the 3rd, 6th, and 8th days and the experiment is terminated on the 10th day. On the 5th and 10th day the cells in one-half the flasks of a given group are enumerated by the modification of the nuclear counting procedure of Sanford *et al.* (14) which is used in this laboratory (12). The number of cells present at the beginning of the experiment is determined by applying the same procedure to a separate group of 3 to 4 duplicate flasks from the same set. Stock solutions of 1 M sodium phosphite are prepared by neutralizing a 30 per cent aqueous solution of purified phosphorous acid (J. T. Baker Chemical Co., Phillipsburg, N. J.; cooled in ice bath) with 10 N sodium hydroxide and diluting to volume with distilled water. These are stored at −20° C. until just prior to use and the final adjustment in pH is made on the completed culture media (Tables II and III) after the addition of phosphite.

[1] Flasks with a surface area of 20 cm.^{2} prepared from square pyrex tubing (2 cm. I.D.). S20 flasks can be purchased from Euclid Glass Engineering Laboratory, Cleveland, Ohio.

RESULTS

The Proliferation of Fibroblasts in Media Containing Phosphite or Bicarbonate and Supplemented with NHS and CEE.—The data presented in Table II compare the growth of 6 strains of fibroblasts in BM1-phosphite medium with that obtained in BM1-bicarbonate medium (see protocol beneath Table II). Growth of individual strains in stoppered flasks is the same whether BM1 is supplemented with phosphite, phosphite plus bicarbonate, or bicarbonate. It is to be noted however, that growth of all strains is reduced by a factor of at least 2 when BM1 is supplemented with phosphite and the flasks remain open to the air by

TABLE II

Proliferation of Fibroblasts in Media Containing Phosphite or Bicarbonate and Whole Serum and Embryo Extract

Cell strain	Inoculum × 10^4	Phosphite flasks open*		Phosphite flasks stoppered		Phosphite + bicarbonate flasks stoppered‡		Bicarbonate flasks stoppered§	
		5 day	10 days	5 day	10 days	5 day	10 days	5 day	10 days
FS4-705	22	7.5	15.8	11.4	24.8	12.5	28.2	11.9	26.4
L-705	20	3.6	12.6	7.0	25.2	7.9	25.7	5.3	20.9
RM3-56	25	3.8	5.1	7.5	12.5	7.0	12.8	7.2	13.1
RS1-56	21	1.9	2.1¶	4.6	8.6	5.0	7.6	4.2	6.7
RT6-56	24	3.8	5.5	5.8	10.8	5.0	11.3	5.9	9.7
U12-705	22	3.0	10.2	12.5	26.8	13.8	26.7	12.8	31.6

* 0.02 M sodium phosphite, pH 7.2; neck of flasks covered with a loosely fitting metal cap.

‡ 0.02 M sodium phosphite, 0.005 M sodium bicarbonate, pH 7.2.

§ 0.02 M sodium bicarbonate, pH 7.2; flasks gassed with mixture containing 5 per cent CO_2, 40 per cent O_2, and 55 per cent N_2.

‖ Increase in cell number referred to inoculum as 1 for period indicated; average values for 2 or more experiments; basal medium 1 employed throughout.

¶ Degenerative changes in the cells evident after 7 to 10 days.

means of loosely fitting metal caps. The pH of the BM1-phosphite medium in the closed flasks does not differ significantly from that in the open vessels at any time in the course of the experiments. Little growth of RS1-56 is obtained under these conditions and after 7 to 10 days, the cells begin to degenerate. On the other hand, strains FS4-705, L-705, RM3-56, RT6-56, and U12-705 can be propagated serially for indefinite periods in BM1-phosphite (open or closed flasks) at rates comparable to those indicated in Table II. These observations indicate that the quantity of CO_2 supplied by the NHS and CEE of BM1, in addition to that produced metabolically by the cells, is not sufficient to promote maximal growth. It is of interest that NHS and CEE in addition to the ingredients of solution S104 do not supply metabolites (at least in sufficient concentration) which substitute for CO_2.

The Failure of Fibroblasts to Proliferate in Open Flasks in Media Containing Phosphite, DHS, and DCEE.—To ascertain whether carbon dioxide is an absolute requirement for the growth of fibroblasts, it was necessary to use media containing DHS and DCEE (BM2 and BM3). The results presented in Table III demonstrate that carbon dioxide is essential for the growth and maintenance of fibroblasts in open flasks containing BM2 or BM3 with added phosphite. In spite of the fact that some growth is obtained under these conditions, the cells of all strains except L-705 and RT6-56 begin to degenerate within 5 to 10 days. Examples of some of the cytologic changes observed are illustrated in Figs. 1 to 6. As might be anticipated from the data on growth in Table III, the degenerative changes are more extensive in the case of strains FS4-705, RM3-56, and RS1-56 than with strain U12-705. Similarly, cytologic alterations in the cells usually are evident earlier and progress more rapidly with time when BM3 is used instead of BM2. Cellular degeneration is observed with strains L-705 and RT6-56 only when special precautions are taken to eliminate carbon dioxide from the medium. For example, growth is reduced and cytologic changes are evident within 10 days when the necks of the flasks are connected to 25 ml. Florence flasks containing pieces of filter paper saturated with 0.15 N sodium hydroxide. It is of particular interest in this connection, that all 6 strains of fibroblasts produce adequate carbon dioxide for maximal growth as demonstrated by the fact that growth in stoppered flasks containing phosphite medium is the same as that obtained in the equivalent bicarbonate medium.

TABLE III

Proliferation of Fibroblasts in Media Containing Phosphite or Bicarbonate and Dialyzed Serum and Dialyzed Embryo Extract

Cell strain	Basal medium	Inoculum	Phosphite flasks open*		Phosphite flasks stoppered		Phosphite + bicarbonate flasks stoppered‡		Bicarbonate flasks stoppered§	
			5 days‖	10 days‖	5 days	10 days	5 days	10 days	5 days	10 days
FS4-705	2	25	2.4	1.9	7.5	12.0	—	—	6.9	10.6
FS4-705	3	22	1.3	1.2¶	3.5	9.9	3.8	10.4	4.5	10.4
L-705	3	19	1.2	3.7	5.0	16.7	5.2	16.7	4.4	15.7
RM3-56	2	24	1.6	0.9¶	7.4	12.0	8.3	14.7	4.1	10.6
RM3-56	3	21	0.8	1.2¶	3.4	5.9	4.0	7.8	3.1	6.4
RT6-56	2	23	2.4	2.8	4.0	8.9	3.6	8.4	3.5	9.0
RT6-56	3	20	2.8	2.0¶	3.4	5.8	3.6	4.9	3.4	5.2
RS1-56	2	26	1.6	1.2¶	3.8	5.5	3.9	5.4	3.1	4.8
U12-705	2	21	1.8	3.9¶	5.1	9.3	4.9	9.9	5.0	10.9
U12-705	3	25	2.4	2.4¶	3.9	6.5	—	—	4.2	6.7

* 0.02 M sodium phosphite, pH 7.2; neck of flasks covered with a loosely fitting metal cap.

‡ 0.02 M sodium phosphite, 0.005 M sodium bicarbonate, pH 7.2.

§ 0.02 M sodium bicarbonate, pH 7.2; flasks gassed with mixture containing 5 per cent CO_2, 40 per cent O_2, and 55 per cent N_2.

‖ Fold-increase in cells for period indicated; average values for 2 or more experiments.

¶ Degenerative changes in the cells evident after 5 to 10 days.

DISCUSSION

The foregoing data demonstrate that carbon dioxide is essential for the growth and maintenance of 6 permanent strains of fibroblasts. Furthermore, mammalian fibroblasts propagated *in vitro* resemble bacteria and other microorganisms in that they produce sufficient metabolic carbon dioxide to satisfy their nutritional requirements as indicated by the fact that the omission of carbon dioxide from the medium is without effect on growth provided the flasks are stoppered. These observations explain why Eagle (15) was unsuccessful in demonstrating a carbon dioxide requirement for strains L and HeLa in experiments with stoppered flasks containing a bicarbonate-free medium. Geyer and Chang (16) demonstrated recently that 2 strains of human epithelial cells (HeLa and conjunctival cells) also require carbon dioxide for growth. This was accomplished by employing potassium hydroxide to adsorb the carbon dioxide contained in the culture vessels. The data presented in Tables II and III indicate that a closed system is required for the practical application of fixed buffers (1, 2) as a substitute for the conventional carbon dioxide–bicarbonate buffer

system in tissue culture media. That is, the use of fixed buffers (provided they are non-toxic) appears to be limited only by the ability of the cells to produce sufficient carbon dioxide metabolically. The results of the present studies are not in accord with the conclusion of Harris (5), that the principal function of carbon dioxide is in the maintenance of the appropriate intracellular pH. This conclusion was based on the failure to obtain outgrowth from explants of chick embryo tissue at pH levels below 7.8 in media free from carbon dioxide, whereas limited growth was observed at higher pH values. It is suggested that these results may be related to the amount of carbon dioxide retained in solution which is a function of the pH of the medium.

Carbon dioxide has long been recognized as an important compound in the nutrition of bacteria (17, 18) and protozoa (19). A biochemical basis for the role of carbon dioxide in nutrition was established when Wood and Werkman (20) demonstrated that it is fixed by heterotrophic bacteria. Since the advent of isotopic tracer techniques, the intermediary metabolism of carbon dioxide has been studied in considerable detail, and it is now recognized that this compound is involved in the synthesis of many important metabolites by tissues of both animal and plant origin (21, 22). The ubiquitous occurrence of carbon dioxide fixation and its important role in cellular biochemistry has prompted Werkman (23) and others to speculate that it is essential for all living cells. The results of these studies in addition to the investigations of others (3–5, 6, 16) are in agreement with this concept in so far as it applies to mammalian cells propagated *in vitro*.

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Explanation of Plate 250

Fig. 1. RM3-56 cells after 5 days in open flasks containing basal medium number 3 supplemented with 0.02 M sodium phosphite. × 120.

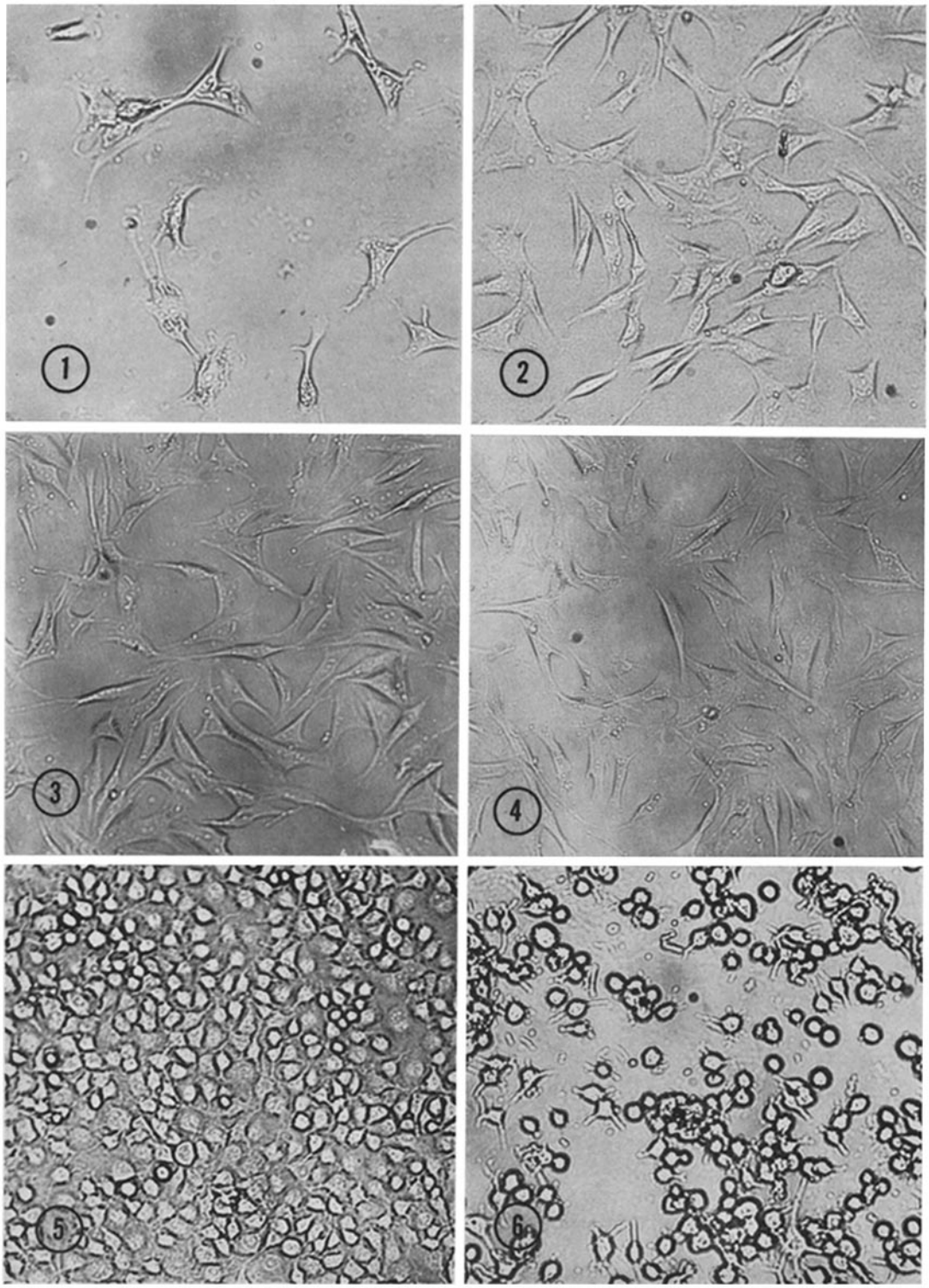
Fig. 2. RM3-56 cells after 5 days in stoppered flasks containing basal medium number 3 supplemented with 0.02 M sodium phosphite. × 120.

Fig. 3. RM3-56 cells after 5 days in stoppered flasks containing basal medium number 3 supplemented with 0.02 M sodium phosphite and 0.005 M sodium bicarbonate. × 120.

Fig. 4. RM3-56 cells after 5 days in stoppered flasks containing basal medium number 3 supplemented with 0.02 M sodium bicarbonate. × 120.

Fig. 5. U12-705 cells after 9 days in stoppered flasks containing basal medium number 2 supplemented with 0.02 M sodium phosphite. × 120.

Fig. 6. U12-705 cells after 9 days in open flasks containing basal medium number 2 supplemented with 0.02 M sodium phosphite. × 120.



(Swim and Parker: Carbon dioxide as nutrient of fibroblasts)