Influence of CO_2 on Respiratory Metabolism of Ehrlich Ascites Tumor.*† (25869)

NIS I. NISSEN (Introduced by J. Kieler)
Fibiger Lab., Biological Division, Copenhagen, Denmark

Although CO_2 has long been considered an unimportant waste product in metabolism of mammalian cells, its significance has been realized more fully during past few years, as a result of the demonstration of CO_2 fixation in cellular synthesis of fatty acids, purines and dicarboxylic acids. Over a long period, *Kieler* and collaborators examined the influence of CO_2 tension on respiratory metabolism of different normal and malignant cells using Cartesian diver technic. These examinations have included the Yoshida rat ascites tumor(1,2,3), Earle's L-strain mouse fibroblasts(1), and normal and malignant human leukocytes(4). Results of experiments on influence of CO_2 upon respiratory metabolism of Ehrlich ascites tumor cells are hereby submitted. Whereas in earlier investigations CO_2 tended to stimulate respiration, the effect upon Ehrlich ascites tumor cells was mainly inhibitory in this respect.

Material and methods. Hypotetraploid and hyperdiploid Ehrlich ascites tumors carried in St/EH and DBA/II mice were employed. Ascites tumor cells were inoculated intraperitoneally and experimented after 2-8 days growth. Mice were sacrificed by cervical fracture and ascites fluid aspirated under sterile conditions. Hemorrhagic fluid was discarded. Stained smears prepared for differential counting showed that 95-99% of cells were tumor cells. Ascites cells were suspended in Ringer-Locke's solution to concentration of 5-6,000 cells/μl. Washing and centrifugation was avoided as this damaged the cells morphologically and lowered respiration. Final cell suspension never contained more than 5% ascites fluid. Ringer-Locke's solution was buffered with tris (hydroxymethyl) aminomethane at concentration of 30 mM. Bicarbonate concentration was adjusted relative to CO_2 in the gas phase to give pH of 7.4. pH value of Ringer-Locke's solution was checked with pH meter before suspension of cells. pH value of final cell suspension was checked with pH paper before introduction into the diver, and in many cases also at end of experiment. No changes in pH could be demonstrated in this way, unless cell concentration exceeded 8000/μl and glucose concentration 5 mM. NaCl concentration varied according to bicarbonate and substrate content to maintain a constant osmotic concentration. Respiration was measured with Cartesian diver technic developed by *Linderstrøm-Lang*(5) and *Holter*(6). The divers were charged at 37°C under water. Solutions were gassed and equilibrated with the gas mixture to be studied during last 24 hours before experiment. Besides CO_2, the gas mixtures contained N_2 and O_2. Concentration of the latter was in all cases 15%. Gassing was repeated after introduction of each seal in the diver. The seals were: bottom seal (0.5 μl 0.15 M bicarbonate solution), lower neck seal (0.5 μl cell suspension), middle neck seal (0.5 μl bicarbonate solution), upper neck seal (0.5 μl paraffin oil), and finally mouth seal provided with hollow glass stopper. A detailed description of filling and gassing methods has recently been given by *Kieler*(3). Oxygen consumption/cell/hour was calculated on basis of initial 3-4 hour period, the linear part of respiration curve.

Results. Results of the influence of CO_2 tension upon endogenous respiration of Ehrlich ascites tumor cells have been summarized in Tables I and II. Two-day-old cells of the tetraploid line were in general not influenced by changes in CO_2 tension (Table I). Six days after transplantation 1% CO_2 had, how-

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TABLE I. Influence of CO_2 on Endogenous Respiration of 2-Day-Old Tetraploid Ehrlich Ascites Tumor Cells in 3 Experiments.

Oxygen consumption/cell/hr ($\mu l \times 10^{-6}$)			
CO_2 % in gas phase			
0	.3	1	5
4.21	4.43	4.32	3.76
4.59		3.70	4.25
4.08	3.94	4.13	4.37

ever, an inhibitory effect upon endogenous respiration of diploid as well as tetraploid line (Table II). Experiments with the diploid line showed that 5% CO_2 did not materially increase this inhibition. This result was a surprise, viewed in connection with our former experiments, where the effect of CO_2 was principally stimulatory to respiration. In these experiments evidence was obtained that the stimulatory effect of CO_2 on respiration of Yoshida cells is caused by increased synthesis of oxalacetate(2,3). An increased oxalacetate synthesis might also be expected to have a moderate stimulatory effect upon respiration of Ehrlich cells, as oxalacetate, added to the medium in 10 mM concentration, had such an effect in a CO_2-free atmosphere (Table III). It is therefore conceivable that lack of stimulatory effect of CO_2 upon respiration of Ehrlich cells is due to lack of pyruvate or phosphoenolpyruvate, substances with which CO_2 can react to form oxalacetate.

To investigate this possibility, CO_2 effect was examined after addition of glucose and pyruvate. Furthermore the relationship between CO_2 effect and oxalacetate and butyrate was studied. Table III shows results of typical experiments. Addition of glucose, pyruvate and oxalacetate did not reduce CO_2 effect significantly, but respiratory stimulation by pyruvate and oxalacetate, usually seen at room air, was abolished in presence of CO_2.

TABLE II. Influence of CO_2 on Endogenous Respiration of 6-Day-Old Ehrlich Ascites Tumor Cells.

	O_2 consumption/cell/hr ($\mu l \times 10^{-6}$)		
	CO_2 % in gas phase		
	0	1	5
Diploid cells (5 exp.)	2.12	1.47 (31% ± 6)*	1.37 (35% ± 14)
Tetraploid cells (6 exp.)	4.04	2.34 (42% ± 11)	

* Figures in parentheses indicate % inhibition by CO_2 ± S.D.

As demonstrated by *Medes and Weinhouse* (7) and others, endogenous respiratory metabolism of Ehrlich ascites tumor cells is concerned mainly with oxidation of fatty acid components. It is therefore interesting that addition of butyrate at concentration of 10 mM is capable of reducing inhibitory effect of CO_2 (Table III) although butyrate, as with other fatty acids(8), has an inhibitory effect upon endogenous respiration.

In view of reports of the significance of biotin in CO_2 fixation, it seemed necessary to exclude the possibility of a lack of biotin in Ehrlich cells. However, neither pretreatment of mice with biotin (25 mg/kg orally or intraperitoneally), nor addition of biotin to the cell suspension had any influence upon CO_2 effect.

TABLE III. Influence of CO_2 on Respiration of Tetraploid Ehrlich Ascites Tumor Cells in Presence of Various Substrates.

			CO_2 % in gas phase		
			0	1	
Age of tumor cells (days)	Substrate	Conc. (mM)	Oxygen consumption/ cell/hr ($\mu l \times 10^{-6}$)		Inhibition (%)
4	Glucose	0	3.02	1.99	34
		1	4.02	3.08	23
		5	2.44	1.79	27
5	Pyruvate	0	3.74	2.07	45
		5	4.96	2.18	56
5	Oxalacetate	0	4.26	2.61	39
		10	5.09	2.24	56
5	Butyrate	0	4.44	2.42	45
		10	3.27	2.92	11

Discussion. Significance of CO_2 tension in respiratory metabolism of animal cells has been little investigated, and the results are partly contradictory. *Root*(9,10,11) examined Arbacia eggs and nervous and muscle tissue and found in all 3 cases an inhibitory effect of CO_2 upon respiration, which agrees with our results. Several other authors found a stimulatory effect of CO_2/HCO_3^- upon respiration. *Danes and Kieler*(1) and *Bicz*(4) reviewed the literature. Our previous examination(1,2,3,4) has shown, for Earle's L-strain mouse fibroblasts, Yoshida ascites tu-

mor cells and normal and malignant leukocytes, a maximal respiration at 0.5-3% CO_2 in the gas phase. Contrary to *Bicz*(4), *Seelich*(12) demonstrated a stimulatory effect upon normal but no effect upon leukemic leukocytes. *Seelich and Letnansky*(13) found endogenous respiration of Ehrlich ascites tumor cells 8% higher in Krebs-Ringer-bicarbonate solution than in Krebs-Ringer phosphate. *Kieler*(3) demonstrated in Yoshida cells a relation between CO_2 effect and metabolic condition of the cells. Cells with a high mitotic coefficient and low respiration showed a great increase in oxygen consumption in presence of CO_2, whereas cells with high respiration and low mitotic coefficient were less stimulated or even inhibited by CO_2.

It is not possible from our experiments to make definite conclusions as to the mechanism of inhibitory effect of CO_2 upon respiration. However, the experiments with butyrate seem to indicate a relationship to fatty acid metabolism. In this connection, *Miller* (14) found a stimulatory effect of bicarbonate upon incorporation of acetate-C^{14} into adipose tissue. *Gibson, Titchener and Wakil*(15) demonstrated in fatty acid synthesis an absolute requirement for bicarbonate, which seemed to play a catalytic role. It is now generally accepted that the initial step in fatty acid synthesis, as pointed out by *Brady*(16), is a fixation of active CO_2 to acetyl CoA with formation of malonyl CoA. In the equilibrium between fatty acid oxidation and fatty acid synthesis, nutritive and hormonal factors are known to play an important role. Whether this equilibrium is influenced by CO_2 remains to be shown.

Summary. The effect of CO_2 on respiratory metabolism of Ehrlich ascites tumor cells was studied with the Cartesian diver technic. In absence of substrate and in presence of glucose, pyruvate or oxalacetate, CO_2 had a pronounced inhibitory effect upon respiration. Only butyrate addition was found to neutralize CO_2 inhibition. The stimulatory effect upon respiration shown for pyruvate, oxalacetate and glucose (at a 1 mM concentration) in a CO_2 free atmosphere, could, when CO_2 was present in the gas phase, be demonstrated only for glucose.

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D-Amino Acids as Source of Non-Specific Nitrogen for Growth of Rats. (25870)

M. RECHCIGL, JR.,* J. K. LOOSLI AND H. H. WILLIAMS
Depts. of Biochemistry and Nutrition and Animal Husbandry, Cornell University, Ithaca, N. Y.

Feeding of essential amino acids alone, each at its required level, will produce only limited growth of rats. Normal growth is obtained by supplementing such regimen with non-es-

* Present address: Lab. of Biochemistry, Nat. Cancer Inst., N.I.H., Bethesda, Md.