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Cartesian Diver Studies on Respiration and Glycolysis of High and Low Sarcoma Lines

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SUMMARY

Two cell types grown in tissue culture, designated high line (NCTC 2472) and low line (NCTC 2555), were studied by Cartesian diver manometric techniques. Studies with NaOH and $NaHCO_3$ as absorbant seals in stoppered microdivers suggest that consistency of respiration of both cell lines may be regulated by the type of seal used in the neck of the divers. Sodium lauroyl sarcosine, at 0.0018 M, inhibits high-line respiration by 65%, and low-line respiration, by only 10%. Both cell lines appear to be almost equally sensitive to 3-nitropropionic acid and to urethan. The most outstanding difference between the two cell lines was their anaerobic glycolysis, of which the low line was approximately one-third that of the high-line cells. Glycolysis in the high-line cells is significantly inhibited by iodoacetic acid and sodium lauroyl sarcosine, whereas sodium lauroyl sarcosine does not significantly influence low-line cells. L-Asparaginase demonstrates no inhibitory effect. It is suggested that both cell lines may have equally functional citric acid cycles and cytochrome systems. The main difference appears to be the more direct dependence of high-line cells for glucose in both glycolytic and respiratory process.

INTRODUCTION

One of the frequent limitations of tumor samples is the amount of tissue available. This is generally true with human biopsies, and it quite frequently is true with early animal tumors. Such small samples do not permit conventional comparative biochemical studies. With the development of the Cartesian diver microrespirometer (14, 19, 20), it became possible to measure the metabolic activity of small quantities of living cells. In view of the extreme sensitivity of the system, it was decided to utilize the Cartesian diver manometric technique, with which conventional manometric measurements can be made on extremely small samples of material.

The present investigation is concerned with an analysis of respiration and glycolysis of high and low sarcoma-producing cell lines and their response to specific metabolic inhibitors. High- and low-line cells possess different glycolytic, enzymatic, and oncogenic properties (24, 26, 29). Upon inoculation into mice, the high line produced sarcomas in 97% of the mice, whereas the low line produced tumors in only about 1%.

In view of the common *in vitro* origin of both high- and low-line cells, their oncogenic differences, and the possibility that CO_2 tensions may influence cellular respiration (3, 11, 18, 23), an attempt was made to reevaluate respiration and glycolysis of the high and low tumor-producing lines with the use of the Cartesian diver principle in hopes that specific differences might be found.

MATERIALS AND METHODS

Culture Procedure. Cell cultures, obtained from the American Type Culture Collection (Rockville, Md.), were maintained at 37° in T-60 or T-30 flasks (Bellco Glass Co., Vineland, N. J.) with Medium NCTC 109 plus 10% fetal calf serum (Grand Island Biological Co., Grand Island, N. Y.) and were gassed with 5% CO_2 plus 95% air, according to procedures described previously (12, 13, 25). For glycolysis experiments, cultures were grown in an atmosphere of 95% N_2 and 5% CO_2 and were intermittently gassed with 95% air and 5% CO_2 every 24 hr. Intermittent gassing with air allowed cells to survive in good morphological condition over a period of 3 or 4 days.

Preparation of Cells for Experiments. Cells to be used for experiments were removed from 4-day-old T-30 cultures with 0.25% trypsin and washed in conditioned medium which had been previously gassed with the desired gas mixture. Following the above procedures, cell viability in loaded divers was found to be approximately 90%, as determined by 0.4% trypan blue exclusion technique.

In glycolysis experiments, similar procedures were followed, except that fresh medium was used instead of conditioned medium. In addition, the medium was supplemented with extra glucose and bicarbonate to give final levels of 0.016 and 0.03 M, respectively (29).

SLS,[2] 3-NPA, urethan, IAA, and L-asparaginase all were obtained from Mann Research Laboratories, New York, N. Y. The L-asparaginase was from *Escherichia coli* and had a specific activity of 25 units/mg. Stock solutions of inhibitors were prepared in 0.9% NaCl solution and were filtered with a Millipore filter. Cells were incubated with L-asparaginase for 24 hr, and other inhibitors were added to cell cultures approximately 2 hr before the experiments.

Microgasometry. Microstandard Cartesian divers with glass

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[2] The abbreviations used are: SLS, sodium lauroyl sarcosine; 3-NPA, 3-nitropropionic acid; IAA, iodoacetic acid.

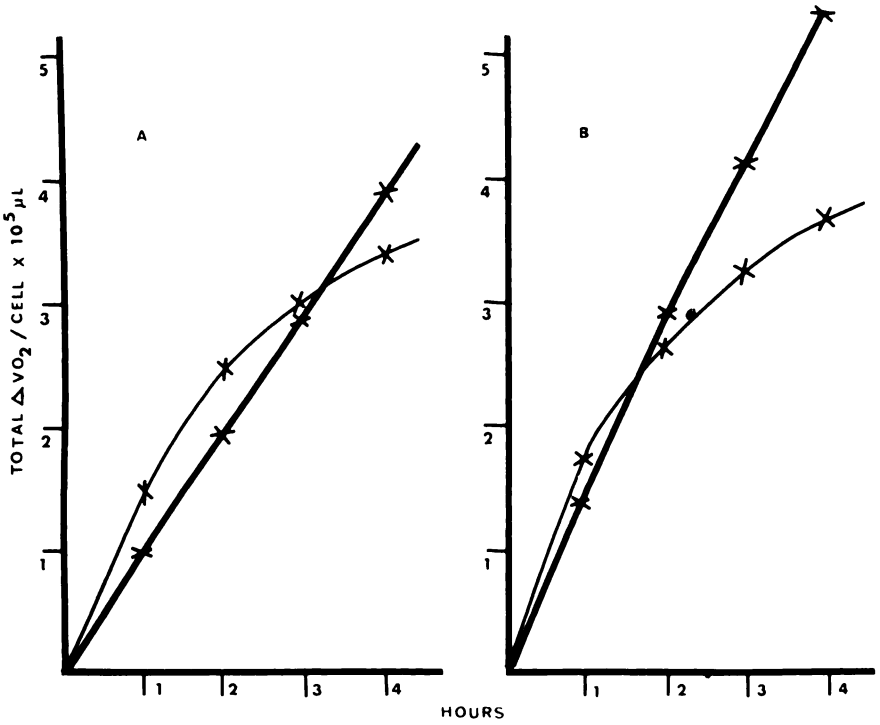

Chart 1. Respiration of high-line (*A*) and low-line (*B*) cells, with 0.1 M NaOH (—) and 0.15 M $NaHCO_3$ (—) used as absorbant seals in stoppered divers. Values were plotted from total average hourly respiration determinations. All determinations were made in 2-day-old conditioned medium. Respiration values are expressed as µl O_2/cell/hr and graphed as a total accumulative average. Each *point* represents an average value from 4 determinations.

stoppers were made from Jena red-stripe capillary tubing, density 2.23, with a total volume of 1 to 2 µl (10). Diffusion of gas from the diver vessel was eliminated by the use of a small glass stopper in the diver vessel neck, in addition to an oil seal. Therefore, it was possible to maintain a reasonably constant CO_2 tension within the diver vessel, thus eliminating variations in cellular metabolism due to extracellular changes in CO_2 tension (11, 19, 20).

The divers were filled at 37° with a special anaerobic diver-filling apparatus (OLE DICH, Hvidore, Denmark), according to the methods described by Linderstrøm-Lang (20) and Holter (14). For respiration studies, loads of the following sequence were made: (*a*) bottom drop of 0.20 µl of $NaHCO_3$ or NaOH; (*b*) Neck Drop 1, 0.20 µl of cell suspension; (*c*) Neck Drop 2, 0.20 µl of $NaHCO_3$ or NaOH. (*d*) Neck Drop 3, 0.20 µl of Klearoil (Sonneborn Petroleum, N. Y.); (*e*) a mouth seal of flotation medium (14) with a glass stopper. In glycolytic studies, the following loads were used: (*a*) Neck Drop 1, 0.25 µl cell suspension; (*b*) Neck Drop 2, 0.25 µl of Klearoil; (*c*) mouth seal and glass stopper. All solutions loaded into divers, with the exception of NaOH, were gassed with the desired gas mixture.

The method used for estimating cell numbers was similar to that of Chakravarty and Zeuthen (9), in that cell counts were made directly in the diver vessel. Three separate counts were made, and an average was calculated.

After loading, the divers were transferred to flotation vessels and allowed to equilibrate for 1 hr. In all determinations, the control divers, filled with every component except cells, were tested simultaneously with the experimental divers. The total ΔV/hr in the control diver was added or subtracted from the total ΔV in the experimental divers in order to obtain a corrected value.

RESULTS

Respiration of High- and Low-Line Cells. Respiration determinations demonstrating the influence of NaOH and $NaHCO_3$ as absorbant seals with high- and low-line cells are summarized in Chart 1. Analysis of average hourly determinations over a 4-hr period shows that respiration was more consistent when $NaHCO_3$ was used as the seal. Respiration in the NaOH diver dropped sharply in 3 to 4 hr, as opposed to more consistent respiration values in the $NaHCO_3$ divers.

Respiration in the Presence of Inhibitors. SLS, at 0.0018 M, reduces respiration in the high-line cells from 1.05 to 0.38 X 10^{-5} µl/cell/hr, a 65% inhibition (Table 2). At the same concentration, respiration in low-line cells is reduced from 1.15 to 1.03 X 10^{-5} µl/cell/hr, a 10% inhibition, whereas a greater concentration of SLS (0.0054 M) produces approximately 87% inhibition in both cell lines (Tables 1 and 2). Both cell lines appear to be almost equally sensitive to 10^{-4} M 3-NPA. Examination of Tables 1 and 2 demonstrates that 3-NPA inhibits respiration of low- and high-line cells 61 and 64%, respectively (0.47 and 0.37 X 10^{-5} µl/cell/hr). The sensitivity of low-line cells to urethan appears to be comparable to high-line sensitivity. Evaluation of Table 2 demonstrates that urethan inhibits high-line respiration approximately 80% (0.22 X 10^{-5} µl/cell/hr). Low-line respiration is inhibited approximately 83% (0.19 X 10^{-5} µl/cell/hr), whereas urethan and succinate in combination inhibit respiration approximately 74% (Table 1). Although the degree of inhibition in the presence of succinate is slightly less than without succinate, it is not significant (9%).

Anaerobic Glycolysis of High- and Low-Line Cells in the Presence of Inhibitors. Comparison of anaerobic glycolysis of

Table 1
Respiration of low-line cells (NCTC 2555) in 2-day-old conditioned medium with and without inhibitors

No. of cells in diver	Inhibitor	Total ΔVO_2/hr X 10^5 µl	ΔVO_2/cell/hr X 10^5 µl	ΔVO_2/cell/hr (mean ± S.E.)
350–1,200 (11 divers)	None	495.1, 384.0	1.14, 1.27	
		650.0, 330.0	1.03, 1.21	
		596.5, 685.0	1.05, 1.02	1.15 ± 0.05
		1539.2, 437.0	1.28, 1.01	
		830.0, 490.0	1.50, 1.18	
		209.0	1.00	
	Controls, 8.95, 7.50, and 6.65 X 10^{-4} ΔV/hr			
300–410 (5 divers)	0.0018 M SLS	462.0	1.00	
		536.8	1.10	
		380.0	1.00	1.03 ± 0.02
		434.6	1.00	
		425.9	1.05	
320–650 (5 divers)	0.0054 M SLS	136.5	0.19	
		104.0	0.10	
		172.5	0.15	0.15 ± 0.01
		118.6	0.15	
		160.5	0.14	
	Controls, 8.95 and 7.50 X 10^{-4} ΔV/hr			
400–520 (5 divers)	10^{-4} M 3-NPA	308.0	0.49	
		341.8	0.52	
		303.0	0.48	0.47 ± 0.02
		202.5	0.48	
		218.5	0.40	
	Control, 10.50 X 10^{-4} ΔV/hr			
420–605 (4 divers)	10^{-3} M succinate	515.0	1.07	
		551.5	1.10	1.13 ± 0.02
		634.5	1.15	
		815.5	1.20	
550–870 (4 divers)	10^{-3} M urethan	232.0	0.19	
		110.0	0.19	0.19 ± 0.01
		134.0	0.18	
		225.5	0.19	
375–650 (5 divers)	10^{-3} M succinate + 10^{-3} M urethan	196.0	0.31	
		202.0	0.36	
		261.5	0.30	0.30 ± 0.02
		173.0	0.26	
		181.5	0.23	
	Controls, 6.65, 7.50, and 8.95 X 10^{-4} ΔV/hr			

high and low lines demonstrates that glycolytic activity of the high line is approximately 0.48 X 10^{-5} µl/cell/hr, whereas the low line is approximately 0.17 X 10^{-5} µl/cell/hr, or only about 35% of that of the high line. Evaluation of the data presented in Table 3 demonstrates that glycolysis in the high line is significantly inhibited by IAA (75%; 0.12 X 10^{-5} µl/cell/hr) and by SLS (40%; 0.29 X 10^{-5} µl/cell/hr). SLS does not appear to reduce significantly the anaerobic glycolysis in the low-line cells (5%; 0.16 X 10^{-5} µl/cell/hr). L-asparaginase demonstrates no inhibitory effect on anaerobic glycolysis. Indeed, high-line cells show an increased anaerobic glycolysis following treatment with L-asparaginase.

DISCUSSION

In the present investigation, both high- and low-line cells exhibited more consistent respiration values when $NaHCO_3$ was used as an absorbant in the divers, compared to values with NaOH. This is seen in the respiration curves as a leveling off during the 3rd or 4th hr in the NaOH divers. Since NaOH

absorbs most of the available CO_2 (20) and isotonic NaOH seals were used (14), it is possible that, as time proceeds, the absorption of available CO_2 is complete. Correlated along with this time period and decrease in available CO_2, the respiration may correspondingly decline in the NaOH divers.

In $NaHCO_3$ divers, a different pattern was presented, as respiration of the cells remained reasonably constant during the course of the experiment. Danes and Kieler (11, 19) considered that this may be accounted for by the constant availability of CO_2 during the course of the experiment and that, therefore, at the termination of the experiment, the cells in the $NaHCO_3$ divers would have greater respiration values, compared to those in the NaOH divers. These results are not surprising in view of the numerous experiments which showed respiratory stimulation by CO_2 and $NaHCO_3$ (2, 3, 11, 19, 27).

The reasons for the stabilizing or stimulatory effect are not clear, but it is thought that these may involve a competition between the oxidative and glycolytic systems for either inorganic phosphate or phosphate acceptor (1). Furthermore, it has been demonstrated that CO_2 may influence the Crabtree effect (11, 19). In this regard, the CO_2 effect may be due to an increased formation of oxaloacetate through a reaction of CO_2 with phosphoenolpyruvate, which in turn may spare ADP.

The inhibition of high-line respiration (65%) by SLS at 0.0018 M, as opposed to only 10% inhibition in low-line cells, is of particular interest. Inhibition of anaerobic glycolysis in high-line cells by SLS suggests that certain enzyme systems, especially hexokinase, may be sensitive to the inhibitor. This is demonstrated by the action of SLS on hexokinase and aldolase and by the fact that concentrations ranging between 0.85 and 5.1 mM SLS progressively reduced phosphate uptake and glucose degradation in intact cells (8). Therefore, it may be inferred that the sum total of oxidative processes in high-line cells may be more dependent upon the direct breakdown of

Table 2

Respiration of high-line cells (NCTC 2472) in 2-day-old conditioned medium with and without inhibitors

No. of cells in diver	Inhibitor	Total ΔVO_2/hr × 10^5 µl	ΔVO_2/cell/hr × 10^5 µl	ΔVO_2/cell/hr (mean ± S.E.)
300–900 (10 divers)	None	1084.3, 308.5	1.14, 0.93	
		660.0, 310.0	1.14, 0.97	
		323.0, 465.0	1.01, 0.93	1.05 ± 0.05
		260.0, 510.5	1.01, 0.92	
		1223.6, 705.3	1.12, 0.92	
	Controls, 6.65, 6.50, and 6.65 × 10^{-4} ΔV/hr			
590–1349 (5 divers)	0.0018 M SLS	603.9	0.44	
		600.8	0.36	
		348.1	0.43	0.38 ± 0.03
		386.0	0.44	
		303.0	0.22	
625–760 (4 divers)	0.0054 M SLS	196.5	0.13	
		188.7	0.14	0.14 ± 0.01
		203.9	0.15	
		179.0	0.13	
	Controls, 9.77 and 11.50 × 10^{-4} ΔV/hr			
366–765 (5 divers)	10^{-4} M 3-NPA	296.0	0.43	
		183.0	0.33	
		236.0	0.35	0.37 ± 0.03
		180.0	0.28	
		425.0	0.47	
	Controls, 6.65 and 6.50 × 10^{-4} ΔV/hr			
507–655 (3 divers)	10^{-3} M succinate	588.0	1.00	
		791.0	1.10	1.08 ± 0.02
		654.0	1.15	
646–1780 (5 divers)	10^{-3} M urethan	418.0	0.19	
		217.5	0.15	
		331.0	0.19	0.22 ± 0.02
		219.0	0.24	
		340.0	0.31	
	Controls, 6.65 and 7.00 × 10^{-4} ΔV/hr			

Table 3

Anaerobic glycolysis of high- and low-line cells in fresh medium supplemented with extra glucose and bicarbonate

Gas phase is 5% CO_2 + 95% N_2.

No. of cells in diver	Inhibitor	Total ΔVCO_2/hr × 10^5 μl	ΔVCO_2/cell/hr × 10^5 μl	ΔVCO_2/cell/hr (mean ± S.E.)
		High-line cells (NCTC 2472)		
700–1700 (8 divers)	None	447.0, 288.0	0.46, 0.55	
		592.0, 1000.0	0.50, 0.55	
		400.0, 157.0	0.46, 0.31	0.48 ± 0.03
		270.0, 263.0	0.47, 0.52	
		Controls, -6.64 and -6.50×10^{-4} ΔV/hr		
800–2000 (5 divers)	0.004 M IAA	182.5	0.10	
		95.0	0.13	
		109.0	0.13	0.12 ± 0.01
		209.4	0.13	
		166.4	0.11	
		Control, -6.65×10^{-4} ΔV/hr		
1750–2110 (4 divers)	0.0054 M SLS	610.0	0.30	
		590.0	0.28	0.29 ± 0.01
		589.0	0.30	
		572.0	0.29	
1200–1300 (3 divers)	25 units L-asparaginase	590.0	0.60	
		501.0	0.55	0.57 ± 0.02
		513.0	0.57	
		Controls -6.50 and -7.00×10^{-4} ΔV/hr		
		Low-line cells (NCTC 2555)		
1400–2600 (5 divers)	None	339.0	0.13	
		412.0	0.22	
		642.0	0.21	0.17 ± 0.03
		250.0	0.10	
		329.0	0.16	
1000–2600 (4 divers)	0.0054 M SLS	413.6	0.13	
		186.0	0.18	0.16 ± 0.03
		329.0	0.16	
		340.0	0.18	
		Controls, -8.93, -6.90, and 7.50×10^{-4} ΔV/hr		

glucose as an energy source, as opposed to a more indirect requirement for low-line cells. In this respect, components of the citric acid cycle may be more dependent upon metabolites formed from the Embden-Meyerhof pathway than upon those from other sources, such as fatty acids. Both cell lines are sensitive to higher concentrations of SLS, as demonstrated by the inhibition induced by SLS at 0.0054 M, and perhaps inhibition of systems other than the Embden-Meyerhof pathway may be involved. Since it is possible that SLS may bind metal ions (21), it may be that respiratory inhibition at higher concentrations of SLS is due to the action on metal ions involved with reactions other than hexokinase or enzymes of the Embden-Meyerhof pathway.

Respiration of both high and low lines is inhibited by urethan to approximately the same extent (80 to 83%). The addition of 10^{-3} M succinate to low-line cells prevented the same degree of inhibition by urethan. However, the degree of protection is slight (9%). These results suggest that both cell lines have functional urethan-sensitive systems. The specificity of the action of urethan may be inferred from the fact that the addition of succinate did not appreciably prevent respiratory inhibition by urethan. In view of these results and of studies by Keilin and Hartree (15–17), one may infer that the urethan-sensitive system probably involves inhibition of hydrogen transfer from succinate to the cytochrome system.

Both cell lines are almost equally sensitive to 3-NPA (61 to 64% inhibition). In view of the fact that 3-NPA is known specifically to inhibit succinic dehydrogenase (28), one may infer that both cell lines have equally functional citric acid cycles, insofar as succinate is concerned.

Carbon dioxide evolution from the high-line cells seems to be due largely to glycolysis, since 75% of the gas evolution is blocked by IAA. IAA is known to inhibit the glycolytic enzyme, 3-phosphoglyceraldehyde dehydrogenase, and the conversion of glucose-^{14}C to $^{14}CO_2$ (22). Therefore, one may assume that anaerobic glycolysis is being determined by the method used here.

Anaerobic glycolysis of high-line cells is much greater than that of the low-line cells, in which activity is approximately 35% that of the high line. Glycolysis of high-line cells is significantly inhibited by SLS (40%); however, low-line cells are not as sensitive to the inhibitor (5%). This suggests that the high-line cells are more dependent upon glucose as an energy source.

Incubation of high-line cells with 25 units of *E. coli* L-asparaginase for 24 hr produced no glycolytic inhibition. Furthermore, no morphological damage could be determined in either high- or low-line cells incubated with the enzyme. According to studies by Broome (4–6) and Broome and Schwartz (7), cells resistant to L-asparaginase are able to supplement asparagine from the medium by systhesis from other components. This may be the case with high- and low-line cells.

In conclusion, respiration and glycolysis measurements were conducted on cell microsamples of high and low tumor-producing lines, with the use of the Cartesian diver, which permits studies at conditions nearest the *in vivo* environment. Thus, it is possible that both cell lines may have equally functional citric acid cycles and cytochrome systems. The main difference appears to be the more direct dependence of high-line cells for glucose as an energy source in both glycolytic and respiratory processes.

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