METABOLIC REACTIONS OF CELLS IN TISSUE CULTURE

I. POLAROGRAPHIC MEASUREMENTS OF ENDOGENOUS RESPIRATION OF CELLS CULTURED *IN VITRO*

LESTER PACKER*, MARCO J. PAPA, JAMES H. RUST, JR. AND SAMUEL J. AJL

Departments of Biochemistry and Bacteriology, Walter Reed Army Institute of Research, Washington, D.C. (U.S.A.)

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SUMMARY

A rapid and simple polarographic method has been found suitable for measurement of the endogenous O_2 consumption in suspensions of liver cells grown in tissue culture. Because of the great sensitivity of this method, it was possible to detect a rate of respiration of 10 mμmoles of O_2 utilized/10 sec.

It was found that the effect of several uncoupling agents and inhibitors on liver tissue culture cells was similar to that reported in studies on liver mitochondria. These findings were best exemplified by the effect of 2,4-dinitrophenol ($1 \cdot 10^{-4}$ *M*), which increased respiration by a factor of six, and antimycin A (0.05 μg/ml), which inhibited respiration of a 10% cell suspension.

INTRODUCTION

The slow growth rate of cells in tissue culture has made it difficult to measure the rate of respiration of cell suspensions with the conventional manometric techniques. In order to circumvent this difficulty, the more sensitive polarographic method of DAVIES AND BRINK[1] was utilized for the measurement of rates of oxygen consumption, and to evaluate the effect of a series of metabolic inhibitors on the endogenous respiration of cells cultured *in vitro*. A similar investigation was made by SIEGEL AND CAILLEAU[2] using specially constructed 2.5-ml Warburg vessels and measuring oxygen consumption over a period of 5 h. The polarographic method appears more amenable to studies of this nature in that it is considerably more sensitive than other methods and the time required for an expt. seldom exceeds several minutes.

It has been found that washed suspensions of mouse-liver cells grown in tissue culture closely resemble liver mitochondria in their behavior toward a variety of compounds known to effect respiration. The results of these expts. are described in this report.

* Present address: Johnson Foundation for Medical Physics, University of Pennsylvania, Philadelphia, Pa.

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MATERIALS AND METHODS

Earle's strain L cells 929 were grown on the glass surface of liter Blake bottles in Eagle's medium containing penicillin, streptomycin, and 5 % horse serum[3]. The culture medium was changed 3 times with the last feeding on the fifth day, 24 h prior to harvest. The cells were collected by decanting the nutrient fluid, scraping them from the glass surface, and suspending in a small amount of buffer containing NaCl (0.10 *M*), KCl (0.005 *M*), and phosphate (0.02 *M* at pH 7.5). The suspension was centrifuged and 1 g of sediment was washed once with approx. 15 ml of buffer and finally resuspended in a total volume of 2 ml.

Expts. were carried out at 26°. in a 1.0-ml reaction mixture containing 0.20 ml of the cell suspension plus 0.80 ml of the aerated buffer to which 0.25 % methylcellulose was added. The latter was used to prevent sedimentation of the cells during the 5-min period required for expts.

Respiration was measured polarographically with the O_2 electrode. A platinum-silver electrode couple polarized at 0.6 V was employed as recommended by DAVIES AND BRINK[1]. The current from the electrodes was recorded with a General Electric Model CE-5 photoelectric potentiometer. Expts. were carried out by inserting the electrodes in a test tube containing the reaction mixture. The test tube was turned at 60 rev./min by a synchronous motor.

RESULTS

The stimulation of respiration by phosphate acceptors and uncoupling agents is well known in mammalian-liver mitochondria[4,5]. It was therefore decided to investigate the effect of these substances in intact liver cells in tissue culture. The effect of 2,4-dinitrophenol (DNP) on the endogenous respiration of a 10 % suspension of mouse-liver L cells is illustrated in Fig. 1. The start of the expt. traces the slow rate of endogenous respiration which is seen to be linear over the 45-sec time interval shown.

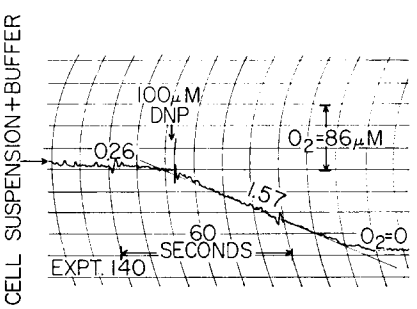

Fig. 1. Effect of 2-4-dinitrophenol on the respiration of Earle's strain L cells. Time moves from left to right and a downward deflection of the trace indicates O_2 consumption. The numbers above the recording refer to the calculated rates of respiration in μmoles O_2 consumed/sec/l which are obtained from the slope of line during each interval. All concns. are given in μmoles/l.

At this point, 10 μl of a 0.01 *M* solution of DNP was added from a micropipette to the tip of a small stirring rod. The rod was introduced into the reaction mixture with rapid stirring for approx. 1 sec. A six-fold increase in the rate of respiration was observed. This fast respiration was maintained at a linear rate for 66 sec after which

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the trace leveled off indicating exhaustion of dissolved O_2. The artifact in the trace just after the addition of the DNP was caused by touching the electrodes with the tip of the stirring rod.

The stimulation of respiration with DNP was further investigated as a function of the concn. of the uncoupler versus the density of the cell suspension. Half-maximal respiratory stimulation was obtained with 9 μM DNP using a 10% cell suspension, while maximum stimulation of respiration (6-fold) was obtained with 100 μM DNP. Using 100 μM DNP, it was demonstrated that higher cell concns. yielded a decreased respiratory stimulation while lower cell concns. produced an increased respiratory stimulation, that is to say, the cell concn. is inversely proportional to the degree of stimulation. The addition of 100 μM DNP to a 10% cell suspension was used as the control for the succeeding expts.

The effect of some other uncoupling agents and phosphate acceptors such as adenosine diphosphate (ADP) and inorganic phosphate were examined for their effect on tissue culture respiration (Table I). The data shows that only DNP, $CdCl_2$ and *p*-nitrophenol were able to stimulate the respiration of these cells as shown by increased ratios in column B/A in Table I. It is of interest that structurally similar compounds such as dibromophenols and *o*-hydroxybenzoate (salicylate) were without effect, although a later addition of DNP as a control in each instance stimulated respiration as seen from the elevated C/A ratios.

TABLE I

RESPIRATION OF EARLE STRAIN L CELLS

(A) Endogenous respiration* μ*mole* O_2*/sec*	*Compound added*	*Concentration mM*	*(B)* Respiration with added compound* μ*mole* O_2*/sec*	*B/A*	*(C)* Respiration with added compound and DNP (0.10mM)*	*C/A*
0.26	2,4-Dinitrophenol	0.10	0.99	3.8	—	—
0.50	2,4-Dibromophenol	0.10	0.35	0.7	0.82	1.6
0.67	2,6-Dibromophenol	0.25	0.53	0.8	0.85	1.3
0.50	Phenol	2.50	0.41	0.8	—	—
0.27	*O*-Nitrophenol	2.50	0.27	1.0	0.55	2.0
0.45	*p*-Nitrophenol	1.00	0.86	1.9	—	—
0.50	2-Hydroxy benzoate	2.50	0.61	1.2	1.08	2.2
0.70	Thyroxine	0.25	0.50	0.7	1.17	1.7
0.32	Gramicidin	0.25	0.35	1.1	0.85	2.7
0.53	Cadmium chloride	0.25	0.99	1.9	0.82	1.6
0.73	Adenosine diphosphate	0.25	0.53	0.7	1.23	1.7
0.31**	Inorganic phosphate	25.00	0.31	1.0	1.40	4.5
0.38	Antimycin A	0.05 μg/ml	0.12	0.3	0.06	0.1
0.31	Sodium azide	0.10	0.00	0.0	0.00	0.0
0.25	Sodium malonate	50.00	0.15	0.6	0.17	0.7

* (A) refers to the rate of endogenous respiration; (B) refers to the rate of respiration in the presence of the added compounds and (C) refers to the rate of respiration in the presence of the added compounds plus 2,4-dinitrophenol (0.10 mM).

** This expt. was carried out with a 10% cell suspension washed in medium containing Tris (hydroxymethyl) aminomethane (0.02 M at pH 7.5) instead of phosphate.

The action of some metabolic inhibitors effective in liver mitochondria was also examined (Table I). It can be seen that low concns. of antimycin A and sodium azide

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strongly inhibited both endogenous respiration and the respiration of the DNP controls. Malonate only slightly inhibited even at a high concn. The range of antimycin A inhibition was documented in another series of expts. and it was found that approx. 0.05 μg/ml were required for 50 % inhibition when respiration was measured immediately after the addition of the inhibitor.

DISCUSSION

The increased sensitivity of respiration measurements of tissue culture cell suspensions by means of a rotating O_2 electrode has made it possible to carefully examine the action of a number of respiratory inhibitors. For example, 9 μM DNP was required to show half-maximal acceleration of respiration, and thus presumably an uncoupling of oxidative phosphorylation[4,5]. The effect of DNP on respiration is observed within seconds after its addition and the ensuing accelerated respiration proceeded at a linear rate. The maximum stimulation of respiration (6-fold) was obtained with 100 μM DNP. These findings are in general agreement with those of SIEGEL AND CAILLEAU[2] who reported a 2.5-fold stimulation with this amount of DNP using the same strain of cells. These workers measured respiration over a 5-h period in specially constructed 2.5 ml Warburg flasks. The explanation of the poorer DNP stimulation obtained by these investigators is probably due to the large number of cells necessarily employed because the findings in this laboratory show that DNP stimulation is inversely related to cell concn. In summary, the effect of DNP on intact cells in tissue culture appears to be similar to that of liver mitochondria.

The inhibition of respiration by azide, antimycin A[5] and malonate[6] observed with cells grown in tissue culture is comparable with the results obtained with mitochondria preparations. Of particular interest is the antimycin A inhibition in less than μg amounts, which indicates that the function of the cytochrome a_3 system has been retained by tissue culture cells.

The lack of measurable effect of ADP, inorganic phosphate, 2,4-dibromophenol, 2,6-dibromophenol, phenol, *o*-nitrophenol and thyroxine remains unexplained. The inability of the uncouplers to penetrate the cells occurs as a likely reason for the lack of effect. Also ADP and phosphate remaining in the cells may mask the effect of the added material. Similar findings have been observed with microbial cells and mouse ascites tumor cells[7].

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