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USE OF A PERFUSION TECHNIQUE FOR MEASUREMENTS OF RESPIRATORY ACTIVITY IN CULTURED CELLS

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SUMMARY

A method for measuring respiratory activity in anchorage-dependent cultured cells has been developed. This method is based on a technique that permits the perfusion of standard plastic culture dishes with attached cells. Basal respiratory activities were studied in two continuous cell lines of neural origin, neuroblastoma C1300 clone $41A_3$ and glioma 138MG. As compared to traditional measurements on detached cells, a fourfold increase in value was obtained. Investigations on membrane permeability suggested that the observed difference could be attributed to alterations in cell membrane integrity. Pretreatment with dibutyryl cyclic AMP, known to induce a morphological and biochemical differentiation in C1300 and 138MG cells, caused in both cell lines an enhanced respiration.

Key words: neural cell lines; respiratory activity; perfusion; polarographic measurements; dibutyryl cyclic AMP.

INTRODUCTION

Measurements of respiratory activity in cultured cells have so far mostly been restricted to methods in which collection of cells in suspension have been a prerequisite. Polarographic (9,14) or micromanometric (8) methods have been utilized. Measurements are preceded by protease treatment of cultures (9), scraping off the cell layer (14) or careful removal of single cells (8). It has been proposed that these methods may give rise to serious impairments of cellular integrity and thereby alterations in respiratory capacity (7). A possible way to measure respiratory activity in intact cells would be to utilize a perfusion technique. This idea has been applied in studies in which a Capillary Culture Unit (CCU) (4) or a culturing vessel related to the Rose-chamber have been utilized (15). The most satisfactory situation, however, would be if measurements could be carried out in an ordinary culture vessel, flask, or dish. One such method employing polarographic recordings in plastic culture flasks has been developed by Hertz and Hertz (7). In this paper we describe a method based on a technique that permits perfusion of cells seeded and grown in ordinary plastic cell culture dishes (11).

MATERIALS AND METHODS

Cell cultures. Stock cultures of neuroblastoma C1300 cells clone $41A^3$ (1) and glioma 138MG cells (12) were grown in Costar plastic flasks in Ham's F10 medium, containing 9% newborn bovine serum, 4% fetal bovine serum, and antibiotics (100 U penicillin and 50 μg streptomycin/ml). Cultures were kept at 37° C in a humidified atmosphere of 4.5% CO_2 in air. Medium was changed twice a week and subcultures were made using 0.25% trypsin in a phosphate buffered salt solution. For experiments cells were seeded into plastic culture dishes (Falcon 3002) at a density of 0.25×10^5 cells/cm^2. Five days later 1.0 m*M* of dibutyryl cyclic AMP (Bt_2-cAMP) was added and cultures incubated for another 6 d.

Instrumentation for respiratory measurements. A special holder for the perfusion chamber (11) and oxygen electrodes was constructed (Fig. 1). This holder was made of polycarbonate plastic (Macrolon). Channels and outlets for electrodes were drilled into the holder. Medium stream can be directed either through the perfusion chamber or through a calibration loop by the aid of built-in valves. Thanks to the transparency of the polycarbonate plastic, air bubbles trapped in the channels can easily be detected during the experiment. The holder arrangement permits a simple introduction of the assembled perfusion chamber into the measuring set-up. The two polarographic oxygen electrodes (YSI 4004 Yellow Springs Instrument Co., Yellow Springs, OH) were operated by two amplifiers (Medelco, Stockholm, Sweden), providing a constant polarization, and a two-channel chart-recorder (Cole-Parmar, Chicago, IL). Electrodes were calibrated with air-saturated water, and zero readings were obtained after reduction with sodium dithionite. Calibration was carried out in a separate stirring chamber. This chamber, constructed as described elsewhere (5), was also used for measuring respiratory activities in scraped off cells.

Inasmuch as polarographic electrodes consume oxygen during measurements, an O_2-gradient is created in the

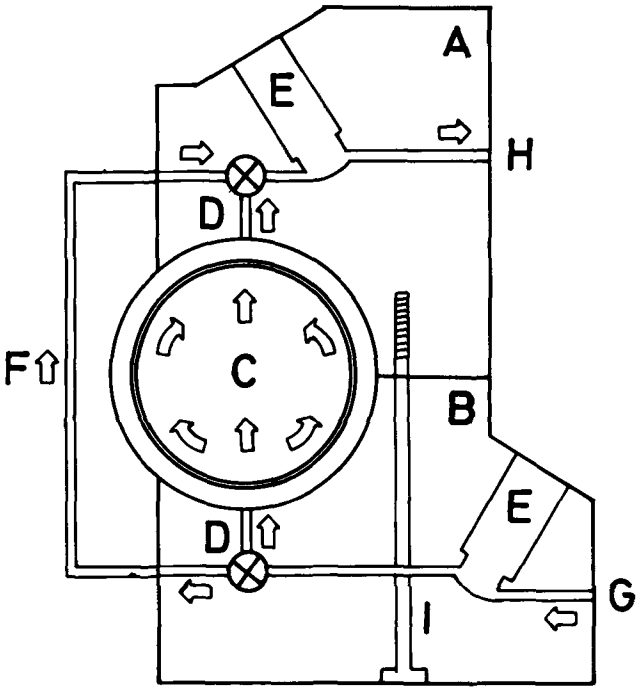

FIG. 1. Holder for electrodes and perfusion chamber. Top part (*A*) and bottom part (*B*) of the holder as well as the perfusion chamber (*C*) are put together by the aid of a threaded pin (*I*). Medium flow can be directed either through the chamber (*C*) or through the calibration loop (*F*) by the built-in valves (*D*). (*E*) represents the electrode outlets, (*G*) is the medium inflow, and (*H*) the outflow.

surrounding medium. To establish a fast equilibrium between electrode and medium it is desirable to keep this gradient as small as possible. In the holder this was achieved by minimizing the medium volume surrounding the electrode. A fast flow of medium resulting in turbulent agitation at the electrode was thereby created. A high-precision peristaltic pump (Ismatec IP-4, Zurich, Switzerland) was used to ensure a stable flow rate through the system. Oxygenation of the perfusion medium was acquired by utilizing silicone tubings (Silastic, Dow Corning, Midland, MI) permeable for oxygen. Holder, tubings, electrodes, and perfusion block were kept at 37° C before experiments. A careful thermoequilibration prevented a subsequent appearance of air bubbles in the medium stream. During an experiment, the holder with electrodes and perfusion chamber tubings and medium reservoir were all immersed into a water bath (Grant SP, Cambridge, UK) to provide temperature stability. The water bath was bubbled with air to ensure an oxygen saturation of the water surrounding the tubings and holder.

Measurments of respiratory activity. In the *perfusion method* all measurements were carried out in a phosphate buffered salt solution (PBSS) containing 150 m*M* NaCl, 3.0 m*M* HCl, 1.0 m*M* $CaCl_2$, 0.6 m*M* $MgCl_2$, 1.6 m*M* KH_2PO_4, 4.3 m*M* Na_2HPO_4, and 7.0 m*M* glucose (300 mOSM, pH 7.3) as perfusion medium. Two culture dishes (Falcon 3002, 21 cm^2) were rinsed three times with 5 ml 37° C PBSS and then mounted on a double perfusion block (11), thus forming a chamber with a total volume of 1.7 ml. The double perfusion block and holder (Fig. 1) with electrodes were assembled and together with tubings and medium reservoirs placed in a water bath. The system was perfused with incubation medium at a rate of 0.5 ml/min. Initially, medium flow was directed through the calibration loop and resulting electrode readings were defined as 100% oxygen content. The medium was then directed through the chamber. The resulting reading gave an initial peak followed by a stable level (Fig. 2). The difference between inlet (Fig. 2 *A*) and outlet (Fig. 2 *B*) electrode readings, represents the oxygen depletion of the perfusion medium as a result of cellular respiratory activity. After a constant reading for about 15 min, medium flow was directed back through the calibration loop.

When measurements were completed the holder was disassembled and the two dishes with attached cells removed from the perfusion block and inspected for morphological integrity in an inverted microscope. Cultures were then trypsinized and the number of cells determined in a Coulter Counter Model B.

For measurements in the *stirring chamber* two culture dishes were rinsed three times with air-saturated 37° C PBSS, and the cell layer scraped off with the aid of a rubber policeman, in a final volume of 1.0 ml. The resulting cell suspension was transferred to the stirring chamber and the decline in oxygen content was recorded by a polarographic oxygen electrode (*see* above). After an initial stabilizing period, a constant decline in readings was obtained from which the respiratory activity was calculated. Cell number was determined from two parallel culture dishes. From scraped dishes additional cells (10%) could be recovered by trypsinization. Corrections of the cell content of the stirring chamber were made accordingly.

Calculation of respiratory activity. Oxygen consumption (*R*) in the perfusion polarographic method was calculated according to the following simplified equation: $R = t \times V_p$ (a − b) × 5.2/ *a* where *t* represents the time, *Vp* the perfusion rate (0.5 ml/min), *a*

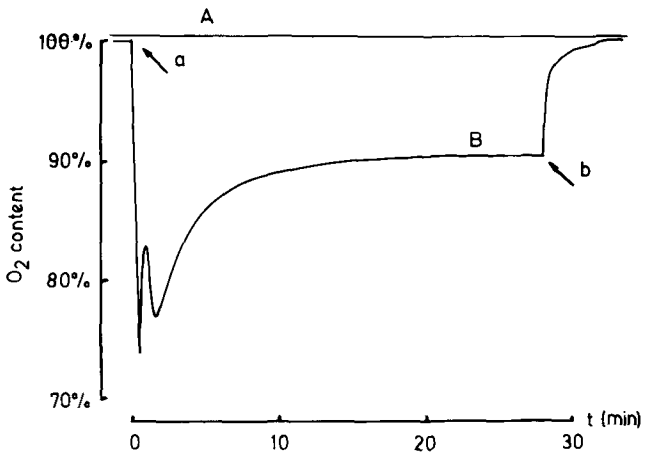

FIG. 2. Polarographic tracing of respiratory activity. *A*, inlet electrode reading; *B*, outlet electrode reading. *Arrows* indicate shifts of medium stream through the chamber (*a*) and through the calibration loop (*b*).

is the inlet and *b* the outlet readings and 5.2 the oxygen content of 1 ml of PBSS at an atmospheric pressure of 760 mmHg (no attempts were made to introduce a correction for day-to-day variations in atmospheric pressure). The obtained *R* value was divided by total cell number and results expressed as $\mu l\ O_2\ h^{-}\ cell^{-}$. Oxygen consumption in the stirring chamber was calculated as described elsewhere (5).

Membrane permeability. Neuroblastoma $41A_3$ cells were incubated at 37° C for 2 h in 4 ml/dish of D-glucose-free PBSS containing 0.5 μCi [^{3}H-2]deoxy-D-glucose (^{3}H-dGlc)/ml (17).

The incubation was terminated by removal of the incubation solution and subsequent washing of the cultures with three 5 ml portions of ice-cold PBSS. The cultures were then either perfused as described above or detached by means of a rubber policeman suspended in ice-cold PBSS, collected on a 0.45 μm Millipore membrane filter (HAWP 02500) in a Millipore Swinnex-25 filter holder, and then perfused. In all experiments the preloaded cultures were perfused at 37° C for 1 h with PBSS containing D-glucose (1.0 mg/ml) at a rate of 1 ml/min. Fractions of the perfusate were collected in scintillation vials every 12 min. At the end of the perfusion, cells were dissolved in 1 ml of 1.0 *M* NaOH and radioactivities determined in Unisolve I scintillation cocktail in a Packard Tri-Carb 2950 liquid scintillation counter. The counting efficiency was 20 to 25%.

Chemicals. Dibutyryl cAMP was obtained from Sigma, St. Louis, MO. An aqueous solution of 2-deoxy-D-1-[^{3}H]glucose (23 μCi/mmol) was purchased from Radiochemical centre, Amersham, UK. Unisolve I was obtained from Koch-Light Laboratories, Colnbrook, UK. Ham's F10 medium, fetal bovine serum, newborn bovine serum, antibiotics, and trypsin were purchased from Flow Laboratories, Irvine, UK.

Results and Discussion

The method described above for measurements of respiratory activity in cell cultures possesses some major advantages: (a) cells remain attached to their substratum during the experiment (*see* below); (b) measurements are carried out at a constant oxygen tension, i. e. cells are not depleting the medium to such an extent that they are forced to change metabolic pathways; and (c) drug effects can be studied during a building up of the desired concentration, to some extent imitating an in vivo situation (10). This latter quality was not made use of in this work but has been in other investigations where the effects of drugs (18), metabolic inhibitors (6), and environmental pollutants (unpublished) on cellular respiration were studied.

Two methods for the measurement of respiratory activity based on perfusion techniques have previously been presented. These utilize as respirometer the CCU (4) and a Rose-chamber related design (15), respectively. Polarographic systems similar to ours have then been applied to the perfusion systems. Although comparable with respect to the overall measuring principle, these methods have the disadvantage of requiring a specialized maintenance of cultures before measurement. A technique presented by Hertz and Hertz (7) utilizes the culture vessel as respirometer. The decline in oxygen content of the culture media is measured polarographically in a plastic cell-culture flask. However, this method has the disadvantage of working in a closed system (11).

As pointed out by Hertz and Hertz (7), mechanical or proteolytic detachment of cultured cells, often employed in other methods, may cause alterations in the membrane permeability for substrates involved in energy metabolism and accordingly give rise to alterations in respiratory activity. To test the susceptibility of the neuroblastoma cell membrane the efflux of [^{3}H]dGlc and [^{3}H]dGlc-6P (17) was studied after mechanical detachment of the cells. Figure 3 shows that radioactivity was slowly released from unmanipulated cells, whereas mechanical detachment caused a dramatic increase in the efflux of [^{3}H]dGlc and

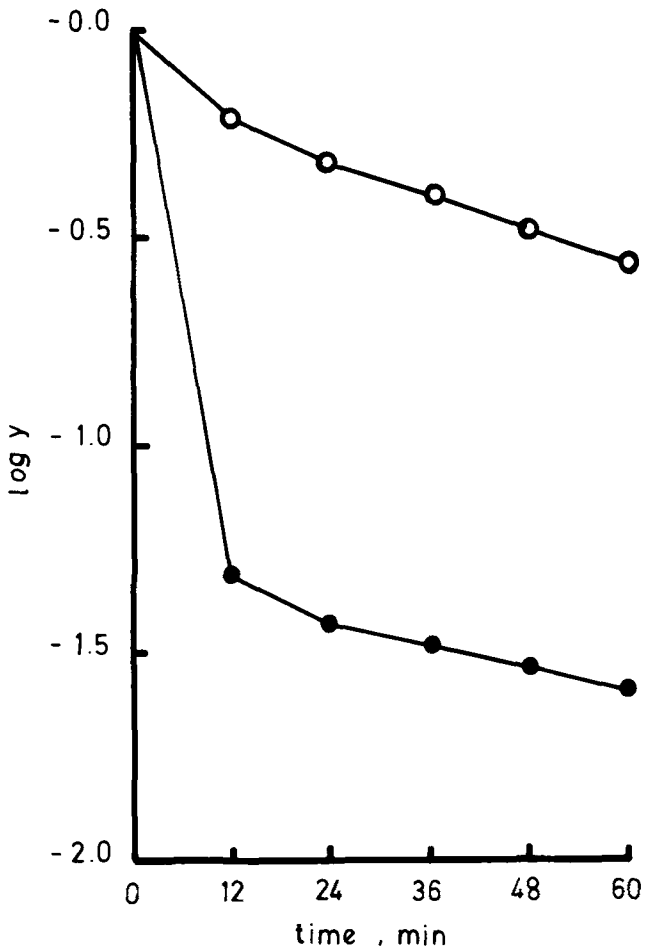

FIG. 3. Efflux of radioactivity from cultured mouse neuroblastoma C1300, clone $41A_3$ cells preloaded with [^{3}H]2-Deoxy-D-glucose. Experimental conditions are given under Materials and Methods. Attached (○○) or mechanically detached (● ●) cultures were perfused at 37° C with PBS containing *D*-glucose (1.0 mg/ml) at a rate of 1 ml/min for 60 min. Twelve-minute fractions of the perfusate were collected. The radioactivity remaining in the cells divided by the amount of radioactivity in the cells at the beginning of the experiment was denoted y and plotted logarithmically vs. time. Each point represents the mean of two independent duplicate experiments.

TABLE 1

GROWTH OF NEURAL CELL LINES[a]

Cell Line	Cell Number ($\times 10^6$ cells/dish)	
	Control	1 m*M* Bt_2-cAMP
$41A_3$	2.33±0.34	1.17±0.13
138MG	2.72±0.18	0.52±0.05

[a]Cell lines were cultured for 5 d. One millimolar dibutyryl cyclic AMP was then added and dishes incubated for another 6 d. Cell numbers are expressed as 10^6 cells/dish and are the mean of 8 to 12 dishes ± SEM.

efflux of [^{3}H]dGlc-6P remained unchanged. Evidently mechanical detachment of at least neuroblastoma cells may cause changes in the membrane permeability for some metabolically important substances. Consequently, the respiratory activities in detached and attached cells were investigated. It was found that scraped-off cells exhibited just one-fourth (24 %± 3) of the respiratory intensity seen for attached cells. These results illustrate the necessity of carrying out measurements of cellular energy metabolism in cells retained on their substratum.

When measuring respiratory activity in plastic cell culture vessels, interference by oxygen leakage into the system can occur (2,7). This oxygen permeability is dependent on the magnitude of the concentration gradient which is built up between the inside and outside of the vessel during an experiment. Investigations of these circumstances show that under the conditions of the present studies, oxygen leakage through the culture dish is negligible (approximately 0.06 μl O_2/h at 40% depletion).

Both neuroblastoma C1300 and glioma 138MG cells cultured in vitro can be induced to undergo morphological differentiation by treatment with certain agents or by alteration of the culture conditions (3,13). Biochemical changes after such treatments indicate a transition toward a differentiated state. For neuroblastoma cells, changes in neurotransmitter metabolism is characteristic (13). In glioma 138MG cells, elevated levels of S100 protein have been observed (16). A characteristic feature of the cellular constituents of the developing mammalian brain is the transition from an anaerobic to an aerobic metabolism, i. e. increasing respiratory activities and a concomitant decrease in lactate formation. With this in mind, we studied the respiratory activity of Bt_2-cAMP differentiated morphologically intact cells (13). Treatment of the neuroblastoma and glioma cells for 6 d with 1 m*M* Bt_2-cAMP resulted in a morphological differentiation as well as a reduction in final cell number as compared to controls (Table 1). These observations are in accordance with those found by others (3,13). As can be seen from Table 2, both neuroblastoma and glioma cells increase their respiratory activity when cultured in the presence of Bt_2-cAMP. The twofold increase found in neuroblastoma cells is in agreement with results obtained for cells induced to undergo morphological differentiation by serum deprivation (8). The threefold increase found in glioma 138MG cells corresponds to the differences seen between young and old cultures, i. e. rapidly dividing and growth inhibited, respectively (16). The basal respiratory activities found for both cell lines are in agreement with the concept of a relatively low respiratory capacity of undifferentiated proliferating cells (13,16). Thus it can be noted that the morphological differentiation caused by Bt_2-cAMP, in addition to the previously found biochemical alterations, gives rise to increased respiratory activities of cultured neuroblastoma and glioma cells. Furthermore the absolute values obtained for respiratory activity coincide with those previously reported. However, it should be pointed out that due to differences in culture conditions and history of the cellular material the validity of such comparisons can be questioned. The registered changes of respiratory activities after Bt_2-cAMP treatment of neuroblastoma and glioma cells constitute one piece of evidence for the usefulness of the method presented in evaluating metabolic activities in anchorage dependent cells cultured in vitro.

TABLE 2

RESPIRATORY ACTIVITIES OF NEUROBLASTOMA $41A^3$ AND GLIOMA 138MG, CULTURED IN PRESENCE AND ABSENCE OF Bt_2-cAMP

Cells	Respiratory Activity[a] (10^{-5} μl O_2 × $cell^{-1}$ × h^{-1})		
	Basal		1 m*M* Bt_2-cAMP, 6 d
Neuroblastoma C1300 clone $41A^3$	0.40 ± 0.06	$P<0.05$[b]	0.78 ± 0.10
Glioma 138MG	0.18 ± 0.02	$P<0.05$[b]	0.63 ± 0.12

[a]Each activity shown is the mean of four to seven experiments ± SEM.

[b]*P*-values are for Student's *t*-test, comparing activities for basal and Bt_2-cAMP treated cultures.

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