Short Communications

SC 2227

Polarographische Messung der O_2-Spannung in Ehrlich-Ascites-Tumoren der Maus

Die Fähigkeit, ohne molekularen Sauerstoff zu überleben, ist wahrscheinlich das wesentlichste Kennzeichen der Tumorzelle, das durch die biochemische Krebsforschung aufgezeigt werden konnte. Während die Anaerobiose der Krebszelle *in vitro* durch zahlreiche Messungen belegt ist, gibt es für eine Anaerobiose *in vivo* bisher nur indirekte Hinweise. Diese wurden erst kürzlich durch WARBURG[1] eingehend abgehandelt.

Die jüngste Entwicklung der polarographischen Messtechnik ermöglicht eine direkte Messung der O_2-Spannung in Ascites-Tumoren. Wir benutzten die Versuchsanordnung von LÜBBERS *et al.*[2], die nach dem Clark-Prinzip mit einer Teflonüberzogenen Pt-Elektrode arbeitet. Die Messungen wurden mit drei verschiedenen Elektroden bei einer Spannung von 0.6 V durchgeführt. Die Eichung erfolgte mit hochgereinigtem, wasserdampf-gesättigtem N_2 und verschiedenen N_2–O_2-Gemischen. 0.02 Torr* O_2 waren mit unserer Versuchsanordnung noch gut nachweisbar. Kleinere Konzentrationen konnten nur geschätzt werden.

Die Ascitestumoren kamen 13–19 Tage nach der Überimpfung zur Messung. Die Punktion der Tumorflüssigkeit erfolgte mit einer 2-ml Injektionsspritze, deren Metallkolben besonders exakt eingeschliffen war. Die Spritze befand sich dabei in einer Hülse aus Plexiglas, aus der nur die Spitze der Injektionsnadel und die Führung des Kolbens herausragte. Dieser Mantel stand unter einem Überdruck an hochgereinigtem N_2 oder Methan, so dass Luftsauerstoff nicht in die Spritze gelangen konnte. Vor der Punktion wurde die Spritze selbst mit N_2 gefüllt, um O_2-Reste zu entfernen. Ebenso wurde durch die Glasapparatur, in der sich die Elektrode befand, so lange reinster, wasserdampf-gesättigter N_2 geleitet, bis das Messgerät 0 anzeigte. Das Punktat wurde durch eine Gummimembran in die Messapparatur eingespritzt. Die Möglichkeit einer nennenswerten Durchmischung des Punktates mit N_2 bestand in unserer Apparatur nicht. Punktion und Füllung der Messkammer nahmen etwa 30 Sek in Anspruch. Die Einstellzeit der Elektrode betrug in unserem Messbereich nur wenige Sekunden.

Bei insgesamt 53 Messungen betrug die O_2-Spannung 0.24 Torr ($\delta = \pm$ 0.24). Der kleinste gemessene Wert war 0.0 Torr, der grösste 1.1 Torr. Bei der Bewertung der Ergebnisse ist zu berücksichtigen, dass die gemessenen Werte eine durchschnittliche O_2-Konzentration von peritoneumnahem und -fernem Ascites bedeuten. Ferner dürfte die O_2-Spannung in den Zellen etwas kleiner sein als in der Tumorflüssigkeit.

Aus unseren Messungen kann geschlossen werden, dass die Ascites-Tumorzelle an der Grenze zwischen aerobem und anaerobem Stoffwechsel lebt. Nach Messungen

* 1 Torr = 1 mm Hg.

von BÄNDER UND KIESE[3] folgt die O_2-Aufnahme von Lebermitochondrien im Bereich kleiner O_2-Drucke in grober Näherung der Funktion

$$\frac{V_{max} - V}{V} \cdot P_{O_2}^{1.4} = K$$

Ohne Berücksichtigung des relativ kleinen Konzentrationsgefälles zwischen Ascitesflüssigkeit und Tumorzelle betrüge die Atmung der Ascites-Tumorzelle *in vivo* bei 0.24 Torr O_2 bei 37° etwa 15 %, bei 0.5 Torr 30 % und bei 1.0 Torr 50 % der maximalen Atmung unter Luft. Diese Werte gelten unter der Voraussetzung, dass die vorher angeführte Gleichung auch für Mitochondrien aus Tumorzellen zutrifft. Es gibt noch ein zweites Argument für die Atmung der Ascites-Tumorzelle *in vivo*: Wir injizierten normalen Mäusen 10–20 ml einer 6 %igen sauerstofffreien Peristonlösung intraperitoneal. Bereits nach einer Stunde einthielten die Punktate aus diesen Tieren etwa 5–10 Torr O_2. Wenn aber die O_2-Spannung in Ascites-Tumoren im Mittel 0.24 Torr beträgt, liegt der Schluss nahe, dass die Tumorzellen den eindiffundierenden O_2 verbrauchen.

Wir nehmen an, dass die Ascites-Tumorzelle, soweit sie sich in der Nähe des Peritoneums befindet, noch einen erheblichen Anteil ihrer Maximalatmung besitzt, während in peritoneumfernen Gebieten die Atmung bis auf 0 absinken kann. Die Ascites-Tumorzelle lebt *in vivo* also fakultativ anaerob, wobei das Ausmass der Atmung durch den Diffusionsweg des O_2 und vielleicht auch anderer Substrate limitiert wird.

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Eingegangen am 8. November, 1962

SC 2276

Volume flow in a series-membrane system

CURRAN AND MCINTOSH[1] have studied net volume flow across a barrier composed of two different membranes arranged in series and separated by a closed compartment. Under proper conditions, net volume flow from one side of the composite membrane system to the other against a water activity gradient was observed. The authors suggested that this observation could be explained in terms of the different reflection[2] and hydrostatic permeability coefficients of the membranes and the solute concentration gradients across the membranes. The present experiments were designed to test this explanation by studying the properties of such a system in greater detail.

The experimental apparatus is illustrated in Fig. 1. It consisted of a lucite chamber divided into three compartments by two different kinds of cellulose mem-

brane which were supported on both sides by wire mesh. Membrane 1 between compartments A and B was Visking dialysis tubing and membrane 2 between compartments B and C was Dupont wet gel. Compartment C was equipped with a graduated pipette to measure volume changes and the central compartment was closed by connection to a mercury manometer so that pressure changes could be observed.

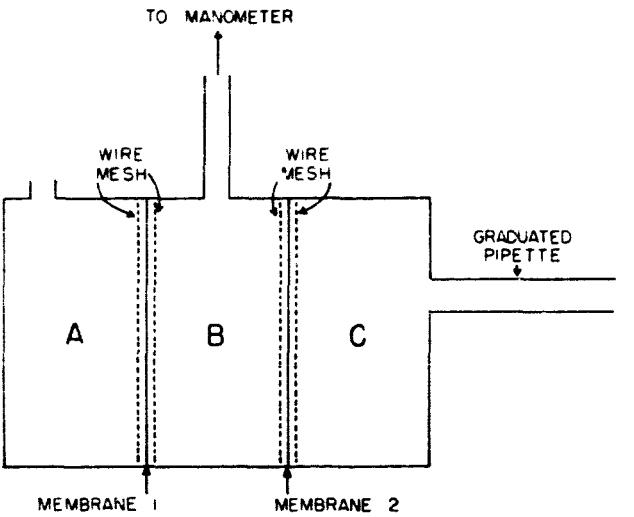

Fig. 1. Apparatus for measuring volume flow and pressure in a three-compartment system.

Compartments B and C were stirred by magnetic stirrers and compartment A by bubbling with air. In some experiments, chamber A was also equipped with a pipette so that volume changes in A and C could be observed simultaneously.

A simple theoretical description of such a three-compartment system can be developed on the basis of the considerations of KEDEM AND KATCHALSKY[2]. The volume flow (J_v) from left to right across each membrane should be given by the following expressions:

$$J_{v1} = L_{p1}(\Delta P_1 - \sigma_1 \Delta\pi_1) \quad (1)$$

$$J_{v2} = L_{p2}(\Delta P_2 - \sigma_2 \Delta\pi_2) \quad (2)$$

in which L_p is the hydrostatic permeability of the membrane and σ is its reflection coefficient. ΔP is the hydrostatic pressure difference and $\Delta\pi$ the osmotic pressure difference across the membrane and the subscripts 1 and 2 refer to the two different membranes. If the system is in a steady state, $J_{v1} = J_{v2} = J_v$ and, since the pressure is the same in both end compartments, $\Delta P_1 = -\Delta P_2 = \Delta P$. Under these conditions, Eqns. 1 and 2 may be solved simultaneously for J_v and ΔP to yield:

$$J_v = \frac{L_{p1}L_{p2}}{L_{p1} + L_{p2}}(\sigma_1\Delta\pi_1 + \sigma_2\Delta\pi_2) \quad (3)$$

$$\Delta P = \frac{L_{p1}\sigma_1\Delta\pi_1 - L_{p2}\sigma_2\Delta\pi_2}{L_{p1} + L_{p2}} \quad (4)$$

The validity of these expressions can be tested if values of L_p and σ for the two membranes are known. These parameters have been measured in the present apparatus using only one membrane at a time placed between compartments B and C. Distilled water was placed on both sides of a membrane ($\Delta\pi = 0$), a pressure was applied and volume flow observed. Under these conditions, $L_p = J_v/\Delta P$. A concentration gradient of sucrose was then applied across the membrane and volume flow again observed with $\Delta P = 0$. From Eqn. 1 or 2, $\sigma = J_v/L_p\Delta\pi$. The values so obtained were $\sigma_1 = 0.075$, $\sigma_2 = 0.019$, $L_{p_1} = 12.6$ μl/min·atm and $L_{p_2} = 60.4$ μl/min·atm.

TABLE I

THEORETICAL AND EXPERIMENTAL VALUES FOR J_v AND ΔP AT VARIOUS SUCROSE CONCENTRATIONS

Initial sucrose concn. in compartment B (M)	J_v (μl/min)		ΔP (mm Hg)	
	Observed	Calculated	Observed	Calculated
0.1	1.1	1.0	30	34
0.2	2.2	2.0	70	69
0.3	3.5	3.2	128	115
0.4	4.2	4.1	156	141
0.5	5.2	5.4	206	190

Using these results, Eqns. 3 and 4 were tested by measuring J_v and ΔP under different conditions and comparing the observed values with those predicted. In these experiments, sucrose solutions of varying concentration were placed in the central compartment and the end compartments were initially filled with distilled water. The system was then observed until a quasi-steady state was reached in which ΔP was constant and the rate of volume decrease in A was equal to the rate of volume increase in B ($J_{v_1} = J_{v_2} = J_v$ = constant). The experiment was then interrupted and the solute concentration in each compartment was determined by measuring the freezing-point depression in order to evaluate the osmotic pressure differences. In all such experiments, there was a net volume flow from compartment A to C and a positive pressure developed in compartment B. The averaged results of 2–5 experiments at each sucrose concentration are summarized in Table I in which observed values of J_v and ΔP are compared with those predicted from Eqns. 3 and 4. The observed and predicted values of both J_v and ΔP agree to within approx. 10 %. Since a conservative estimate of the errors in determining the L_p's and σ's indicates that the calculated values of J_v and ΔP could be in error by at least 7 %, the agreement may be considered satisfactory.

These results indicate that Eqns. 1 and 2, derived on the basis of the thermodynamics of irreversible processes, offer an adequate phenomenological description of volume flow across membranes at several different values of pressure and concentration gradients. They further confirm the explanation suggested by CURRAN AND MCINTOSH[1] for the observation that net volume flow may occur from compartment A to C in the absence of, or against, a water activity gradient. As indicated by Eqns. 3 and 4, such an effect requires that the values of σ be different for the two membranes since Δw_1 and Δw_2 have opposite signs, and that a positive pressure exist in the region between the membranes.

CURRAN AND MCINTOSH[1] suggested that a series-membrane arrangement could play a role in water transfer in biological systems and might explain the link between active solute transport and water movement observed in such tissues as intestine[2] and gall bladder[4]. If an active transport system maintained solute concentration higher in compartment B than in A or C, net water transfer from A to C could occur against a water activity gradient. The theoretical implications of such a system have been developed in detail by PATLAK, GOLDSTEIN AND HOFFMAN[5] using expressions similar to Eqns. 1 and 2 as a starting point. This mechanism of water movement requires barriers of different σ's arranged in series with active solute transport across one barrier into a region which cannot expand indefinitely as fluid enters it. General structural arrangements which might satisfy these requirements can be observed in a number of tissues. For example, ITO[6] has pointed out that the parietal cells in the gastric mucosa of several species have morphological characteristics, observed with the electron microscope, which could fit this model. The present experiments indicate that the theoretical description of volume flow across a series-membrane system is valid and lend additional support to the suggestion that such a system might explain some of the rather puzzling observations concerning water movement across biological membranes.

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Received February 4th, 1963

* Supported in part by a Public Health Service Research Career Program award (AM-K3-5456) from the National Institute of Arthritis and Metabolic Diseases.

SC 2264

Structure of humic acid
IV Electron-paramagnetic-resonance studies*

Recently[1] the existence of stable free radicals in soil humic acid has been established in this laboratory by electron-paramagnetic-resonance measurements. An analysis of the shape and the temperature dependence of the spectrum led us to conclude that two radical species might be present: a semiquinone-type radical and a quinhydrone-type radical. We now wish to present evidence which strongly suggests the existence of quinhydrone and quinone moieties, as well as semiquinone radicals.

* See refs. 1–3 for Papers I–III in this series. The work described in the present paper was presented at the 142nd National Meeting of the American Chemical Society, September 1962.

When sodium metal (5.0 g) was added in small amounts to a suspension of 1.0 g of humic acid (extracted from a podzol B forest soil) in 50 ml of absolute ethanol, an evolution of gas occurred over a period of 1 h. The resulting suspension was filtered and the brown sodium humate dried; the unpaired-spin concentration of the product was 25 times greater than that of the original acid. Treatment of humic acid with $NaOC_2H_5$ under similar conditions resulted in a sodium humate with a radical concentration 15 times greater than the original sample. The sodium salt of humic acid recovered from an aqueous solution also showed a marked increase in unpaired-spin concentration over the free acid. Acidification of the sodium humate led to a product with lower radical content than the original humic acid.

TABLE I

EFFECT OF VARIOUS TREATMENTS OF HUMIC ACID ON UNPAIRED-SPIN CONCENTRATION

*Chemical treatment of humic acid**	*Spins g*** ($\times 10^{18}$)
None	1.4
Sodium salt from ethanol	23.0
Sodium salt from water	10.0
Sodium–alcohol reduction (salt)	35.0
Sodium–alcohol reduction, free acid	0.6

* Extracted from forest podzol-B horizon, Cheshire (Great Britain).
** Spin concentration estimated by comparison with diphenyl picryl hydrazyl. Number of radicals assumed to be proportional to signal height times (signal width)^2.

TABLE II

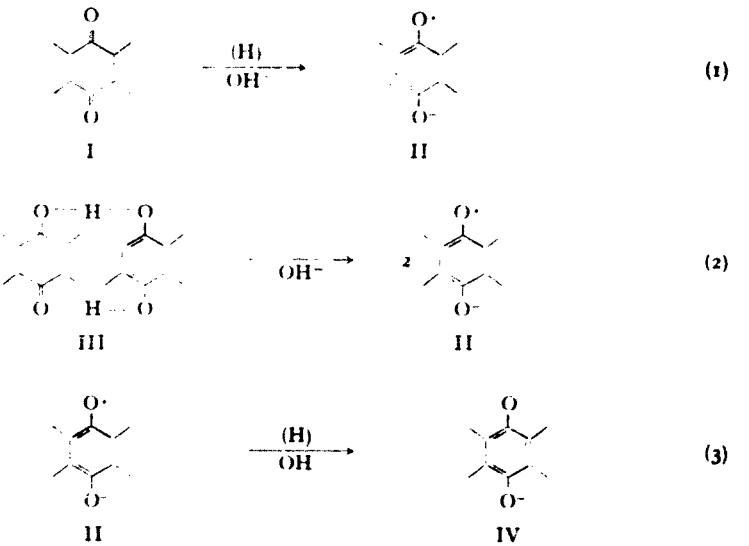
SPIN CONCENTRATIONS AS A FUNCTION OF TIME

Sample	*Time (min)*	*Spins/g* ($\times 10^{18}$)
1	Untreated	1.47
2	10	8.55
3	20	9.21
4	30	34.40
5	45	6.38
6	60	12.50
7	90	12.50
8	120	11.20
9	150	11.25
10	180	7.90
11	210	9.20
12	240	5.90

We can accommodate these results to a humic acid model which contains quinone (I) moieties coexistent with semiquinone (II) and quinhydrone (III) moieties. It is well known[4–6] that reduction of quinones in basic media leads to stable semiquinone-radical ions. Semiquinone-radical ions may also be formed from diamagnetic quinhydrones by simple conversion to the salt[5]. The increase in radical content which we observed after chemical reduction could be due in part to the formation of the quinhydrone salt and in part to the reduction of a quinone function. Additional support for this proposed model comes from the fact that the spectrum for solid sodium *p*-biphenoquinhydrone (*g*, 2.0044; line width, 5 Gauss)[5] is very similar to that for

solid sodium humate. Furthermore, a strong infrared-absorption band at 1550 cm^{-1} in sodium humate corresponds to that reported for the sodium salt of biphenoquinhydrone and other model quinhydrone salts[8].

The chemical transformations of reduction and basification of the proposed model humic acid moieties are illustrated below. Although *p*-benzoquinoid structures are described here, chemical evidence[7] indicates that *o*-benzoquinoid moieties exist in humic acid also.



(1)

(2)

(3)

Although Reactions 1 and 2 produce an increase in semiquinone-radical ions, it is also possible that these ions (or semiquinones existing in humic acid prior to chemical treatment) may be reduced to the diamagnetic phenolate (IV) as in Step (3). Thus, if all three reactions are possible, one would anticipate at least one (and probably more) maximum in spin concentration as a function of time of reduction or amount of reducing agent. Our studies with chemical reduction of humic acid confirms this expectation. In Table II are listed the spin concentrations of humic acid samples, aliquots of which were periodically removed from a heterogeneous reaction mixture containing sodium and alcohol.

The initial rapid increase in radical concentration is most likely a result of Reaction 2, whereas the subsequent variation in spin concentration could be caused by competition between Reactions 1 and 3.

High-resolution studies of aqueous alkaline solutions of humic acid reveal hyperfine structure in the spectra which is not evident in the solid samples. Because of its unsymmetrical shape, the spectrum could represent the superposition of absorptions by two or more radical species. A similar assignment was made for the spectrum of the parent humic acid[1]. It is significant that reduction does not alter the general shape of the electron-paramagnetic resonance spectrum, indicating that no marked change in radical species occurs.

The accumulated evidence is consistent with a humic acid model containing quinone, semiquinone and quinhydrone moieties. Current studies in this laboratory

are concerned with the electron-paramagnetic resonance and infrared analysis of humic acid fractions obtained by controlled oxidation and reduction. The purpose of these studies is to determine the nature and concentration of the quinoid and phenolic moieties.

We wish to acknowledge the support of the American Chemical Society, Petroleum Research Fund, Grant No. 970-A4 and the U.S. Atomic Energy Commission, Grant No. AT(11-1)908 for part of this work.

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Received December 21st, 1962