

RELEASE FROM THE CRABTREE EFFECT BY HYPOXIC CELL RADIOSENSITIZERS

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Summary.—The Crabtree effect can be observed when the O₂ consumption of tumour cells or of mammalian cells grown in culture is measured in physiological medium containing glucose.

The effect of 2 hypoxic cell radiosensitizers, misonidazole and NDPP, on the O₂ consumption of Ehrlich ascites tumour cells was compared in media with and without glucose.

A stimulatory effect on O₂ consumption was found for 5–20mM misonidazole as well as for 0.5mM NDPP, both in media containing 10⁻²M glucose.

Thus glucose induced a Crabtree effect in Ehrlich tumour cells, expressed as 38–45% inhibition of O₂ consumption relative to that in the same medium without glucose. The stimulatory effect of misonidazole and NDPP on O₂ utilization in medium with glucose undoubtedly appeared as a release from the Crabtree effect.

IN A RECENT PAPER we reported that misonidazole (MIS) significantly increases the O₂ consumption and decreases the respiratory-control ratio of guinea-pig liver mitochondria (Mustea *et al.*, 1978). The respiratory-control ratio is a measure of the extent of coupling between respiration and phosphorylation. Chance (1959) has defined it as the ratio of "the respiratory rate in the presence of added ADP to the rate obtained following its (ADP) expenditure". We regarded this behaviour of MIS as rather similar to that of the oxidative-phosphorylation uncouplers.

In this work we present additional new arguments supporting our hypothesis. Starting from the findings that oxidative-phosphorylation uncouplers such as 2,4-dinitrophenol (DNP) and dicumarol release from the Crabtree effect (Loomis & Lipman, 1948; Wenner & Weinhouse, 1955; Chance & Hess, 1956; Racker, 1956; Ram *et al.*, 1963), we attempted to use this test in investigating 2 hypoxic-cell radiosensitizers, MIS and NDPP, reported to have a stimulatory effect on O₂ consumption (Durand *et al.*, 1976; Mustea *et al.*,

1978). We preferred these 2 radiosensitizers mainly because of their good solubility in water.

It is known that the Crabtree effect concerns the *in vitro* inhibition of the O₂ consumption of tumours by addition of glucose (Crabtree, 1929) but some exceptions were also reported. Thus, in some experimental cancers such as the Walker carcinoma 256 and the DBA murine ascitic thymoma, as well as in some human neoplasms, there was no Crabtree effect (Elliott & Baker, 1935; Levy *et al.*, 1953; Kiricuta *et al.*, 1965; Mustea, 1974). On the other hand, the Crabtree effect has been observed in some normal tissues (retina, cartilage) and in a series of facultative anaerobes and yeasts (Locker & Spitzzy, 1956; Cohen, 1957; Noell, 1958; de Deken, 1966; Mustea & Muresian, 1967*a*, 1967*b*).

MATERIALS AND METHODS

All experiments were performed on Ehrlich ascites tumour cells, ELD hyperdiploid strain, propagated by routine inoculation of 4 × 10⁶ cells into NMRI mice. The cells were har-

vested 10–15 days later, when they were in the plateau phase of growth.

The O₂ consumption was measured polarographically using an O₂ Clark electrode housed in a 3.8ml closed reaction vessel and maintained at 37°C. The potential at the O₂ electrode was -0.6 V. The cells were suspended in Tyrode medium free of Ca²⁺ and glucose, Krebs–Ringer phosphate medium, and in the same media with 10⁻²M glucose added.

Misonidazole (MIS; Ro-07-0582, 1-(2-nitro-1-imidazolyl)-3-methoxy-2-propanol) was a gift from Roche Products Ltd, England, and NDPP (β N,N-dimethyl-p-nitropropionophenone HCl) was a gift from Dr L. Revesz of the Karolinska Institute, Stockholm. DNP (2,4-dinitrophenol) was provided by Fluka A.G., Switzerland. The compounds were dissolved in saline and added to the reaction media to obtain the concentrations reported to have a stimulatory effect on the O₂ consumption of the cells (Ram *et al.*, 1963; Durand *et al.*, 1976; Mustea *et al.*, 1978).

The effect of the above-mentioned chemicals on the O₂ consumption of Ehrlich ascites tumour cells was expressed as a relative O₂ utilization ratio (OUR) obtained by dividing the rate of O₂ consumption after addition of the chemical by the initial rate measured in its absence. When the effect of glucose on the rate of O₂ consumption was determined, OUR was calculated as:

$$\frac{\text{O}_2 \text{ consumption rate with glucose added}}{\text{Endogenous O}_2 \text{ consumption rate}}$$

Release from the Crabtree effect (RCE) was calculated according to the formula:

$$\text{RCE \%} = \frac{\text{OUR}_{\text{gd}} - \text{OUR}_{\text{g}}}{1 - \text{OUR}_{\text{g}}} \times 100$$

$$\text{OUR}_{\text{gd}} = \frac{\text{O}_2 \text{ consumption rate with drug and glucose added}}{\text{Endogenous O}_2 \text{ consumption rate}}$$

$$\text{OUR}_{\text{g}} = \frac{\text{O}_2 \text{ consumption rate with glucose}}{\text{Endogenous O}_2 \text{ consumption rate}}$$

A graphic demonstration of the application of this formula is shown in Fig. 2.

RESULTS

In glucose-free media, MIS and NDPP produced an inhibition of the rate of O₂ consumption of Ehrlich ascites tumour cells (OUR < 1, Table I).

Glucose in a concentration of 10⁻²M induced a Crabtree effect measured as a pronounced decrease of the rate of O₂ consumption. The OURs were 0.55 in Tyrode medium (inhibition 45%) and 0.62 in Krebs–Ringer phosphate medium (inhibition 38%) respectively (Figs. 1 and 2).

The inhibition of O₂ consumption by glucose was partially reversed by both radiosensitizers, and also by DNP. In all cases the OURs were > 1 (Fig. 1, 2; Table I). If the rate of O₂ consumption

TABLE I.—The effect of misonidazole (MIS), NDPP and DNP on relative oxygen utilization ratio (OUR) of Ehrlich ascites tumour cells (5 × 10⁶/ml)

Compound	mM	OUR			
		Tyrode medium		Krebs–Ringer medium	
		– glucose*	+ glucose†	– glucose*	+ glucose†
MIS	5		1.08 ± 0.022 (8)		
	10	0.92 ± 0.03 (10)	1.19 ± 0.047 (9)	0.81 ± 0.086 (7)	1.2 ± 0.037 (4)
	20		1.28 ± 0.061 (10)		
NDPP	0.5	0.96 ± 0.023 (4)	1.26 ± 0.082 (4)	0.88 ± 0.043 (6)	1.25 ± 0.095 (4)
DNP	0.1	1.18 ± 0.106 (6)	1.68 ± 0.078 (9)	0.85 ± 0.05 (7)	1.13 ± 0.046 (5)

* O₂ consumption rate with drug added
Endogenous O₂ consumption rate

† O₂ consumption rate with drug and glucose added
O₂ consumption rate with glucose added

The numbers in brackets = No. of determinations.

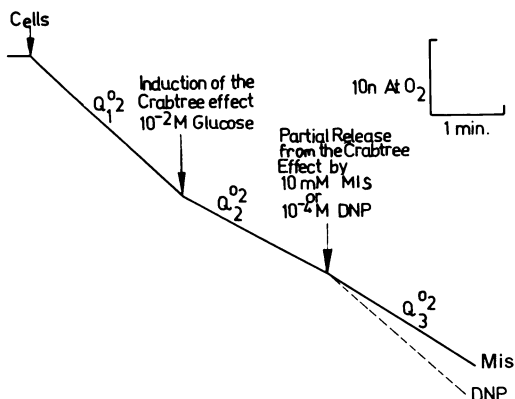


FIG. 1.—Polarographic registration of O₂ consumption in Ehrlich ascites tumour cells suspended in glucose-free Tyrode medium. The Crabtree effect was induced by addition of 10⁻²M glucose. Partial release from the Crabtree effect was obtained by adding 10mM misonidazole or 10⁻¹mM DNP. Ehrlich tumour cell density was 5 × 10⁶/ml.

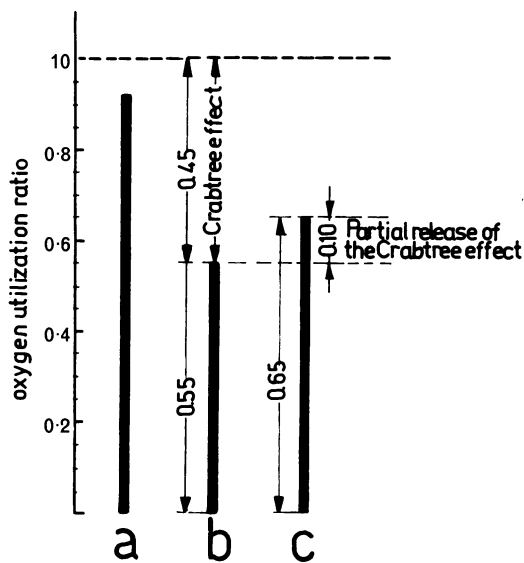


FIG. 2.—Graphic analysis of the oxygen utilization ratios for Ehrlich ascites tumour cells as influenced by:

- a—10mM MIS
- b—10⁻²M glucose
- c—10mM MIS + 10⁻² M glucose

$$RCE \% = \frac{0.65 - 0.55}{1 - 0.55} \times 100 = 22.22\%$$

Endogenous rate of O₂ consumption in Tyrode medium free of glucose = 1.0.

in the presence of glucose and radiosensitizer was related to the initial rate of O₂ consumption in media without glucose and radiosensitizer (endogenous rate), we obtained the OURs listed in Table II. These data show that the rate of O₂ consumption in the presence of glucose and radiosensitizers in this study was below the endogenous rate (Fig. 2).

The data on the release from the Crabtree effect by MIS, NDPP and DNP are included in Table II. In addition correlation between drug concentration and magnitude of release was also obtained for MIS (Table II).

DISCUSSION

It was presumed that the effect of hypoxic radiosensitizers on cellular res-

piration may contribute to their effectiveness, in addition to electron-affinity and cytotoxicity. Chemical compounds which inhibit O₂ utilization could enhance radiation response by increasing oxygenation of the tumours. On the other hand, compounds stimulating O₂ consumption may alter their effectiveness by enlarging the hypoxic regions of the tumours (Biaglow & Durand, 1976; Durand *et al.*, 1976, 1978; Haynes & Inch, 1976). According to these criteria it appears to be of practical

TABLE II.—Release from the Crabtree effect (RCE) in the presence of MIS, NDPP and DNP

Compound	mM	Tyrode medium		Krebs-Ringer medium	
		OUR*	RCE	OUR*	RCE
MIS	5	0.59	8.88	0.74	31.57
	10	0.65	22.22		
	20	0.70	33.33		
NDPP	0.5	0.69	31.11	0.77	39.47
DNP	0.1	0.92	82.00	0.70	21.05

* $\frac{\text{O}_2 \text{ consumption rate with drug and glucose added}}{\text{Endogenous rate of O}_2 \text{ consumption}}$

importance to know the effect of hypoxic cell radio sensitizers on O₂ utilization. The recent investigations permit us to divide radiosensitizers in 2 groups: respectively with inhibitory, and with stimulatory effects on O₂ utilization. It is pertinent to mention that the stimulatory effects for most of these compounds was only seen when glucose was present in the reaction medium (Biaglow *et al.*, 1975, 1978; Mustea *et al.*, 1978). The requirement for glucose in stimulating O₂ utilization was interpreted as being due to exhaustion of endogenous substrates necessary for respiration (Biaglow *et al.*, 1978). Our results indicate another possible mechanism and interpretation. Glucose induces in the majority of tumours and in some cell cultures a Crabtree effect expressed as an inhibition of O₂ consumption (see Ibsen, 1961 for a review; Biaglow *et al.*, 1969). For Ehrlich ascites tumour cells this inhibition was reported to be 25–57% of endogenous O₂ consumption, depending upon experimental conditions (Ibsen, 1961; Mustea, 1974). The uncoupling agents of oxidative phosphorylation reverse the inhibition of O₂ consumption by glucose. MIS and NDPP have proved to have similar properties to the uncouplers regarding release from the Crabtree effect. Therefore their stimulating of O₂ consumption in media containing glucose produces a marked release from the Crabtree effect. Taking our results into consideration and the attempts of various authors to find a correlation between the effectiveness of hypoxic sensitizers and their effects on oxygen utilization, we consider it necessary to re-examine the stimulatory effects on O₂ consumption observed in media containing glucose.

The Crabtree effect is regarded as a regulatory mechanism between the glycolytic and respiratory compartments, and is dependent on the level of adenine nucleotides (Belitzer, 1936; Chance & Hess, 1959). The mechanism of action of hypoxic radiosensitizers on the Crabtree effect ought to be investigated, taking into account the action of these drugs on

adenine factors limiting respiration (NAD⁺, ATP), as well as the activity of the enzymes reported as having a predominant part in release from the Crabtree effect (Yamada, 1968; Asami & Yamada, 1969). In connection with the latter aspect, determinations of the effect of hypoxic radiosensitizers on ATP-ase activity are now being carried out in our laboratory.

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