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Evaluation of the kinetics of the intracellular reduction of 2,6-dichlorophenolindophenol in normal and transformed hepatocytes measured by amperometric methods

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Amperometric methods were used to study the kinetics of intracellular reduction of 2,6-dichlorophenolindophenol (DCIP) in normal and transformed hepatocytes with glucose and succinate as substrates. The curves showing the formation of $DCIP_{red}$ as a function of time were biphasic, the first part obeying the equation of a pseudo-first-order reaction, the final part corresponding to Michaelis-Menten kinetics. A statistical method was used to estimate pseudo-first-order rate constants k as well as K_m and V_{max} values. At saturating glucose concentrations k, K_m and V_{max} values were higher in normal compared to transformed cells. Decreasing glucose concentrations revealed lowered saturation concentrations in tumour cells compared to normal cells. With succinate as substrate for hepatocytes, k values were higher than with glucose, while K_m and V_{max} were about the same. Hepatoma cells did not metabolize succinate. k values could be attributed to intracellular dehydrogenase activities including cytosolic and mitochondrial processes. Differences in pseudo-first-order rate constants between normal and tumour cells may therefore represent characteristic alterations associated with transformation.

Introduction

In a previous study the intracellular enzymatic reduction of 2,6-dichlorophenolindophenol (DCIP) was investigated in normal and transformed hepatocytes in order to obtain an insight into the redox behaviour of such cells [1,2]. From the data obtained it had been suggested that DCIP can be reduced by intracellular NAD(P)H. It is well known that the activity of NAD(P)H-linked enzymes is different in tumour cells and normal cells [3–5]. Accordingly, both the reducing capacity of the cells as well as the kinetics of DCIP reduction were found to be different in transformed compared to normal hepatocytes [1,2]. NAD(P)H can be formed intracellularly in different metabolic pathways such as glycolysis and the pentose phosphate pathway localized in the cytosol and by enzymes of the tricarboxylic acid cycle localized in the mitochondria. Furthermore, $FADH_2$, which is the coenzyme linked to succinate dehydrogenase, may also contribute to DCIP reduction. In order to discriminate between the various reactions leading to NAD(P)H and $FADH_2$, glucose and succinate were used as substrates in the experiments reported in the present paper. The formation of the reduced form of DCIP ($DCIP_{red}$) was measured by the amperometric methods described previously [1,2] and the kinetics of the different reactions were evaluated quantitatively. The kinetic constants obtained are discussed in terms of intracellular enzyme activities of normal and transformed cells.

Materials and Methods

Amperometric measurements

The electrochemical apparatus and the experimental conditions were the same as described in Refs. 1 and 2. A jacketted polarographic cell was provided with a rotating gold disk electrode (Beckman Instruments 188501) at a constant speed of 50 rps, geometric surface 0.302 cm^2, a double junction reference electrode, Ag/AgCl, satd. KCl/ phosphate-buffered saline (Metrohm, Model 6.0726.100), a Pt-auxiliary electrode, and a gas inlet. The temperature was kept constant at $37 \pm 0.1°C$ throughout. The voltage applied to the electrodes was under potentiostatic control using a Wenk-

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ing potential control amplifier PCA 72L. The voltage was adjusted to a constant value of + 0.2 V by a voltage scan generator (Wenking VSG 72). The formation of the reduced form of DCIP ($DCIP_{red}$) was followed by measuring the anodic current at the preselected voltage as a function of time. The polarographic cell was filled with phosphate-buffered saline to yield a final volume of 6.5 ml. Glucose was added in varying concentrations ($1 \cdot 10^{-4}$ to $2 \cdot 10^{-2}$ M) from stock solutions of 1.0 M and 0.1 M, respectively. Oxygen was removed from the solution by purging with nitrogen. After purging, the solution was kept under a nitrogen layer and the cells were added from a concentrated cell suspension (typically 10^7 cells/ml) to yield a final concentration of $2.5 \cdot 10^6$ cells per total volume of 6.5 ml. The small amount of oxygen added together with the cell suspension was consumed by respiration, the consumption of oxygen being followed in the limiting current range of the cathodic reduction of oxygen at about −0.6 V. When the solution was free of oxygen, DCIP was added from a predeaerated (by purging with nitrogen) stock solution of $5 \cdot 10^{-3}$ M in phosphate-buffered saline to obtain final concentrations of $(2–5) \cdot 10^{-4}$ M. Similar experiments were performed using succinate as substrate ($2 \cdot 10^{-2}$ M).

Preparation of the cell suspensions

Normal hepatocytes were isolated from fed or 20-h-starved male Sprague-Dawley rats (250–350 g body weight, Zentralinstitut für Versuchstierzucht, Hannover, F.R.G.) according to Seglen's method [6] with modifications as described earlier [7]. The cells were washed and taken up in phosphate-buffered saline and kept in ice until used. Morris hepatoma 3924A was taken from the 'Tumorbank' of the German Cancer Research Center and adapted to tissue culture as described [5]. Culture conditions were as reported [1,5]. The establishing of C_1I, a non-tumorigenic hepatocyte-derived cell line, has been published previously [3]. This cell line reveals metabolic properties which are comparable to those observed in preneoplastic hepatocytes [8] or slowly growing hepatomas [3,5], but as yet does not grow after transplantation in athymic nude mice. The cells were grown in modified Ham's F12 medium + 10% fetal calf serum [3]. Both cell lines (MH3924A and C_1I) were harvested by trypsinization. Cells were counted with a Neubauer hemocytometer and stock cell suspensions were adjusted to 10^7 cells/ml with phosphate-buffered saline. Protein was determined according to Heinzel's method [9].

Reagents

DCIP sodium salt (E. Merck, Darmstadt, F.R.G.) was used without further purification taken into account the residue on evaporation of 13.5%. All other substances were used reagent grade. Phosphate-buffered saline: 0.2 g KCl, 0.2 g KH_2PO_4, 1.15 g $Na_2HPO_4 \cdot H_2O$, 8.0 g NaCl made up to 1 litre with H_2O (pH 7.2).

Evaluation of the measurements

Formation of the reduced form of DCIP ($DCIP_{red}$) was measured as anodic current $x(t)$ as function of time and was analyzed statistically by the function $y(t) = \ln(a/a - x(t))$ where a is the initial $DCIP_{ox}$ concentration. The curves obtained were found earlier to be biphasic [2], suggesting pseudo-first-order kinetics in the initial part of the curve and Michaelis-Menten kinetics in the second phase. They were evaluated according to the following biphasic model.

$$y(t) = \begin{cases} k \cdot t & t < B \\ \frac{V_{max}}{K_m} \cdot t - \frac{x(t)}{K_m} & t \geq B \end{cases} \quad (1)$$

with first-order reaction kinetics $k \cdot t$ up to a break point B and subsequent Michaelis-Menten kinetics in the integrated form of

$$y(t)/t = (V_{max}/K_m) - (x(t)/t) \cdot (1/K_m).$$

Reaction kinetic parameters k, K_m and V_{max} describe overall metabolic reactions and therefore they represent apparent values.

For some reactions, where the second phase of $DCIP_{red}$ formation was not reached ($B = \infty$) first-order reaction kinetics were assumed to hold. Because of some nonlinear effects observed at very small times, where $x(t)$ as well as $y(t)$ were small, this reaction was modelled as

$$y(t) = k \cdot t \qquad t \geq A \quad (2)$$

where A was a left break point near 0. Determination of the parameters k, K_m and V_{max} in the biphasic equation, Eqn. 1, was obtained by a nonlinear curve fitting (nonlinear regression) technique as described in Ref. 10. A direct search method was used thereby to minimize the least-squares criterion:

$$\sum_{i=1}^{n} (y_i - y(t_i))^2$$

as a function of the four unknown parameters (k, B, K_m, V_{max}), when $y_i = \ln(a/a - x_i)$ was obtained from the measured $DCIP_{red}$ at time t_i and $y(t_i)$ was the model function from Eqn. 1 evaluated at time t_i. An estimate of the statistical precision was obtained by the jackknife technique [11], according to which standard errors of k, K_m and V_{max} were calculated.

Determination of the first-order reaction parameter, k, in model 2 was obtained by simple linear regression subsequently to an estimation of the break point A by change-point regression [12] based on a residual sum of

squares criterion. As an estimate of the precision of k, the standard deviation of k was calculated by simple linear regression [13] applied to model 2.

Combination of estimates obtained from repeated experiments were calculated by a variance-weighted mean of the estimates obtained in each experiment, separately for each parameter [14]. As estimate of the statistical precision of those combined estimates, we used the standard deviation of the variance-weighted mean as given by Cochran (Ref. 14; see also Ref. 15).

Results

Table I summarizes the data obtained when the substrates glucose and succinate were kept constant at a concentration ($2 \cdot 10^{-2}$ M) which was found to be saturating for the process measured (see Table III). DCIP was kept constant at a concentration ($2 \cdot 10^{-4}$ M) which was below the concentration found earlier to be inhibiting [1]. Mean values and standard deviations of pseudo-first-order rate constants, K_m and V_{max} values are shown in Table I, these having been obtained from various cell preparations. Since a certain variability between cell preparations was observed, particularly in the case of hepatocytes, the kinetic constants from the incubations with varying DCIP and glucose concentrations were determined using only individual cell preparations. The data shown in Tables II and III represent means ± S.D. of at least two experiments using one individual cell preparation which is a typical one out of three investigated ones.

Table I shows that the pseudo-first-order-rate constant obtained with glucose as substrate is higher with normal compared to transformed cells. With succinate the k value is even higher compared to glucose in normal cells, but not measurable in transformed hepatocytes (data not shown: the reaction of MH3924A cells with succinate as substrate was very low; this is in accordance with the low succinate dehydrogenase activity found with biochemical assays in many tumours). The amount of cellular protein added with the $2.5 \cdot 10^6$ cells in any experiment was 1.3 (C_1I cells) and 0.91 mg (MH3924A). V_{max} recalculated in terms of the protein content yielded similar values for the different cell types (data not shown).

Table II shows the pseudo-first-order rate constants with glucose and succinate as substrates as a function of the concentration of DCIP. Pseudo-first-order rate constants were found to decrease with increasing DCIP concentrations pointing to an inhibitory effect of high DCIP concentrations. Therefore, for all measurements with varying substrate concentrations, only one single DCIP concentration was used ($2 \cdot 10^{-4}$ M).

The results described up to now were obtained from measurements with saturating glucose concentrations. In order to reveal differences between normal and transformed cells with respect to saturation concentrations for glucose, kinetic data were also collected with decreasing glucose concentrations. When normal hepatocytes were isolated from well-fed animals, kinetic constants as a function of glucose concentration could not be obtained. Although the cells were prepared and stored in a glucose-free phosphate-buffered saline (the same as with the other cell types used), the blank values, i.e., the values with no substrate added, were about the same as the values with the highest glucose concentration added. This indicated a high endogenous substrate concentration in the hepatocytes which probably originated from glycogen degradation and which could only be eliminated by starvation of the animals before isolation of the hepatocytes. This, however, caused a pronounced decrease in the rate of glucose consumption by the cells, which was probably due to the shift from glucose breakdown to gluconeogenesis in hepatocytes of starved rats. It can be concluded that with hepatocytes from starved rats the measurement of the kinetics within the time scale chosen did not yield useful information.

The data obtained from Morris hepatoma and C_1I cells are shown in Table III. In the case of the Morris hepatoma cells a biphasic function could be observed in the concentration range from $1 \cdot 10^{-2}$ M to $7.5 \cdot 10^{-4}$ M glucose. In the same range of glucose concentration the DCIP added ($2 \cdot 10^{-4}$ M) was reduced to 100% (Table III). With decreasing substrate concentrations that part of the curve which obeyed Michaelis-Menten kinetics gradually disappeared. At glucose concentra-

TABLE I

Pseudo-first-order rate constants k, K_m and V_{max} values calculated from the reduction of DCIP with respect to time in 6.5 ml phosphate-buffered saline containing $2.5 \cdot 10^6$ cells, $2 \cdot 10^{-4}$ mol/l DCIP and 0.02 mol/l substrate (glucose or succinate as indicated)

The data represent overall means ± S.D. from several cell preparations.

Cell type	Substrate	Number of cell preparations investigated	k (s^{-1})	K_m ($mol \cdot l^{-1}$)	V_{max} ($mol \cdot l^{-1} \cdot s^{-1}$)
MH 3924A	glucose	6	$5.2 \cdot 10^{-3} \pm 1.8 \cdot 10^{-4}$	$1.9 \cdot 10^{-5} \pm 1.5 \cdot 10^{-6}$	$5.2 \cdot 10^{-7} \pm 1.3 \cdot 10^{-7}$
Hepatocytes	glucose	12	$6.7 \cdot 10^{-3} \pm 1.2 \cdot 10^{-4}$	$7.5 \cdot 10^{-5} \pm 1.7 \cdot 10^{-5}$	$1.1 \cdot 10^{-6} \pm 1.4 \cdot 10^{-7}$
Hepatocytes	succinate	2	$1.3 \cdot 10^{-2} \pm 1.5 \cdot 10^{-3}$	$8.7 \cdot 10^{-5} \pm 1.5 \cdot 10^{-6}$	$1.1 \cdot 10^{-6} \pm 3.3 \cdot 10^{-7}$

TABLE II

Pseudo-first-order rate constants, k (s^{-1}), calculated from the initial phase of the reduction of DCIP versus time in 6.5 ml of PBS containing $2.5 \cdot 10^6$ cells and 0.02 mol/l substrate (glucose and succinate as indicated) as a function of the concentration of DCIP

The data represent means ± S.D. of at least two measurements in individual cell preparations.

DCIP ($mol \cdot l^{-1}$)	MH3924A, glucose	Hepatocytes, glucose	Hepatocytes, succinate
$2 \cdot 10^{-4}$	$5.2 \cdot 10^{-3} \pm 1.8 \cdot 10^{-4}$	$6.7 \cdot 10^{-3} \pm 1.2 \cdot 10^{-4}$	$1.3 \cdot 10^{-2} \pm 1.5 \cdot 10^{-3}$
$3 \cdot 10^{-4}$	$3.8 \cdot 10^{-3} \pm 0.3 \cdot 10^{-3}$	$1.5 \cdot 10^{-3} \pm 0.7 \cdot 10^{-4}$	$8.5 \cdot 10^{-3} \pm 0.5 \cdot 10^{-3}$
$4 \cdot 10^{-4}$	$1.9 \cdot 10^{-3} \pm 0.7 \cdot 10^{-3}$	$0.45 \cdot 10^{-3} \pm 0.2 \cdot 10^{-4}$	$6.5 \cdot 10^{-3} \pm 0.7 \cdot 10^{-3}$
$5 \cdot 10^{-4}$	$0.75 \cdot 10^{-3} \pm 0.3 \cdot 10^{-4}$	not determined	$5.0 \cdot 10^{-3} \pm 0.9 \cdot 10^{-3}$

tions lower than $5 \cdot 10^{-4}$ M, only first-order reaction constants are given. A similar behaviour was observed for the C_1I cells. The range of glucose concentration in which the biphasic function was obtained was, however, smaller, i.e., from $1 \cdot 10^{-2}$ to $1 \cdot 10^{-3}$ M only.

Discussion

The significance of the pseudo-first-order rate constant obtained from the initial part of DCIP-reduction may be explained as follows.

The processes involved in the DCIP reduction are the following:

$$\text{substrates} + \begin{Bmatrix} \text{NAD(P)} \\ \text{FAD} \end{Bmatrix} \xrightarrow{\text{dehydrogenase}} \begin{Bmatrix} \text{NAD(P)H} \\ \text{FADH}_2 \end{Bmatrix} + \text{metabolites} \quad (3)$$

$$\begin{Bmatrix} \text{NAD(P)H} \\ \text{FADH}_2 \end{Bmatrix} + \text{DCIP}_{ox} \xrightarrow[\text{DCIP-oxidoreductase}]{\text{(DCIP-DT-diaphorase)}} \begin{Bmatrix} \text{NAD(P)} \\ \text{FAD} \end{Bmatrix} + \text{DCIP}_{red} \quad (4)$$

The concentrations of the oxidized and reduced forms of DCIP inside the cells are determined by the enzymatic reactions 3 and 4. Reaction 3 rather than 4 is considered to be rate-limiting. The involvement of an NAD(P)H-DCIP-complex in DCIP reduction (reaction 4) has been suggested earlier [1]. Such an NAD(P)H-dependent reductase of DCIP has also been found in the phagocytic vesicles of polymorphonuclear leucocytes [16]. Furthermore, a DT-diaphorase (DCIP) which reduces quinones has been found to reveal a high activity in liver [17] and in tumours [18]. Intracellular enzyme kinetics of artificial electron acceptors were investigated only recently by Kreysa and Krämer [19]. Hence, pseudo-first-order rate constants obtained from our calculations are considered to reflect the overall activity of NAD(P)H-forming and -consuming enzymes in the cells. Different k values are obtained for the different cell types and the different substrates used, under otherwise identical conditions. Table I shows that hepatocytes exhibit a slightly higher NAD(P)H formation rate compared to MH3924A cells when glucose was used as substrate.

TABLE III

Pseudo-first-order rate constants (k), K_m and V_{max} values ± S.D. calculated from the reduction of DCIP versus time in 6.5 ml PBS containing $2 \cdot 10^{-4}$ mol/l DCIP and $2.5 \cdot 10^6$ cells using different cell types and varying glucose concentrations

The values are obtained from individual cell suspensions.

Cell type	Glucose concentration ($mol \cdot l^{-1}$)	k (s^{-1})	K_m ($mol \cdot l^{-1}$)	V_{max} ($mol \cdot l^{-1} \cdot s^{-1}$)	DCIP reduced (%)
C_1I	$1 \cdot 10^{-2}$	$3.7 \cdot 10^{-3} \pm 0.2 \cdot 10^{-3}$	$1.9 \cdot 10^{-5} \pm 0.3 \cdot 10^{-5}$	$4.5 \cdot 10^{-7} \pm 0.1 \cdot 10^{-7}$	100
	$5 \cdot 10^{-3}$	$4.0 \cdot 10^{-3} \pm 0.4 \cdot 10^{-3}$	$2.4 \cdot 10^{-5} \pm 0.3 \cdot 10^{-5}$	$5.5 \cdot 10^{-7} \pm 0.2 \cdot 10^{-7}$	100
	$2.5 \cdot 10^{-3}$	$3.1 \cdot 10^{-3} \pm 0.1 \cdot 10^{-3}$	$2.9 \cdot 10^{-5} \pm 1.3 \cdot 10^{-5}$	$4.3 \cdot 10^{-7} \pm 0.6 \cdot 10^{-7}$	100
	$1 \cdot 10^{-3}$	$6.1 \cdot 10^{-3} \pm 0.8 \cdot 10^{-3}$	$3.0 \cdot 10^{-5} \pm 1.9 \cdot 10^{-5}$	$8.5 \cdot 10^{-7} \pm 1.8 \cdot 10^{-7}$	58
	$1 \cdot 10^{-4}$	$0.6 \cdot 10^{-3} \pm 0.5 \cdot 10^{-4}$	-	-	37
Morris hepatoma 3924A	$1 \cdot 10^{-2}$	$2.8 \cdot 10^{-3} \pm 0.2 \cdot 10^{-3}$	$1.5 \cdot 10^{-5} \pm 0.5 \cdot 10^{-5}$	$3.0 \cdot 10^{-7} \pm 0.3 \cdot 10^{-7}$	100
	$5 \cdot 10^{-3}$	$3.5 \cdot 10^{-3} \pm 0.6 \cdot 10^{-3}$	$1.8 \cdot 10^{-5} \pm 1.1 \cdot 10^{-5}$	$4.1 \cdot 10^{-7} \pm 0.6 \cdot 10^{-7}$	100
	$2.5 \cdot 10^{-3}$	$2.4 \cdot 10^{-3} \pm 0.2 \cdot 10^{-3}$	$2.7 \cdot 10^{-5} \pm 2.5 \cdot 10^{-5}$	$2.9 \cdot 10^{-7} \pm 0.8 \cdot 10^{-7}$	100
	$1 \cdot 10^{-3}$	$2.2 \cdot 10^{-3} \pm 0.1 \cdot 10^{-3}$	$3.5 \cdot 10^{-5} \pm 1.8 \cdot 10^{-5}$	$2.7 \cdot 10^{-7} \pm 0.5 \cdot 10^{-7}$	100
	$7.5 \cdot 10^{-4}$	$1.6 \cdot 10^{-3} \pm 0.1 \cdot 10^{-3}$	$5.1 \cdot 10^{-5} \pm 0.8 \cdot 10^{-5}$	$2.5 \cdot 10^{-7} \pm 0.1 \cdot 10^{-7}$	100
	$5 \cdot 10^{-4}$	$5.0 \cdot 10^{-4} \pm 1.0 \cdot 10^{-4}$	-	-	80
	$2.5 \cdot 10^{-4}$	$3.9 \cdot 10^{-4} \pm 0.2 \cdot 10^{-4}$	-	-	40
	$1.25 \cdot 10^{-4}$	$1.8 \cdot 10^{-4} \pm 0.1 \cdot 10^{-4}$	-	-	25

The data obtained with succinate as substrate indicate that also the mitochondrial enzymes can be involved in DCIP reduction. The reaction rate obtained from incubation of hepatocytes with succinate was even higher than with glucose. This indicates that succinate dehydrogenase is measured directly under these conditions. The extremely low response to succinate of MH3924A cells can be explained with their very low succinate dehydrogenase activity [20,21].

The K_m values listed in Tables I and III calculated from the second part of the curves are comparable to K_m values known for substrates and coenzymes of enzymes of glycolysis and pentose phosphate pathway, e.g., glyceraldehyde-3-phosphate dehydrogenase (K_m for NAD: $1.3 \cdot 10^{-5}$ M, for glyceraldehyde 3-phosphate: $9.0 \cdot 10^{-5}$ M) and glucose-6-phosphate dehydrogenase (K_m for NADP: $2.3 \cdot 10^{-6}$ M, for glucose 6-phosphate $3.3 \cdot 10^{-5}$ M), see [22,23]. We, therefore, assume that the NAD(P)H-generating enzymes of glycolysis and pentose phosphate pathway are reflected by our method.

The enzymes catalyzing glucose phosphorylation, on the other hand, show higher K_m values; glucokinase: $K_m = (5\text{–}8) \cdot 10^{-3}$ M, hexokinase: $K_m = 1$ to $2 \cdot 10^{-4}$ M [24,25]. Glucose phosphorylation in Morris hepatoma is performed almost exclusively by hexokinase [5], in hepatocytes almost exclusively by glucokinase [25]. The data given in Table III show that only at glucose concentrations where the respective glucose phosphorylating enzymes are saturated the Michaelis-Menten type of kinetics is obeyed. The results show that glucose concentrations yielding Michaelis-Menten kinetics are lower in Morris hepatoma cells compared to immortalized but not tumorigenic C_1I cells and much lower compared to normal hepatocytes. This is consistent with the finding that in hepatic tumour cells glucose metabolism is maintained at lower glucose concentrations than in hepatocytes [26].

Conclusions

The kinetic data obtained in the present paper reveal that pseudo-first-order rate constants calculated from the initial part of the curve can be attributed to intracellular dehydrogenase activities. Extra- and intramitochondrial NAD(P)H-$FADH_2$/DCIP complexes could be shown to be involved in these processes. Significant differences in k-values were found between normal and transformed hepatocytes, whereby transformed cells showed lower reaction rates than normal cells. The K_m values deduced from the final part of the curve were found to correspond to enzymes of glucose metabolism.

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