Poly(Adenosine Diphosphate Ribose) Metabolism and Regulation of Myocardial Cell Growth by Oxygen

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Control of the rate of cardiac cell division by oxygen occurs most probably by altering the redox state of a control substance, e.g. $\text{NAD}^+ \rightleftharpoons \text{NADH}$. NAD^+ (and not NADH) forms poly(ADP-ribose), an inhibitor of DNA synthesis, in a reaction catalysed by poly(ADP-ribose) polymerase. Lower partial pressure of oxygen, which increases the rate of division, would shift $\text{NAD}^+ \rightarrow \text{NADH}$, decrease poly(ADP-ribose) synthesis, and increase DNA synthesis. Chick-embryo heart cells grown in culture in 20% O_2 (in which they divide more slowly than in 5% O_2) did exhibit greater poly(ADP-ribose) polymerase activity (+83%, $P < 0.001$) than when grown in 5% O_2 . Reaction product was identified as poly(ADP-ribose) by its insensitivity to deoxyribonuclease, ribonuclease, NAD glycohydrolase, Pronase, trypsin and micrococcal nuclease, and by its complete digestion with snake-venom phosphodiesterase to phosphoribosyl-AMP and AMP. Isolation of these digestion products by Dowex 1 (formate form) column chromatography and paper chromatography allowed calculation of average poly(ADP-ribose) chain length, which was 15–26% greater in 20% than in 5% O_2 . Thus in 20% O_2 the increase in poly(ADP-ribose) formation results from chain elongation. Formation of new chains also occurs, probably to an even greater degree than chain elongation. Additionally, poly(ADP-ribose) polymerase has very different K_m and V_{max} values and pH optima in 20% and 5% O_2 . These data suggest that poly(ADP-ribose) metabolism participates in the regulation of heart-cell division by O_2 , probably by several different mechanisms.

The vigorous rate of myocardial cell division that occurs in the foetus declines rapidly after birth. In rat heart all muscle cells have ceased to divide by the third to sixth week (Enesco & LeBlond, 1962; Rumyantsev, 1965; Sasaki *et al.*, 1970). We believe that the low arterial $p\text{O}_2$ (partial pressure of O_2) (20–25 mmHg) that normally occurs in the foetus is essential for the rapid rates of growth and cell division that occur *in utero* and that the abrupt increase in arterial $p\text{O}_2$ that accompanies birth and spontaneous respiration is largely responsible for the subsequent rapid decline in the rate of myocardial cell division. Support for this hypothesis came from studies in our laboratory on the effects of different O_2 concentrations on growth of cardiac muscle in tissue culture (Hollenberg, 1971). Decreased ambient O_2 increased the rates of division of cardiac cells, whereas progressively higher O_2 concentrations inhibited such cellular proliferation. The ability of O_2 to alter the rate of cardiac cell division was also demonstrated *in vivo* in the newborn rat. Short periods of hyperoxia decreased cell proliferation (Honbo & Hollenberg,

1973), whereas exposure to decreased oxygenation for the first 3 weeks after birth augmented cell division (Hollenberg *et al.*, 1976). Our hypothesis and experimental findings, which demonstrated the importance of O_2 in modifying mitotic activity, were also consistent with several other theories in which mitosis is inhibited when the cell is in a relatively oxidized state. According to Szent-Gyorgi (1968) cell division is regulated by electron acceptors such as α -oxo aldehydes or derivatives of glyoxal. Such substances inhibit cell division when they act as oxidizing agents; when they act as reducing agents they promote cell division. Edgar (1970) suggested that the ascorbate system (ascorbic acid $\rightleftharpoons$ dehydroascorbic acid) plays a major biochemical role in regulating cell division. Thus in our experiments (where decreased O_2 increases the rate of division of heart cells in tissue culture and in newborn rats) O_2 probably acts to alter the redox state of a control substance (e.g. $\text{NAD}^+ \rightleftharpoons \text{NADH}$). NAD^+ , in addition to its better-known participation in dehydrogenase reactions in which it is not consumed, serves as a precursor for poly(ADP-ribose), an inhibitor of DNA synthesis (Hayaishi, 1976). Poly(ADP-ribose) synthesis is mediated by a polymerase that splits off nicotinamide from NAD^+ and

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simultaneously polymerizes its ADP-ribose moiety to form the homopolymer poly(ADP-ribose) (Chambon *et al.*, 1963; Fujimura *et al.*, 1967; Nishizuka *et al.*, 1967, 1969; Ueda *et al.*, 1975). Approximately 90% of the total activity of poly(ADP-ribose) polymerase is associated with the chromatin (Nishizuka *et al.*, 1969). The physiological role of poly(ADP-ribose) and the ADP-ribosylation of chromosomal proteins in nuclear function remains unclear to date, despite several reports showing their close relationship to DNA metabolism (Kidwell & Burdette, 1974; Roberts *et al.*, 1975; Claycomb, 1976). The recent discovery of this system offered a potential mechanism by which O_2 theoretically could regulate cell division. Low oxygen partial pressure, by converting NAD^+ into NADH [which cannot act as substrate for poly(ADP-ribose) polymerase] may decrease the precursor pool of NAD^+ and could eventually decrease poly(ADP-ribose) synthesis.

The studies reported in the present paper were undertaken to clarify whether poly(ADP-ribose) and its polymerase participate in the regulation of cardiac-cell division by O_2 in tissue culture.

Experimental

Materials

AMP, cyclic AMP, ATP, calf thymus DNA, nicotinamide, NMN, ADP-ribose, adenosine, NAD^+ , Pronase and NAD glycohydrolase were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Ribonuclease, deoxyribonuclease and snake-venom phosphodiesterase were bought from Worthington Chemical Co., Freehold, NJ, U.S.A. Triton X-100 and [adenine- $U-^{14}C$]NAD were purchased from Amersham/Searle, Chicago, IL, U.S.A. Dowex 1 (X2; 200–400 mesh; chloride form) was purchased from Bio-Rad Laboratories, Richmond, CA, U.S.A., and converted into the formate form.

Preparation and culture of chick heart cells

The ventricles from hearts of white Leghorn chick embryos (6–9 days old) were finely minced and treated with 0.05% trypsin (Grand Island Biological Co., Grand Island, NY, U.S.A.) in Ca^{2+} - and Mg^{2+} -free Hanks solution (for composition see catalogue of Grand Island Biological Co.) for 2 min at 37°C. The supernatant fraction containing blood cells and debris was discarded and the tissues were additionally treated with 0.25% trypsin for 10 min at 37°C. The treated cells were centrifuged at 320g for 10 min and the supernatant fraction was discarded. The pelleted cells were suspended in NCI growth medium (Irvine Scientific, Irvine, CA, U.S.A.) (for composition see catalogue of Grand Island Biological Co.), counted on a haemocytometer slide, and further diluted with

the growth medium to standard concentrations. Tissue-culture dishes (100 mm × 20 mm; Corning Glass Works, Corning, NY, U.S.A.) containing 12 ml of NCI medium with 20% foetal calf serum were inoculated with 7.5×10^5 – 1.7×10^6 cells. They were incubated in specified gas environments at 37°C in plastic chambers (30 cm × 30 cm × 30 cm) placed within a CO_2 incubator (Forma Scientific, Marietta, OH, U.S.A.). The gas mixture used consisted of constant 5% CO_2 (to maintain pH), either 20% or 5% O_2 , and the remainder N_2 . The premixed gases were led into the incubator and bubbled into the individual plastic chambers through a layer of water at the bottom of chamber. On day 6 of culture the cells were washed with 1 mM-Tris/HCl (pH 7.5), harvested in the same buffer containing 0.1% Triton X-100, and homogenized with several strokes of a hand-held glass homogenizer with a Teflon pestle.

Assay of poly(ADP-ribose) polymerase

Activity of poly(ADP-ribose) polymerase was assayed in a homogenate of cultured chick heart cells by measuring the incorporation of [^{14}C]NAD $^+$ at 10 min at 30°C into an acid-insoluble product. The standard reaction mixture contained the following in a total volume of 0.1 ml: 50 mM-Tris/HCl (pH 7.5), 15 mM-KCl, 15 mM-MgCl $_2$, 10 mM-NaF, 0.1 mM-dithiothreitol, 3 mM-cyclic AMP, 10 μ g of DNA, 96.15 μ M-[^{14}C]NAD $^+$ (67 μ Ci/ μ mol) and 50–100 μ g of protein [assayed by the method of Lowry *et al.* (1951), with bovine serum albumin as standard]. Reactions were initiated by addition of [^{14}C]NAD $^+$ and incubated at 30°C for 10 min in a water bath. The reaction was stopped by adding 2 ml of ice-cold 10% (w/v) trichloroacetic acid. The acid-insoluble material was collected on Millipore filters and washed with 4 × 5 ml of ice-cold 5% trichloroacetic acid to remove all acid-soluble radioactivity. The filters were dried, placed in scintillation vials containing 15 ml of Bray's (1960) solution {4 g of PPO (2,5-diphenyloxazole) and 200 mg of dimethyl-POPOP [1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene], 60 g of naphthalene, 100 ml of methanol and 20 ml of ethylene glycol per litre of dioxan} and counted for radioactivity in a Beckman LS-350 liquid-scintillation counter. Corrections for quenching were made by using automatic external standardization. The results were corrected for zero-time controls. The specific activity of poly(ADP-ribose) polymerase is expressed as pmol of [^{14}C]NAD $^+$ incorporated/min per mg of protein.

Preparation of poly(ADP-ribose)

The synthesis and isolation of poly(ADP-ribose) were carried out by a modification of the method of

Burzio *et al.* (1975). Large-scale preparation of poly(ADP-ribose) was carried out in the same reaction mixture as for the poly(ADP-ribose) polymerase assay described above: 8–10 mg of protein from cells grown in 20% or 5% O₂ was used in a final volume of 6 ml and all reactants were increased proportionately. Maximum polymerization was achieved at 30°C by incubating the mixture for 30 min. Assessment of the maximum reaction was done by withdrawing samples at various times and measuring the trichloroacetic acid-precipitable radioactivity as described for the poly(ADP-ribose) polymerase assay. The reaction was stopped by the addition of ice-cold trichloroacetic acid to a final concentration of 10% and keeping the mixture for 6 h at –20°C. The precipitate was collected by centrifugation at 27000g for 30 min, washed with 2 × 15 ml of 5% trichloroacetic acid and with 3 × 15 ml of 95% (v/v) ethanol. The precipitate was resuspended in 0.1 M-NaOH and incubated for 60 min at 37°C. The mixture was neutralized with conc. HCl. To the mixture Tris/HCl (pH 7.5), MgCl₂ and CaCl₂ solutions were added to final concentrations of 50 mM, 10 mM and 1 mM respectively. To eliminate the contaminating nucleic acids, deoxyribonuclease and pancreatic ribonuclease were each added to a final concentration of 100 µg/ml and the mixture was incubated for 60 min at 37°C. Pronase was added to a final concentration of 50 µg/ml and the mixture further incubated for 60 min at 37°C. The solution was centrifuged at 15000g for 15 min to remove any insoluble material formed. The supernatant was deproteinized by extraction with chloroform/3-methylbutan-1-ol (24:1, v/v) three times (Marmur, 1961). Then 0.1 vol. of 25% (w/v) potassium acetate, pH 5.0, was added to the aqueous layer and the polymer was precipitated by the addition of 3 vol. of cold 95% (v/v) ethanol. After 24 h at –20°C the mixture was centrifuged at 27000g for 30 min and the precipitate was dissolved in 1 ml of 10 mM-Tris/HCl (pH 7.4)/5 mM-MgCl₂ and stored at –20°C. Final recovery of ¹⁴C radioactivity in poly(ADP-ribose) was 78–82%. This material was characterized by treatment with various enzymes.

Determination of average chain length

Average chain length of poly(ADP-ribose) was determined by the methods of Shima *et al.* (1969) and Miller (1975). After treatment with deoxyribonuclease, ribonuclease and NAD glycohydrolase the acid-insoluble polymer was treated with snake-venom phosphodiesterase. This cleaves each poly(ADP-ribose) chain into many soluble phosphoribosyl-AMP units and a single AMP unit. These compounds were then separated by Dowex 1 (formate form) column chromatography and by paper chromatography and their respective radioactivities measured. Chain length is expressed as the average

number of adenine residues per chain and was calculated by the following equation:

Chain length =

$$\frac{\text{c.p.m. in AMP} + \text{c.p.m. in phosphoribosyl-AMP}}{\text{c.p.m. in AMP}}$$

Poly[¹⁴C](ADP-ribose) (1 × 10⁵ c.p.m. in reaction product from cells grown in 20% O₂ and 6.55 × 10⁴ c.p.m. in reaction product from cells grown in 5% O₂), was incubated for 60 min at 37°C with 10 µg of snake-venom phosphodiesterase in a total of 0.3 ml of 100 mM-Tris/HCl (pH 7.0)/10 mM-MgCl₂. The mixture was then analysed by column and paper chromatography. The mixture was applied together with markers (adenosine, AMP, ADP-ribose and ATP) to a column (1 cm × 25 cm) of Dowex 1 (X2; formate form). Elution was carried out in a stepwise manner with 30 ml of water, 30 ml of 0.05 M-formic acid, 50 ml of 0.5 M-formic acid, 50 ml of 1.0 M-formic acid, 60 ml of 4.0 M-formic acid and 125 ml of 6.0 M-formic acid/0.4 M-ammonium formate. Fractions (3 ml) were collected and assayed for radioactivity. Descending single-dimensional chromatography was also carried out on the mixture in the solvent system isobutyric acid/propan-1-ol/water/aq. 25% NH₃ (75:25:25:2.5, by vol.). Authentic non-radioactive markers were chromatographed and located under a u.v. lamp. The radioactivity was determined in 1 cm strips by placing them in scintillation vials containing 10 ml of scintillation fluid (4 g of PPO, 200 mg of POPOP/litre of toluene) and counting them in a Beckman liquid-scintillation counter.

Results

Poly(ADP-ribose) polymerase activity

Incubation of a homogenate of cultured chick-embryo heart cells with [¹⁴C]NAD⁺ labelled in the adenine moiety results in the incorporation of radioactivity into an acid-insoluble fraction that has been identified as poly(ADP-ribose). Use of boiled homogenate eliminates all ¹⁴C incorporation. DNA, Mg²⁺ and a thiol compound (dithiothreitol) are essential for full enzyme activity (Janakidevi & Koh, 1974; Hilz & Stone, 1976). The acid-insoluble product was insensitive to the actions of deoxyribonuclease, ribonuclease, NAD glycohydrolase, micrococcal nuclease, Pronase and trypsin, but was completely solubilized (above 99%) by the action of snake-venom phosphodiesterase (Table 1). The acid-soluble products were used to determine average chain length of poly(ADP-ribose), as described below.

The time course of [¹⁴C]NAD⁺ incorporation at 30°C from cells grown in 20% and 5% O₂ is shown in Fig. 1, and is complete by 30 min. Optimum temperature for the reaction is 30°C (Fig. 2). How-

Table 1. Enzymic digestion of the reaction products
Reaction components and isolation procedure for the product are described in the text. Isolated reaction products were treated with the indicated enzymes in the appropriate buffers (Hasegawa *et al.*, 1967) and incubated for 60 min at 37°C. The reaction was stopped by adding cold 10% trichloroacetic acid, the acid-insoluble material collected on a Millipore filter, washed with cold 5% trichloroacetic acid and the remaining radioactivity determined by liquid-scintillation counting.

Enzymes ($\mu\text{g/ml}$)	Remaining acid insoluble radioactivity (c.p.m.)	
	20% O ₂	5% O ₂
None	8036	8221
Ribonuclease (100)	8052	8198
Deoxyribonuclease (100)	8105	8200
Micrococcal nuclease (100)	8000	8301
Pronase (50)	8097	8240
Trypsin (100)	7988	8285
Snake-venom phosphodiesterase (40)	45	58
NAD glycohydrolase (100)	8010	8250

Fig. 1. Poly(ADP-ribose) polymerase activity of cells grown in (○) 20% and (●) 5% O₂ as a function of time
Enzyme activity was assayed as described in the text: 100 μg of protein was used in each determination.

ever, pH optima differ, depending on the concentration of O₂ in which the cells are grown (Fig. 3): in 20% O₂ activity reaches a peak at about pH 7 and then falls off sharply, whereas in 5% O₂ a broad plateau of activity exists from pH 7 to pH 9. In cells grown in 20% O₂ (in which cells divide more slowly) net radioactivity in acid-insoluble product is 162% greater than in cells grown in 5% O₂ (224 versus 87 pmol of NAD incorporated/30 min per mg of protein; $P < 0.02$). These values for radioactivity represent a net balance between synthesis by poly(ADP-ribose) polymerase and degradation by poly(ADP-ribose) glycohydrolase. Thus cyclic AMP, a

Fig. 2. Poly(ADP-ribose) polymerase activity of cells grown in (○) 20% and (●) 5% O₂ as a function of temperature
Enzyme activity was assayed as described in the text at pH 7.5.

Fig. 3. Poly(ADP-ribose) polymerase activity of cells grown in (○) 20% and (●) 5% as a function of pH
Enzyme activity was assayed at 30°C as described in the text.

potent inhibitor of glycohydrolase, was added to the reaction mixture to assess more exactly polymerase activity. Addition of cyclic AMP to the reaction mixtures increased [¹⁴C]NAD⁺ incorporation into

Fig. 4. Double-reciprocal plot of rate of poly(ADP-ribose) formation at different NAD concentrations

Enzyme activity was assayed at 30°C and pH 7.5 as described in the text. ○, Cells grown in 20% O₂: $K_m = 43.4 \mu\text{M}$; $V_{max} = 250 \text{ c.p.m.}$ ●, Cells grown in 5% O₂: $K_m = 500 \mu\text{M}$; $V_{max} = 25 \text{ c.p.m.}$

acid-precipitable product to a similar extent in both sets of reaction mixtures (by 113 and 100 pmol/30 min per mg of protein in 20% and 5% O₂ respectively). Thus, with cyclic AMP added to the reaction mixture, [¹⁴C]NAD⁺ incorporation increased 83% in cells grown in 20% O₂ compared with those grown in 5% O₂ (337 versus 187 pmol/30 min per mg of protein; $P < 0.001$). At these different O₂ concentrations the K_m and V_{max} of poly(ADP-ribose) polymerase differ significantly (Fig. 4). In 20% O₂ the K_m is 11.5 times lower (0.043 versus 0.5 mM) and the V_{max} 10 times greater (250 versus 25 c.p.m.) than in 5% O₂. This suggests that in cells grown in 20% O₂ poly(ADP-ribose) polymerase possesses greater affinity for NAD⁺, which together with an increase in the maximum velocity of the reaction result in increased enzyme activity in the assay *in vitro*.

Determination of average chain length of poly(ADP-ribose)

The chain length of the homopolymer formed *in vitro* from cells grown in 20% and 5% O₂ was determined by complete digestion of the polymer with snake-venom phosphodiesterase, which yields from each chain one AMP molecule and a variable number of repeating phosphoribosyl-AMP units, depending on chain length. By this approach one could determine whether the increased poly(ADP-ribose) being synthesized in the incubation *in vitro* with cells grown in 20% versus 5% O₂ was due to formation of new chains or primarily to elongation of chains. AMP and phosphoribosyl-AMP were separated on a Dowex column and the average chain length was determined from the ratio of the total radioactivity in

Fig. 5. Chromatography of the snake-venom phosphodiesterase-digested products of poly(ADP-ribose) on a column (1 cm × 25 cm) of Dowex 1 (X2, formate form) (a) Cells grown in 20% O₂; (b) cells grown in 5% O₂. Elution was carried out in a stepwise manner (indicated by the arrows from left to right) with water, 0.05 M-formic acid, 0.5 M-formic acid, 1 M-formic acid, 4 M-formic acid and 6 M-formic acid/0.4 M-ammonium formate respectively. Fractions (3 ml) were collected and assayed for radioactivity (●) and A_{260} (---). The recovery of radioactivity was 100% in (a) and 96% in (b); it was eluted mainly in the AMP and phosphoribosyl-AMP peaks. ADP-ribose was used as a marker for phosphoribosyl-AMP.

phosphoribosyl-AMP to that in AMP. The radioactivity profiles for samples grown in 20% and 5% O₂ show a high degree of similarity (Fig. 5). Two major radioactive peaks were eluted, and corresponded to AMP and ADP-ribose markers. The radioactivity under these two peaks accounted for the total radioactivity applied to the column. These results suggest that the products synthesized from enzymes derived from cells grown in both O₂ concentrations were similar and were AMP and phosphoribosyl-AMP. Average chain length of poly(ADP-ribose) synthesized *in vitro* in two experiments used by Dowex chromatography for the 20%- and the 5%-O₂ reaction products were 16 and 13, and 20 and 17 respectively

Table 2. Average chain length of isolated products

Preparation of samples and procedure for column chromatography are described in the legend to Fig. 5. Procedure for paper chromatography is described in the text and the legend to Fig. 6.

	Column chromatography			Paper chromatography		
	Radioactivity (c.p.m.)		Average chain length $\left(\frac{1+b}{a}\right)$	Radioactivity (c.p.m.)		Average chain length $\left(\frac{1+b}{a}\right)$
	AMP (a)	Phospho- ribosyl- AMP (b)		AMP (a)	Phospho- ribosyl- AMP (b)	
Expt. 1						
20% O ₂	6936	103026	15.9	721	11094	16.4
5% O ₂	4926	58236	12.8	700	8366	13.0
Expt. 2						
20% O ₂	5000	94128	19.8			
5% O ₂	6180	100006	17.2

Fig. 6. Paper chromatography of phosphodiesterase digests of poly(ADP-ribose)

Poly[¹⁴C](ADP-ribose) (20000 c.p.m.) from cells grown in 5% O₂ (b) and 20% O₂ (c) was digested with snake-venom phosphodiesterase, concentrated by freeze-drying, applied to the paper (Whatman no. 1), chromatographed and processed as described in the text. NAD⁺ (32000 c.p.m.) (a) was also similarly processed. AMP and ADP-ribose were used as markers.

(Table 2). Paper chromatography was used to separate AMP and phosphoribosyl-AMP (Fig. 6), and similar chain lengths of 16 and 13 (for 20% and 5% O₂ respectively) were obtained (Table 2). Thus it appears that the increased activity of poly(ADP-ribose) polymerase in 20% O₂ results in an incubation product with slightly greater chain length. The striking increase in [¹⁴C]NAD⁺ incorporation into cells grown in 20% O₂ versus 5% O₂ (162% greater) contrasts

with only a 15–26% increase in chain length and suggests that chain initiation also is probably augmented in 20% O₂.

Discussion

The ability to augment rates of cell proliferation in tissue culture as one progressively lowers ambient O₂ below 20% is a phenomenon widely distributed in Nature. Such regulation of cell division by O₂ has been observed in almost all cell types studied to date (Hollenberg, 1971; Brosemer & Rutter, 1961; Rueckert & Mueller, 1960; Taylor *et al.*, 1971, 1974; Albers & Bierman, 1976). The present studies used two different concentrations of O₂ (20% and 5%) to vary the rate of division of chick-embryo heart cells grown in tissue culture. At the higher O₂ concentration poly(ADP-ribose) polymerase activity increased by 83%, whereas cell growth was inhibited by 15–30%. This inverse relationship of poly(ADP-ribose) polymerase activity and rate of cell proliferation that we noted in the present studies has been found by many authors in different model systems (see review by Hilz & Stone, 1976). In rat cardiac muscle, polymerase activity increased during post-natal development (when mitotic activity falls sharply) and was inversely related to DNA synthesis and activity of DNA polymerase α (Claycomb, 1976). Burzio & Koide (1970, 1972) reported that NAD⁺ markedly decreases [³H]dTTP incorporation and template activity of chromatin in normal and regenerating rat liver. Although others have also shown this same phenomenon in different systems (Brightwell & Shall, 1971), this inhibition is not observed in lymphoma and hepatoma cells (Lehmann & Shall, 1972; Burzio & Koide, 1972). Moreover, template activity may be enhanced and not decreased by poly(ADP-ribose), as shown in HeLa cells (Roberts *et al.*, 1973, 1974; Smulson *et al.*, 1975). In further

attempts to define its physiological role, various investigators have shown that during phases of the cell cycle poly(ADP-ribose) polymerase activity and poly(ADP-ribose) concentration appear to fluctuate significantly. Most agree that the G₂ (or G₁) phase is associated with the highest and the S phase associated with the lowest polymerase activity (Miwa *et al.*, 1973; Shall *et al.*, 1974; Kidwell & Watts, 1974; Smulson *et al.*, 1975; Kidwell & Mage, 1976). What these reports as well as our own present studies leave unclear is whether fluctuations in activity of poly(ADP-ribose) polymerase and concentration of poly(ADP-ribose) result in changes in DNA synthesis and cell proliferation or are just associated with them. Moreover, by focusing on the mechanism by which the partial pressure of O₂ modifies cell proliferation the present studies add one additional complexity. If indeed ADP-ribosylation of nuclear proteins is directly and functionally involved in nuclear replication, does O₂ act through this system to modify mitotic activity, or does it act by some completely independent mechanism? O₂ may well increase the nuclear [NAD⁺]/[NADH] ratio and thereby increase poly(ADP-ribose) polymerase activity and poly(ADP-ribose) synthesis. The concentration of NAD (which is low in proliferating tissue and high in resting tissue) was proposed as a controlling factor for cell division (Morton, 1958). In cardiac muscle, too, NAD concentration increased as cell proliferation ceased (Claycomb, 1976). For NAD to control poly(ADP-ribose) synthesis the intracellular concentrations would have to be limiting with respect to its affinity for poly(ADP-ribose) polymerase. The K_m values found in the present studies (0.043 mM-NAD⁺ in 20% and 0.5 mM-NAD⁺ in 5% O₂) agree with values reported for other tissues, which range from 0.07 to 0.3 mM-NAD⁺. NAD⁺ concentrations vary from 0.1 to 1 mM depending on the type of tissue and its proliferative behaviour (Hilz & Stone, 1976). Thus, since the K_m values are so close to the intracellular NAD concentrations, regulation of poly(ADP-ribose) polymerase by change in concentration of NAD⁺ substrate remains a distinct possibility. Moreover, the partial pressure of O₂ by altering the [NAD⁺]/[NADH] ratio would tend to amplify such regulation.

The greatly different K_m and V_{max} values as well as different pH optima found with different O₂ concentrations in the present study suggest yet other possible modes of regulation of poly(ADP-ribose) synthesis by O₂. Such kinetic differences may result from induced conformational change of the enzyme, the presence of cofactors, inhibitors, activators or modifiers. However, since the effects of O₂ take several days to become manifest, we consider that the most attractive and likely explanation for the different kinetics is that the change in O₂ concentration causes induction of isoenzymes that have different functional

characteristics. Although much less than the 11-fold difference in K_m values found in the present studies, Kidwell & Burdette (1974) found the K_m for polymer synthesis in HeLa cells to be 0.42 mM-NAD⁺ in S-phase nuclei and 0.75 mM-NAD⁺ in G₂-phase nuclei. On this basis they suggested the presence of two or more polymerase activities. Dungan *et al.* (1974) also postulated the existence of multiple forms of poly(ADP-ribose) polymerase, based on differences in the properties of crude 'soluble' and 'particulate' enzyme preparations. The occurrence of two very different polymerase activities in 20% and 5% O₂ in our studies as shown by differences in values for K_m , V_{max} , and pH optima is somewhat analogous to the behaviour of lactose dehydrogenase. When cultured chick-embryo heart cells are subjected to hypoxic conditions a shift of isoenzymes occurs from aerobic to anaerobic forms (which contain a greater proportion of M subunits) (Lindy & Rajasalmi, 1966). Thus our data suggest that poly(ADP-ribose) metabolism participates in the regulation of heart-cell division in culture by O₂, possibly by altering polymerase activities as well as concentration of NAD⁺ substrate.

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