Poly(Adenosine Diphosphate Ribose) Polymerase Activity and Nicotinamide Adenine Dinucleotide in Differentiating Cardiac Muscle*

By WILLIAM C. CLAYCOMB

*Department of Cell Biophysics, Baylor College of Medicine,
Houston, Tex. 77025, U.S.A.*

(Received 18 August 1975)

Poly(ADP-ribose) polymerase activity in nuclei isolated from differentiating cardiac muscle of the rat has been characterized and its activity measured during development. Optimum enzyme activity is observed at pH 8.5. Poly(ADP-ribose) polymerase is inhibited by ATP, thymidine, nicotinamide, theophylline, 3-isobutyl-1-methylxanthine and caffeine and stimulated by actinomycin D. The activity measured under optimal assay conditions increases during differentiation of cardiac muscle and is inversely related to the rate of DNA synthesis and to the activities of DNA polymerase α and thymidine kinase. When DNA synthesis and the activity of DNA polymerase α are inhibited in cardiac muscle of the 1-day-old neonatal rat by dibutyryl cyclic AMP or isoproterenol, the specific activity of poly(ADP-ribose) polymerase measured in isolated nuclei is increased. The concentration of NAD⁺ in cardiac muscle increases during postnatal development. In the adult compared with the 1-day-old neonatal rat the concentration of NAD⁺ relative to fresh tissue weight, DNA or protein increased 1.7-fold, 5.2-fold or 1.4-fold respectively. The concentration of NAD⁺ in cardiac muscle of the 1-day-old neonatal rat can be increased by approx. 20% by dibutyryl cyclic AMP. These data suggest that NAD⁺ and poly(ADP-ribose) polymerase may be involved with the repression of DNA synthesis and cell proliferation in differentiating cardiac muscle.

The eukaryotic cell nucleus contains an enzyme which catalyses a reaction involving the sequential transfer of the ADP-ribose moiety of NAD⁺ to histones and other chromosomal proteins, resulting in the formation of a homopolymer of ADP-ribose residues [poly(ADP-ribose)] and the release of nicotinamide (Chambon *et al.*, 1963, 1966; Fujimura *et al.*, 1967; Nishizuka *et al.*, 1967, 1969; Ueda *et al.*, 1975). Poly(ADP-ribose) polymerase has been identified as the enzyme catalysing this reaction. Approx. 90% of the total activity of this enzyme 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 is unclear. Results of several investigators suggest that poly(ADP-ribose) may be involved with the regulation of DNA replication and cell proliferation (Burzio & Koide, 1970, 1971; Smulson & Rideau, 1972; Smulson *et al.*, 1971, 1975; Roberts *et al.*, 1973, 1974; Kidwell & Burdette, 1974). The chemistry and possible biological functions of

poly(ADP-ribose) and poly(ADP-ribose) polymerase have been reviewed (Sugimura, 1973; Honjo & Hayaishi, 1973) and were the subject of two recent meetings [*Proceedings of the Fogarty International Center Symposium on Poly(ADP-Ribose)*, 1974; *Proceedings of the Seminar on Poly(ADP-Ribose) and ADP-Ribosylation of Protein*, 1975].

DNA synthesis and hence cell proliferation in terminally differentiating cardiac muscle of the rat decrease progressively after birth and essentially cease by the middle of the third week of postnatal development (Claycomb, 1973, 1975a). Temporally correlated with this loss in synthetic activity is an almost complete loss in activity of DNA polymerase α and thymidine kinase (Claycomb, 1973, 1975a; Gillette & Claycomb, 1974). The activity of DNA polymerase β remains relatively constant during postnatal development (Claycomb, 1975a). The studies reported in the present paper were undertaken to determine what relationship, if any, the activity of poly(ADP-ribose) polymerase has to cell proliferation and cell differentiation in cardiac muscle and to investigate further the regulation of DNA synthesis in this tissue. A preliminary account of some of these observations has been reported (Claycomb, 1975b).

* This is Paper III in a series concerning biochemical aspects of cardiac-muscle cell proliferation and cell differentiation. Gillette & Claycomb (1974) is the preceding paper of this series.

Experimental

[adenine-2,8- ^3H]NAD $^+$ (sp. radioactivity 3.46 Ci/mmol) was purchased from New England Nuclear (Boston, Mass., U.S.A.); [methyl- ^3H]thymidine (sp. radioactivity 15.8 Ci/mmol) and [^3H]dTTP (sp. radioactivity 30 Ci/mmol) were from Amersham/Searle (Arlington Heights, Ill., U.S.A.); isoproterenol was from Winthrop Laboratories (New York, N.Y., U.S.A.); theophylline was from Nutritional Biochemicals (Cleveland, Ohio, U.S.A.); 3-isobutyl-1-methylxanthine was from Aldrich Chemical Co. (Milwaukee, Wis., U.S.A.); cyclic AMP, dibutyryl cyclic AMP (6-*N*,2'-*O*-dibutyryl-adenosine 3':5'-cyclic monophosphate), cyclic GMP, dibutyryl cyclic GMP (2-*N*,2'-*O*-dibutyryl-guanosine 3':5'-cyclic monophosphate), NAD $^+$ and yeast alcohol dehydrogenase (395 units/mg of protein) were from Sigma Chemical Co. (St. Louis, Mo., U.S.A.); actinomycin D was from Sigma or Merck, Sharp and Dohme (West Point, Pa., U.S.A.); and Eagle's minimum essential medium containing Hanks' salt base was from Microbiological Associates (Bethesda, Md., U.S.A.).

Timed-pregnant rats were obtained from Holtzman (Madison, Wis., U.S.A.) on the 14th day of gestation. They were housed in individual cages and maintained on water and standard laboratory chow *ad libitum*. Neonatal rats were raised in litters of ten.

Isolation of nuclei

Neonatal rats were killed by decapitation without anaesthesia. The hearts were quickly removed, atria and large vessels trimmed away, and the ventricles were rinsed in 0.15 M-NaCl, blotted dry and weighed. A 5% (w/v) homogenate of minced ventricular cardiac muscle was prepared in ice-cold 10 mM-Tris/HCl (pH 7.0) containing 300 mM-sucrose, 2 mM-MgCl $_2$, 4 mM-EGTA [ethanedioxybis(ethylamine)-tetra-acetic acid] and 1 mM- β -mercaptoethanol (homogenizing buffer) with a glass homogenizer fitted with a motor-driven Teflon pestle (30–45 s at maximum speed). The homogenate was filtered through four layers of cheesecloth and centrifuged at 750 g for 10 min at 4°C. The crude nuclear pellet was washed twice with homogenizing buffer containing 0.5% Triton X-100. The pellet was suspended with a glass stirring rod in 30 ml of homogenizing buffer containing 2.4 M-sucrose, and nuclei were sedimented by centrifugation at 58 000 g for 60 min at 4°C in a Beckman SW 25.1 rotor. The purified nuclei were suspended in 1 mM-Tris/HCl, pH 7.5, containing 300 mM-sucrose and 1 mM- β -mercaptoethanol at a final DNA concentration of 200–400 $\mu\text{g}/\text{ml}$. The nuclear preparations were free of whole cells and cytoplasmic contamination as judged by light- and electron-microscopy. The protein/DNA ratio of nuclei isolated from cardiac muscle at different times

during development ranged between 2.22 and 3.83; the mean $\pm$ S.E.M. of 59 preparations was 3.05 ± 0.07 .

Assay of poly(ADP-ribose) polymerase

Poly(ADP-ribose) polymerase activity was assayed by measuring the initial rate of incorporation of [^3H]NAD $^+$ into an acid-insoluble product. The standard reaction mixture contained the following in a total volume of 0.25 ml: 100 mM-Tris/HCl (pH 8.5); 2.5 mM-MgCl $_2$; 1 mM- β -mercaptoethanol; 0.29 nmol (2×10^6 d.p.m.) of [^3H]NAD $^+$ and nuclei (approx. 5 μg of nuclear DNA). Reactions were initiated by addition of nuclei. The incubation was carried out at 25°C for 2.5 min in a water bath with continuous shaking, and stopped by addition of 2 ml of ice-cold 10% (w/v) trichloroacetic acid. The acid-insoluble material collected by suction filtration on Whatman GF/C glass-fibre filters was washed with 20 ml of ice-cold 5% (w/v) trichloroacetic acid. The filters were placed in scintillation vials, dried in an oven and counted for radioactivity in a Packard Tri-Carb liquid-scintillation spectrometer in 10 ml of a scintillation fluid containing 4 g of PPO (2,5-diphenyl-oxazole) and 0.05 g of dimethyl-POPOP [1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene] per litre of toluene. Corrections for quenching were made by using automatic external standardization. The results were corrected for zero-time controls, which were less than 50 d.p.m. above background. Unless otherwise indicated, the specific activity of poly(ADP-ribose) polymerase is expressed as pmol of [^3H]ADP-ribose incorporated per μg of DNA in 2.5 min at 25°C. Values reported in this paper were obtained by using nuclei that had been isolated and frozen. Enzyme activity was similar in freshly isolated nuclei and in nuclei that had been frozen and thawed once or twice. An appreciable loss of enzyme activity was observed when nuclei were frozen and thawed repeatedly.

Assay of DNA synthesis *in vivo*

DNA synthesis in cardiac muscle (as determined by [^3H]thymidine incorporation into DNA) was measured as previously described (Claycomb, 1975a).

Assay of DNA synthesis *in vitro*

Tissue slices of ventricular cardiac muscle were prepared with a Stadie-Riggs tissue slicer. Approx. 150 mg of tissue was incubated for 2 h at 37°C in 5 ml of Eagle's minimum essential medium with [^3H]thymidine (5 $\mu\text{Ci}/\text{ml}$) in a 25 ml Erlenmeyer flask. At the end of the incubation, the tissue was homogenized in 2.0 ml of 0.15 M-NaCl, and [^3H]thymidine incorporation into DNA was measured as previously described (Claycomb, 1975a). [^3H]Thymidine incorporation under these incubation conditions is linear with time for at least 4 h.

Assay of DNA polymerase

DNA polymerase α activity was determined as previously described (Claycomb, 1975a) with activated DNA as the primer-template.

Extraction and assay of NAD⁺

The concentration of NAD⁺ in extracts of cardiac muscle was measured by using alcohol dehydrogenase and following the change in extinction at 340 nm. The extraction procedure is modified from one used previously (Claycomb & Kilsheimer, 1969). Samples of tissue (0.5 and 1.0 g) were homogenized in 4.0 ml of ice-cold 0.15 M-NaCl. Portions of the homogenate were taken for protein and DNA determinations. To the remainder of the homogenate enough 70% (w/v) HClO₄ was added to make a final concentration of 10%. The homogenate was chilled on ice for 45 min and centrifuged at high speed in a clinical centrifuge. The pH of the decanted supernatant fraction was adjusted to 8.0 with 5 M-K₂CO₃ and centrifuged to remove insoluble KClO₄.

The cuvette contained the following in a total volume of 3.65 ml: 2 mmol of ethanol; 500 μ mol of glycylglycine, pH 9.5; 3.0 ml of the tissue extract; and

Fig. 1. Poly(ADP-ribose) polymerase activity as a function (a) of nuclear DNA concentration and (b) time of incubation

Reactions were carried out under standard conditions as described in the text by using nuclei isolated from cardiac muscle of 3-day-old neonatal rats. In determining the time-course, 6.2 μ g of nuclear DNA was used.

Fig. 2. Effect of pH on the activity of poly(ADP-ribose) polymerase

Reactions were carried out under standard conditions by using nuclei (5.7 μ g of DNA) isolated from cardiac muscle of 5-day-old neonatal rats. The final concentration of Tris/HCl was 100 mM.

0.125 mg of alcohol dehydrogenase, freshly prepared in 10 mM-Tris/HCl, pH 8.0. The reaction was initiated by addition of enzyme and was complete within 1.0 min. The change in extinction at 340 nm was measured at 22°C in a Gilford model 2000 spectrophotometer. The reaction was linear with an NAD⁺ concentration over the range 10–1000 nmol. All assays were carried out within 6 h of killing the animals. Results are corrected for loss of NAD⁺ during the extraction procedure, which was approx. 55%. In all experiments recovery was determined by adding a known amount of NAD⁺ to the initial tissue homogenate.

Chemical assays

DNA was measured by the Burton (1956) modification of the diphenylamine reaction with calf thymus DNA as a standard. Protein was determined by the procedure of Lowry *et al.* (1951), with crystalline bovine serum albumin as a standard.

Results

Characterization of poly(ADP-ribose) polymerase

The activity of poly(ADP-ribose) polymerase in purified nuclei is linear for the first 5 min of incubation at 25°C and directly proportional to nuclear DNA concentration up to at least 18 μ g (Fig. 1). The pH optimum for the reaction is 8.5 (Fig. 2). The pH optimum for poly(ADP-ribose) polymerase has been reported to be 8.0 in nuclei isolated from rat liver and 8.6 in nuclei from bovine spleen and Ehrlich ascites cells (Fujimura *et al.*, 1967; Nakazawa *et al.*, 1968; Dungan *et al.*, 1974).

Table 1. *Effect of various compounds on poly(ADP-ribose) polymerase activity*

Reactions were carried out by using nuclei prepared from cardiac muscle of either 3- or 5-day-old neonatal rats. Standard reaction conditions are given in the text. DNA content per reaction mixture was approx. 5.0 μ g. Each value is the mean of three to six determinations.

Reaction mixture	Enzyme activity (% of control)
Complete (control)	100
Omissions	
Mg ²⁺	78
β -Mercaptoethanol	59
Additions	
NaF (4 mM)	95
KCl (50 mM)	96
ATP (5 mM)	84
Nicotinamide (1 mM)	2
Thymidine (1 mM)	5
Cyclic AMP (1 mM)	97
Dibutyl cyclic AMP (1 mM)	94
Cyclic GMP (1 mM)	92
Dibutyl cyclic GMP (1 mM)	94
5'-AMP (1 mM)	92
Isoproterenol (1 mM)	96
Theophylline (1 mM)	6
3-Isobutyl-1-methylxanthine (1 mM)	18
Caffeine (1 mM)	15
Actinomycin D (20 μ g/ml)	128

The effect of various compounds on the activity of poly(ADP-ribose) polymerase is summarized in Table 1. Mg²⁺ and β -mercaptoethanol are both needed for full enzymic activity. The optimum Mg²⁺ concentration was determined to be 2.5 mM. Several investigators (Fujimura *et al.*, 1967; Nishizuka *et al.*, 1967; Lehmann & Shall, 1972; Müller *et al.*, 1974; Lehmann *et al.*, 1974) include NaF and KCl in their reaction mixtures for assay of poly(ADP-ribose) polymerase. These compounds had little effect on cardiac-muscle poly(ADP-ribose) polymerase activity. ATP inhibited slightly. Since NAD⁺ can be formed in isolated nuclei from ATP and NMN (Fujimura *et al.*, 1967), this inhibition by ATP may be due to a dilution of [³H]NAD⁺ in the reaction mixture by formation of non-radioactive NAD⁺. Nicotinamide, an end product of the ADP-ribosylation reaction, and thymidine inhibited almost completely. Inhibition by these compounds has also been noted by others (Fujimura *et al.*, 1967; Burzio & Koide, 1970; Clark *et al.*, 1971; Preiss *et al.*, 1971). 5'-AMP, cyclic AMP, dibutyl cyclic AMP, cyclic GMP, dibutyl cyclic GMP and isoproterenol had little effect on enzyme activity. The methylxanthines theophylline, 3-isobutyl-1-methylxanthine and caffeine greatly inhibit

the activity of poly(ADP-ribose) polymerase. Actinomycin D was found to stimulate activity. This stimulation was observed with actinomycin D obtained from two different commercial sources.

Activity of poly(ADP-ribose) polymerase during postnatal development

The specific activity of poly(ADP-ribose) polymerase in nuclei isolated from cardiac muscle at various times during postnatal development is shown in Fig. 3. Enzyme activity more than doubles between day 1 and day 16, and a further increase in activity is observed in nuclei isolated from cardiac muscle of the adult. This same pattern is also observed if enzyme activity is expressed relative to nuclear protein or if the incubation time for the reaction is extended to 5 min.

Effect of dibutyl cyclic AMP and isoproterenol on poly(ADP-ribose) polymerase, DNA synthesis and DNA polymerase α

The rate of DNA synthesis decreases (Claycomb, 1975a) as intracellular concentrations of cyclic AMP increase (Novák *et al.*, 1972) in cardiac muscle of the rat during early postnatal development. Previous work has shown that dibutyl cyclic AMP and isoproterenol inhibit DNA synthesis in differentiating cardiac muscle when administered *in vivo* or when added to tissue slices *in vitro* (Claycomb, 1975b,c). Therefore it was decided to determine what effect inhibition of DNA synthesis would have on the activity of poly(ADP-ribose) polymerase. Results of a representative experiment are summarized in

Fig. 3. *Poly(ADP-ribose) polymerase activity in nuclei isolated from cardiac muscle during postnatal development and from the adult*

Reactions were carried out under standard conditions by using between 4 and 8 μ g of nuclear DNA.

Table 2. Effect of dibutyryl cyclic AMP and isoproterenol on poly(ADP-ribose) polymerase activity, DNA synthesis and activity of DNA polymerase α in differentiating cardiac muscle

Newborn rats were injected subcutaneously with either dibutyryl cyclic AMP (25 mg/kg) together with theophylline (50 mg/kg), or isoproterenol (25 mg/kg) together with theophylline (50 mg/kg). Theophylline was injected along with dibutyryl cyclic AMP and isoproterenol, since it had been determined previously that it potentiated the effect of both of these agents (W. C. Claycomb, unpublished work). Control litter-mates received 0.15 M-NaCl. All injections were made in 0.1 ml at approx. 6:00 p.m. Then 15 h later the animals in which DNA synthesis *in vivo* was being measured were injected subcutaneously with [³H]thymidine (2 μ Ci/kg), and 1 h later, all of the animals were killed and the various assays carried out as described in the text. Hearts from 15 to 20 animals were pooled to isolate nuclei for each poly(ADP-ribose) polymerase determination.

	Poly(ADP-ribose) polymerase activity (% of control*)	DNA synthesis <i>in vivo</i> (% of control†)	DNA synthesis <i>in vitro</i> (% of control‡)	DNA polymerase α activity (% of control§)
Dibutyryl cyclic AMP	162	19	42	56
Isoproterenol	149	14	36	64

* Control activity was 0.092 pmol of [³H]ADP-ribose incorporated/ μ g of DNA.

† Control [³H]thymidine incorporation was 1092 d.p.m./ μ g of DNA.

‡ Control [³H]thymidine incorporation was 5268 d.p.m./ μ g of DNA.

§ Control activity was 29.1 pmol of [³H]dTMP incorporated/mg of protein.

Table 3. Concentrations of NAD⁺, DNA and protein in cardiac muscle of the developing and adult rat

The concentrations of NAD⁺, DNA and protein in samples of fresh tissue were determined as described in the text. Values are the means $\pm$ S.E.M. The numbers of individual determinations are given in parentheses. Adults were females that weighed between 250 and 325 g. One-day-old neonatal rats were injected with dibutyryl cyclic AMP (25 mg/kg) together with theophylline (50 mg/kg) at approx. 6:00 p.m. Control litter-mates received 0.15 M-NaCl. Then 16 h later the animals were killed and 10–30 hearts were pooled for each NAD⁺ determination. Further details are given in the legend to Table 2 and in the text.

Postnatal development (days)	NAD ⁺ content			DNA (mg/g wet wt.)	Protein content	
	(nmol/g wet wt.)	(nmol/mg of DNA)	(nmol/mg of protein)		(mg/g wet wt.)	(mg/mg of DNA)
1 (12)	232 $\pm$ 14	73.5 $\pm$ 3.7	1.72 $\pm$ 0.08	3.24 $\pm$ 0.11	139 $\pm$ 10	43
1 + Dibutyryl cyclic AMP (7)	279 $\pm$ 17*	87.7 $\pm$ 4.6*	2.08 $\pm$ 0.11*			
8 (9)	317 $\pm$ 14	91.7 $\pm$ 4.5	1.97 $\pm$ 0.12	3.38 $\pm$ 0.18	153 $\pm$ 9	45
16 (6)	340 $\pm$ 25	100.7 $\pm$ 7.9	2.20 $\pm$ 0.08	3.25 $\pm$ 0.20	159 $\pm$ 16	49
22 (4)	372 $\pm$ 26	136.5 $\pm$ 15.4	2.25 $\pm$ 0.12	2.77 $\pm$ 0.21	166 $\pm$ 17	60
Adult (12)	402 $\pm$ 22	379.4 $\pm$ 18.5	2.34 $\pm$ 0.13	1.04 $\pm$ 0.13	183 $\pm$ 7	176

* $P < 0.05$; P values calculated by Student's t test.

Table 2. For comparison, the rate of DNA synthesis measured *in vivo* and *in vitro* and the activity of DNA polymerase α were also determined in this same experiment. Both dibutyryl cyclic AMP and isoproterenol increase the specific activity of poly(ADP-ribose) polymerase measured in isolated nuclei and inhibit DNA synthesis and the activity of DNA polymerase α . The stimulation of poly(ADP-ribose) polymerase in ten separate experiments ranged between 126 and 263% of control activity, with a mean of 156 for dibutyryl cyclic AMP and a mean of 140 for isoproterenol. A similar degree of stimulation was observed if the specific activity of the enzyme was expressed relative to nuclear protein. In animals older than 3–4 days the stimulation of poly(ADP-ribose) polymerase by

dibutyryl cyclic AMP and isoproterenol was not as pronounced.

Concentrations of NAD⁺, DNA and protein in cardiac muscle during development

Tissue concentrations of NAD⁺ in cardiac muscle increase during postnatal development and can be increased by approx. 20% in the 1-day-old neonatal rat by the administration of dibutyryl cyclic AMP along with theophylline (Table 3). The growth pattern and DNA and protein content of cardiac muscle should be considered when expressing tissue concentrations of NAD⁺, or for that matter any other cellular metabolite. The concentration of NAD⁺ expressed on a DNA or per-cell basis is 5.2-fold higher in cardiac muscle of the adult compared with the

1-day-old neonatal rat (Table 3). If the data are expressed on a wet-tissue-weight or protein basis the increase is 1.7-fold or 1.4-fold respectively. Growth of cardiac muscle during early development is due primarily to cell proliferation (Goss, 1966). In cardiac muscle of the rat DNA synthesis essentially ceases by the middle of the third week of postnatal development and subsequent growth is due to cell enlargement (Claycomb, 1975a; Sasaki *et al.*, 1968). The data in Table 3 reflect this change in growth pattern. DNA content per g of fresh muscle is approx. 3-fold less in tissue from the adult compared with that from 1-day- to 2-week-old neonatal rats. Assuming that the amount of DNA per cell does not change during development this would suggest that there are fewer cells per g wet weight of tissue in the adult. This is supported by histometric determinations which show that cardiac-muscle cells of the adult rat are approx. 10 times larger than those of the 1-day-old neonatal rat (Sasaki *et al.*, 1968). It is also noteworthy that the amount of protein per cell (mg of protein/mg of DNA) is approx. 4-fold higher in cardiac muscle of the adult compared with the 1-day-old neonatal rat (Table 3). This is due to the acquisition of the contractile proteins during muscle differentiation.

Discussion

Data presented in this paper show that the activity of poly(ADP-ribose) polymerase and the intracellular concentration of NAD^+ in differentiating cardiac muscle of the rat are inversely related to the rate of DNA synthesis and cell proliferation. The activity of poly(ADP-ribose) polymerase and the synthesis of poly(ADP-ribose) have been reported to be positively correlated (Colyer *et al.*, 1973; Lehmann *et al.*, 1974), negatively correlated (Smulson & Rideau, 1972; Smulson *et al.*, 1975; Brightwell *et al.*, 1975), or not correlated at all (Hilz & Kittler, 1971) with the DNA-synthetic activity of isolated nuclei or with the rate of DNA synthesis and cell proliferation in tissues or cells in culture. NAD^+ decreases $[^3\text{H}]\text{dTTP}$ incorporation and template activity of chromatin towards exogenous DNA polymerase in nuclei isolated from normal and regenerating tissue; this inhibition is not observed in nuclei isolated from hepatoma cells, lymphoma cells or lymphocytes stimulated to proliferate with phytohaemagglutinin (Burzio & Koide, 1970, 1971, 1972, 1973; Lehmann & Shall, 1972; Nagao *et al.*, 1972). The ADP-ribosylation of proteins in nuclei isolated from HeLa cells is reported to enhance template activity towards endogenous or exogenous DNA polymerase (Roberts *et al.*, 1973, 1974).

The increase in intracellular concentrations of NAD^+ and poly(ADP-ribose) polymerase activity in

cardiac muscle during the first few weeks after birth may be related to the decline in DNA synthesis. During this period of postnatal development there is a 30–40-fold decrease in the rate of $[^3\text{H}]\text{thymidine}$ incorporation into DNA (Claycomb, 1975a). The further increase in poly(ADP-ribose) polymerase activity and NAD^+ concentration after the second week of postnatal development may be concerned with terminal cell differentiation and enlargement and with the increased number of mitochondria in the enlarged cardiac-muscle cell. It is entirely possible, however, that the increased activity of poly(ADP-ribose) polymerase observed in nuclei isolated from cardiac muscle of the adult reflects a change in the ratio of muscle to non-muscle cells. Approx. 58% of the total cell population of the heart of the 1-day-old neonatal rat are cardiac-muscle cells; this changes to 50% 14 days after birth (Sasaki *et al.*, 1968). Growth of the heart after the third week of postnatal development is due primarily to cardiac-muscle cell enlargement and to some extent to the slow accumulation of non-muscle cells. In the adult only 20–25% of the total number of cells in the heart are actually myocytes (Morkin & Ashford, 1968; Sasaki *et al.*, 1968); the remaining 75–80% are fibroblasts, circulating erythrocytes and cells comprising nerve and vascular tissue. Therefore in cardiac muscle the assignment of a biological function or biochemical parameter to a specific cell type or to an organelle isolated from a specific cell type is difficult. This, however, is a problem encountered in studies of all heterogeneous tissues.

Biosynthesis and turnover of NAD^+ apparently occur in the nucleus (Nishizuka *et al.*, 1969; Rechsteiner & Catanzarite, 1974), although it has been reported that mitochondria are capable of synthesizing NAD^+ and also contain an enzyme system which transfers the ADP-ribose moiety of NAD^+ to proteins (Kun *et al.*, 1975; Grunicke *et al.*, 1975). The function of NAD^+ in the nucleus is unclear. Morton (1958) suggested that NAD^+ is important in the control of cell division. Low intracellular concentrations of NAD^+ are characteristic of rapid cell division in several tissues and NAD^+ concentrations increase as cell proliferation ceases (Caiger *et al.*, 1962; Burch & Von Dippe, 1964; Clark & Greenbaum 1966; Jacobson *et al.*, 1974). This is also true of differentiating cardiac muscle. The ADP-ribosylation of proteins in nuclei has been reported to be proportional to the concentration of NAD^+ (Roberts *et al.*, 1974). Therefore NAD^+ may function in the repression of DNA synthesis by chemically modifying chromosomal or other nuclear proteins and enzymes by ADP-ribosylation.

Finally, it remains to be determined whether the increase in NAD^+ concentration and activity of poly(ADP-ribose) polymerase in cardiac muscle, both during development and by dibutyl cyclic

AMP, result in, or are a result of, an inhibition of DNA synthesis.

I thank Susan Bryde for her conscientious technical assistance. This investigation was supported by grants from the American Heart Association, the Muscular Dystrophy Associations of America and by Grant HL 17269-01 from the National Institutes of Health, and was carried out during the tenure of an Established Investigatorship from the American Heart Association.

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