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The Synthesis of Nicotinamide-Adenine Dinucleotide and Poly (Adenosine Diphosphate Ribose) in Various Classes of Rat Liver Nuclei

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1. The activities of NMN adenylyltransferase and an enzyme that synthesizes poly (ADP-ribose) from NAD were investigated in the various classes of rat liver nuclei fractionated by zonal centrifugation. 2. The highest specific activities of these two nuclear enzymes occur in different classes of nuclei. In very young and in mature rats it was shown that a correlation exists between DNA synthesis and NMN adenylyltransferase activity, but in rats of intermediate age this correlation is less evident. The highest activities of the enzyme that catalyses formation of poly (ADP-ribose) are in the nuclei involved in the synthesis of RNA. 3. The significance of these results in relation to NAD metabolism is discussed.

Nuclei are readily fractionated in the type A zonal rotor (Johnston, Mathias, Pennington & Ridge, 1968*a*). A study of the synthesis of nucleic acids by using this technique showed that rat liver nuclei may be separated into four classes (Johnston, Mathias, Pennington & Ridge, 1968*b*). Stromal nuclei (2nS) synthesize little RNA or DNA. Both diploid and tetraploid parenchymal nuclei (2nP, 4nP) are active in the synthesis of RNA, whereas the synthesis of DNA is concentrated in a fourth class of nuclei, intermediate in size between the diploid and tetraploid. The capacity to incorporate orotic acid *in vivo* into RNA was found to parallel, in general, the distribution of RNA polymerase.

The importance of the study of the enzymes of nucleic acid synthesis in nuclei is obvious. However, it has been claimed that many other enzymes occur in nuclei (Siebert, 1968), including several involved in the metabolism of NAD. Two enzymes of importance in this respect, which appear to be exclusively of nuclear location, are ATP–NMN adenylyltransferase (NAD pyrophosphorylase, EC 2.7.7.1) and an enzyme that uses NAD to make a polymer of ADPribose, called here poly (ADP-ribose) polymerase (Chambon, Weill & Mandel, 1963; Chambon, Weill, Doly, Strosser & Mandel, 1966; Nishizuka, Ueda, Nakazawa & Hayaishi, 1967; Reeder, Ueda, Honjo, Nishizuka & Hayaishi, 1967; Fujimura, Hasegawa & Sugimura, 1967; Sugimura, Fujimura, Hasegawa & Kawamura, 1967). Although NMN adenylyltransferase has long been recognized as a nuclear enzyme (Hogeboom & Schneider, 1952; Branster & Morton, 1956) the significance of its location has remained obscure. The role of poly (ADP-ribose) polymerase and of its product is even more enigmatic. As a preliminary to studies on the function of these enzymes we have investigated their distribution in the various nuclear classes that are separable by zonal centrifugation.

MATERIALS AND METHODS

Animals. Norwegian hooded rats were used except where stated. They were fed *ad libitum* on Purina chow.

Isolation of nuclei. The nuclei were prepared from rat liver by a modification of the method of Widnell & Tata (1964) and separated into classes by zonal centrifugation as described by Johnston *et al.* (1968*a*). The gradient was recovered in fractions (12·5ml.) and the nuclei from each zonal fraction were harvested by centrifugation at 2000*g* for 10min. at 4° and washed by resuspension in 0·25M-sucrose–1mM-$MgCl_2$.

Reagents. ATP, NMN, alcohol dehydrogenase and tris were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, W. Germany; bovine pancreatic deoxyribonuclease (electrophoretically pure) was from Sigma (London) Chemical Co. Ltd., London S.W.6; bovine pancreatic ribonuclease (four-times crystallized) was from Seravac Laboratories, Maidenhead, Berks.; phosphodiesterase (*Bothrops atrox*) was from BDH (Chemicals) Ltd., Poole, Dorset; DEAE-cellulose (Whatman DE 52) was obtained from W. and R. Balston Ltd., Maidstone, Kent; [*adenine*-8-^{14}C]ATP, [5-^{3}H]orotic acid and [6-^{3}H]thymidine were obtained from The Radiochemical Centre, Amersham, Bucks.

Preparation of [^{14}C]*NAD.* [^{14}C]NAD was prepared from NMN and [*adenine*-8-^{14}C]ATP by using yeast NMN adenylyltransferase (Kornberg, 1950). It was purified by two successive runs on DEAE-cellulose columns with a linear

gradient of 0–0·1 M-NH_4HCO_3 (Chaykin, 1965). On DEAE-cellulose paper, run with 0·1 M-tris–HCl buffer, pH 8·0, the product was 97% pure on a radioactivity basis. The [^{14}C]-NAD had specific radioactivities of 500–830 d.p.m./nmole.

Enzyme assays. NMN adenylyltransferase was assayed by a method similar to that of Kornberg (1950). The incubation mixture contained 1·5 μmoles of NMN, 2·5 μmoles of ATP, 150 μmoles of nicotinamide, 50 μmoles of glycylglycine–KOH buffer, pH 7·4, 7·5 μmoles of $MgCl_2$ and nuclei in a total volume of 0·5 ml. After incubation for 20 min. at 37° the reaction was stopped by adding 0·5 ml. of 10% (w/v) trichloroacetic acid at 0° or by freezing in a methylCellosolve–solid CO_2 mixture and the product was stored overnight at −40°. After removal of the precipitate by centrifugation, the supernatant was neutralized with 2 M-NaOH and the NAD content determined in an 0·5 ml. sample by the method of Klingenberg (1965). The specific activity of NMN adenylyltransferase is expressed as μmoles of NAD synthesized/hr./10^9 nuclei.

The production of NAD was a linear function of the numbers of nuclei throughout and beyond the range of those actually used in the assay of the various fractions. In the procedure for the assay of NMN adenylyltransferase we have established that the concentration of nicotinamide employed (0·3 M) was sufficient to inhibit completely any degradation of NAD formed in the assay.

Poly (ADP-ribose) polymerase was assayed as described by Nishizuka *et al.* (1967), but with a higher concentration of [^{14}C]NAD in the incubation mixture, which contained 60 nmoles of [^{14}C]NAD, 7·5 μmoles of $MgCl_2$, 1 μmole of NaF, 1 μmole of 2-mercaptoethanol, 15 μmoles of KCl, 25 μmoles of tris–HCl buffer, pH 7·4, and sonicated nuclei in a total volume of 0·25 ml. After incubation for 15 min. at 37° the reaction was stopped with 1 μmole of unlabelled NAD followed immediately by 2 ml. of ice-cold 5% (w/v) trichloroacetic acid. The mixture was then filtered on a Whatman GF/C glass-fibre filter and dried, and its radioactivity was counted in a liquid-scintillation spectrometer. The specific activity of poly (ADP-ribose) polymerase is expressed as nmoles of [^{14}C]ADP-ribose incorporated/15 min./10^9 nuclei. Nuclei recovered from the zonal fractions, suspended in 0·1 ml. of 0·01 M-tris–HCl buffer, pH 7·4, were sonicated for 30 sec. with the MSE 100 w ultrasonic disintegrator with a titanium vibrator microprobe (end diam. $\frac{1}{8}$ in.). We have found that if nuclei are sonicated before the assay of poly (ADP-ribose) polymerase the incorporation from [^{14}C]NAD is increased by 50%.

Injections. Radioactive compounds diluted in 0·9% NaCl were injected intraperitoneally 60–90 min. before death.

Measurement of radioactivity. This was done by using the Beckman or Intertechnique liquid-scintillation spectrometer, in 15 ml. of a scintillation fluid containing 4 g. of 2,5-bis-(5-*tert.*-butylbenzoxazol-2-yl)thiophen (CIBA Ltd., Horsham, Sussex) in 1 l. of toluene. Radioactivity in nuclei was solubilized when necessary by the method of MacGregor & Mahler (1967). Methanol (0·5–1 ml.) was included in each vial to eliminate destruction of the scintillator by Hyamine. The counting efficiency was 23% for ^{3}H and 89% for ^{14}C.

Counting of nuclei. Nuclei were counted immediately after dilution in 0·9% NaCl for injection (Evans Medical Ltd., Speke, Liverpool), in a Coulter model F counter with a 100 μm. orifice tube (aperture setting, 64; attenuation, 0·707; threshold, 8).

RESULTS

NMN adenylyltransferase. Initial experiments on adult rats revealed that, although activity was present in the 2nP and 4nP nuclei, the highest specific activity occurred in a peak between these classes of nuclei and in a region just beyond the 4nP zone. Since we had previously shown that nuclei in the zone between 2nP and 4nP were active in DNA synthesis (Johnston *et al.* 1968*b*) we investigated both the incorporation of [^{3}H]thymidine and the specific activity of NMN adenylyltransferase in fractions across the gradient. The results are shown in Fig. 1(*a*). The correlation between DNA synthesis and NMN adenylyltransferase activity is evident. Fig. 1(*a*) also shows that a second zone of DNA synthesis occurs ahead of the 4nP zone and accompanies the further rise of the specific activity of NMN adenylyltransferase. The second zone of DNA synthesis beyond the 4nP peak suggests the synthesis of octaploid nuclei, of which a small number occur in rats. Studying, by radioautography, nuclei labelled with [^{3}H]thymidine for 2–2$\frac{1}{2}$ hr., Carriere (1969) found them to be of larger diameter than the modal size for diploids or tetraploids but not yet to have reached tetraploid or octaploid size. She suggested that enlargement lagged behind DNA synthesis (Carriere, 1969). This is precisely analogous to the situation we describe here for DNA synthesis.

To investigate the NMN adenylyltransferase situation more fully we studied rats of body weight 25, 60 and 120 g. The results are shown in Fig. 1. In 25 g. rats (Fig. 1*d*), although some correlation exists between NMN adenyltransferase activity and incorporation of [^{3}H]thymidine, there is a substantial peak of enzyme specific activity in nuclei sedimenting more slowly than those involved in DNA synthesis but ahead of the main diploid peak. The 60 g. stage is particularly complex. The transition of liver parenchyma cells from a diploid to a predominantly tetraploid state has entered its most rapid phase, and at the same time binucleate diploid cells have reached their maximum incidence, which may be 35–50% of the total cell population (Carriere, 1969). In the 60 g. rats (Fig. 1*c*) there is a substantial amount of NMN adenylyltransferase activity associated with the diploid nuclei in the absence of significant DNA synthesis. However, the peak of DNA synthesis that has now emerged partially overlaps a peak of enzyme activity. At the 120 g. stage (Fig. 1*b*) the mature pattern has already been established with the heightening of the specific activity between the 2n and 4n peaks.

Two experiments were also carried out with albino rats of 35 g. and 200 g. body weight. The adults showed the same pattern as the adult Norwegian hooded rats. The 35 g. rats (17 days *post*

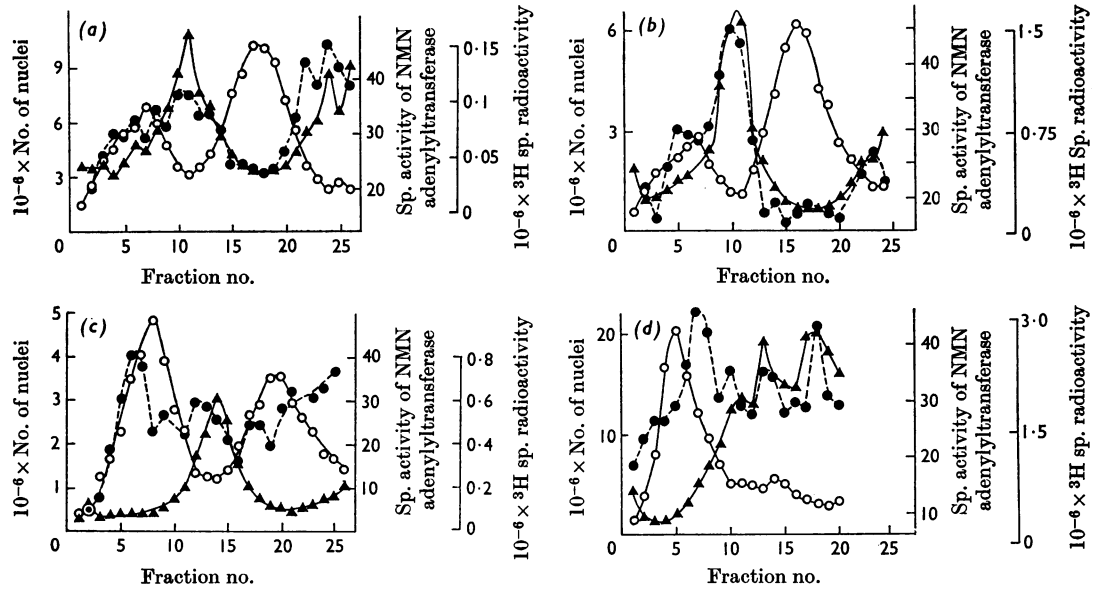


Fig. 1. Distribution of NMN adenylyltransferase activity and the synthesis of DNA *in vivo* in various classes of rat liver nuclei. Norwegian hooded rats of (*a*) 200g., (*b*) 120g., (*c*) 60g. and (*d*) 25g. body wt. were used. The following injections of [^{3}H]thymidine (13·7–25·0c/m-mole) were given: two 200g. rats, 100μc each; three 120g. rats, 50μc each; six 60g. rats, 20μc each; twelve 25g. rats, 30μc each. Nuclei from 12g. wet wt. of liver were used in each experiment. Sedimentation is from left to right. The peak of the diploid nuclei lies between fractions 5 and 10 and the tetraploid between fractions 15 and 20. Fractions were 12·5ml. each; 7ml. was used for the NMN adenylyltransferase assay, 4ml. for measurement of ^{3}H radioactivity and the remainder for Coulter counting. Fraction 1 is at an effluent volume of approx. 220ml., which is at 8·0cm. from the axis of rotation and corresponds to 23·5% (w/w) sucrose and a density at 5° of 1·097. Fraction 25 is at 11·5cm. from the axis (32·6% sucrose, density 1·14). ●, NMN adenylyltransferase activity (μmoles of NAD synthesized/hr./10^9 nuclei); ▲, [^{3}H]thymidine incorporation (c.p.m./10^9 nuclei); ○, no. of nuclei in each NMN adenylyltransferase assay.

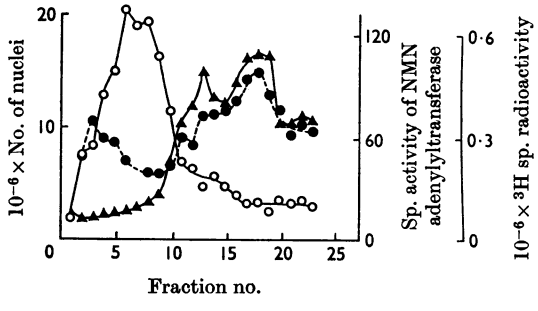


Fig. 2. Distribution of NMN adenylyltransferase activity and the synthesis of DNA *in vivo* in rat liver nuclei from 35g. albino rats. 12g. wet wt. of liver was used for the preparation of nuclei. Conditions were as given for Fig. 1. ●, NMN adenylyltransferase activity (μmoles of NAD synthesized/hr./10^9 nuclei); ▲,[^{3}H]thymidine incorporation (c.p.m./10^9 nuclei). ○, no. of nuclei in each NMN adenylyltransferase assay.

partum), which although heavier were in fact younger than the 25g. Norwegian hooded rats (21 days *post partum*), gave the result shown in Fig. 2. The fact that a reasonable correlation exists between DNA synthesis and NMN adenylyltransferase at this younger stage appears to reinforce the suggestion that the later dissociation of these two features of the nuclear profile may be characteristic of the main phase of the transition from diploid to tetraploid. The point should be made that these patterns, although complex, are nevertheless reproducible.

To show that the patterns of NMN adenylyltransferase were not due to removal of NAD by NAD glycohydrolases or by poly (ADP-ribose) polymerase, a control experiment was performed by incubating each fraction obtained from a zonal run of nuclei from 250g. rats with 0·1μmole of NAD under conditions identical with those of the assay, i.e. in the presence of nicotinamide. No loss of NAD was observed in any fraction when the amount of NAD was assayed by using alcohol dehydrogenase by the method of Klingenberg (1965). In another control experiment the endogenous NAD concentrations in the fractions across the gradient were shown not to contribute signficantly (approx. 2% of NAD synthesized) to the profile of NMN adenylyltransferase.

It might be argued that the activity seen under, for instance, the tetraploid peak is due to overlap from both the slower- and the faster-moving zones of high NMN adenylyltransferase activity. This was shown not to be the case in an experiment with adult rats, in which the central portion of the tetraploid peak (about two-thirds of the total) was purified by re-running it on a zonal gradient (Johnston *et al.* 1968*a*). Since the resulting tetraploid nuclei still retained 75% of their NMN adenylyltransferase specific activity, this strongly suggests that these nuclei do contain substantial NMN adenylyltransferase activity.

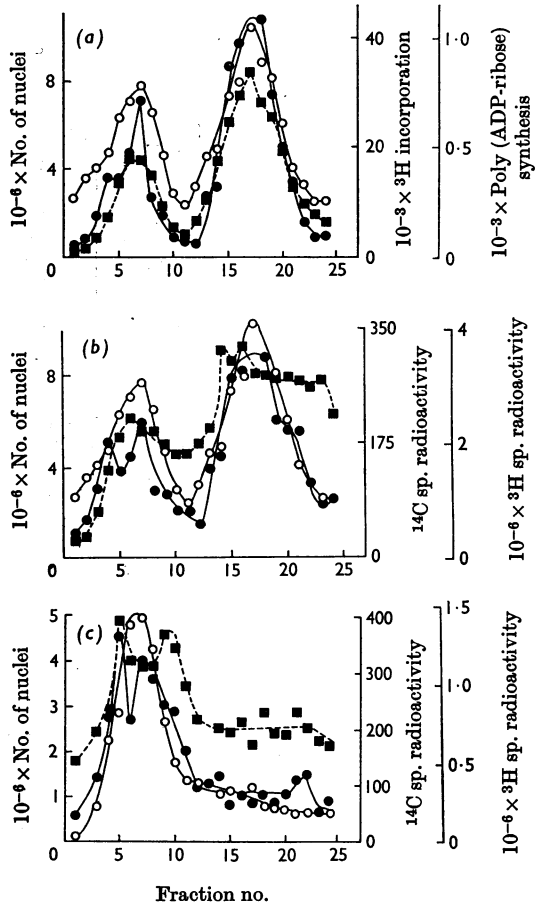


Fig. 3. Distribution of poly (ADP-ribose) polymerase activity and the synthesis of RNA *in vivo* in various classes of rat liver nuclei. The following injections of [^{3}H]orotic acid (1c/m-mole) were given: three 150g. rats, 25μc each; twelve 25g. rats, 6μc each. Two 4ml. portions of each fraction were taken, for the enzyme assay and for the determination of ^{3}H radioactivity; the remainder was used for Coulter counting. Nuclei from 12g. of liver were used in (*a*) and from 11g. in (*c*). (*a*) RNA and poly (ADP-ribose) synthesis in nuclei from 150g. rats: ●, ^{14}C radioactivity (c.p.m.) incorporated into poly (ADP-ribose); ■, ^{3}H radioactivity (c.p.m.) incorporated; ○, no. of nuclei in 4ml. of fraction. (*b*) and (*c*) Specific radioactivities of [^{3}H]RNA and [^{14}C]poly (ADP-ribose) in (*b*) 150g. rats by using data from (*a*) and in (*c*) 25g. rats: ●,^{14}C specific radioactivity (nmoles of [^{14}C]ADP-ribose incorporated/15min./10^9 nuclei); ■, ^{3}H specific radioactivity (c.p.m./10^9 nuclei); ○, no. of nuclei in 4ml. of fraction.

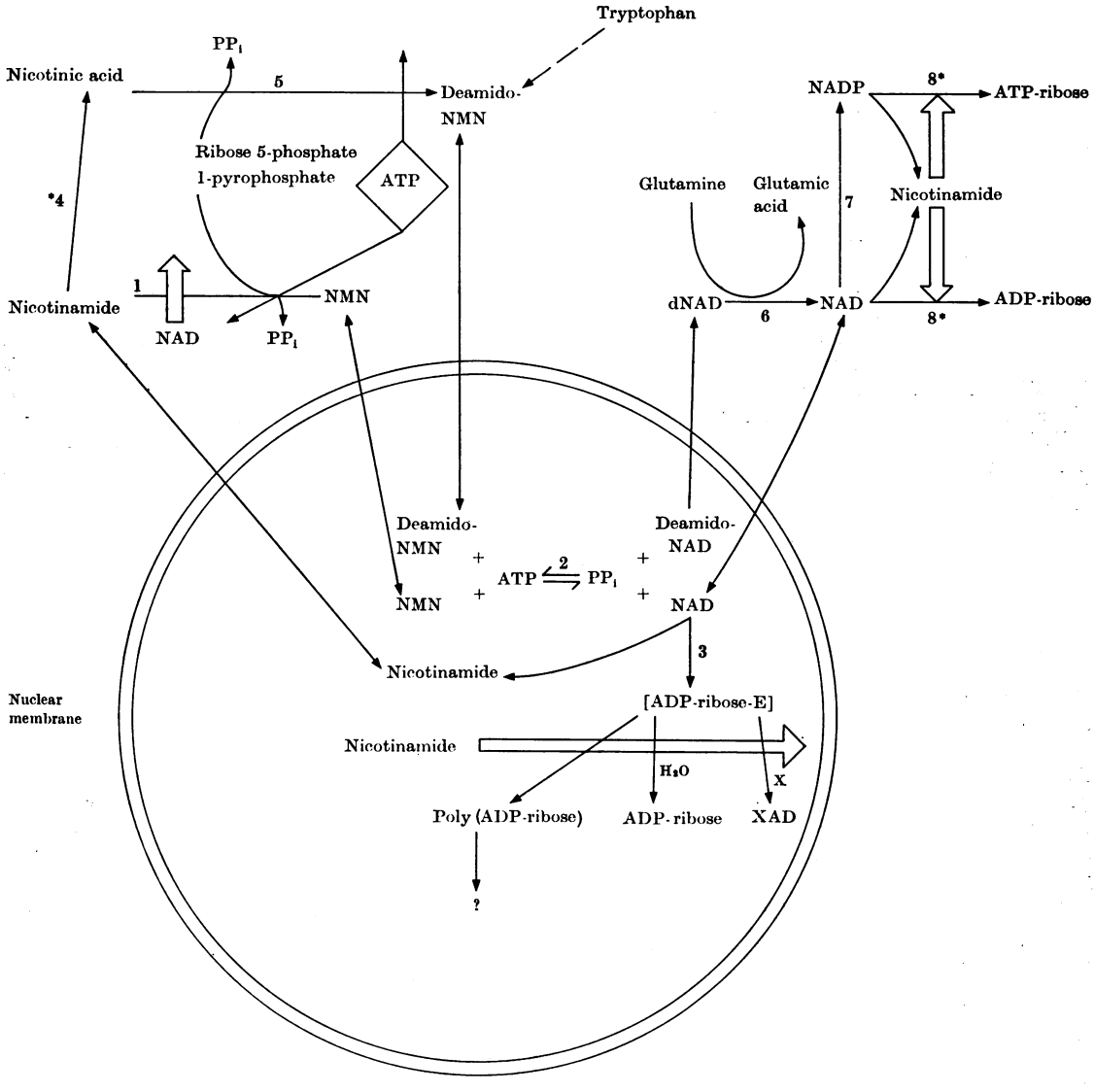
Poly (ADP-ribose) polymerase. In 150g. rats the profile of poly (ADP-ribose) polymerase was closely aligned with that for the incorporation of [^{3}H]orotic acid and hence RNA synthesis (Fig. 3*a*). The peaks of specific activity for the polymerase are within the diploid and tetraploid zones (Fig. 3*b*) and out of phase with the regions of highest NMN adenylyltransferase activity. The specific activity for poly (ADP-ribose) polymerase in the diploid zone has been observed in three experiments to split into two peaks. This is also seen with 25g. rats (Fig. 3*c*), where synthesis RNA and poly (ADP-ribose) is again associated with the bulk of the nuclei.

The radioactive product was identified as poly (ADP-ribose) by essentially the methods of Nishizuka *et al.* (1967). Thus incubation of the product (approx. 1nmole in 1ml. of 0·25M-tris–HCl buffer, pH 7·4) at 37° for 4hr. with ribonuclease (500μg./ml.), deoxyribonuclease (500μg./ml.) plus 0·03M-magnesium chloride or 0·6M-potassium hydroxide caused no loss of acid-insoluble radioactivity, whereas snake-venom phosphodiesterase (pyrophosphatase; see Butler, 1955) (250μg./ml.) rendered all but 5% soluble. Further, when the enzyme assay was carried out with deoxyribonuclease added at zero time, the incorporation fell by 80%. Finally, it was shown that incorporation from [^{14}C]NAD was decreased to 49% of the control value on addition of an equimolar amount of unlabelled NAD to the incubation mixture.

DISCUSSION

The central role of the nucleus in formation and degradation of NAD is apparent from a consideration of the metabolic pathways summarized in Scheme 1. The actual flux through separate pathways will be controlled by a multiplicity of factors, among which compartmentalization may be critical. It is against this background that distribution of NMN adenylyltransferase and poly (ADP-ribose) polymerase must be viewed.

The results with older rats indicate a high specific activity of NMN adenylyltransferase in nuclei engaged in the synthesis of DNA. Greenbaum & Pinder (1968) observed that in mammary glands the specific activity of NMN adenylyltransferase approximately doubles during the lactation cycle at precisely the same time as the doubling of the



Scheme 1. Pathways of NAD metabolism in liver tissue. The enzymes involved are: (1) NMN pyrophosphorylase (NMN–pyrophosphate phosphoribosyltransferase, EC 2.4.2.12); (2) deamido-NAD pyrophosphorylase (ATP–nicotinate mononucleotide adenylyltransferase, EC 2.7.7.18) and NMN adenylyltransferase (ATP–NMN adenylyl-transferase, EC 2.7.7.1); (3) poly (ADP-ribose) polymerase or nuclear NAD glycohydrolase (EC 3.2.2.5), or both; (4) nicotinamide deamidase; (5) nicotinate mononucleotide pyrophosphorylase (nicotinate mononucleotide–pyrophosphatephos phoribosyltransferase, EC 2.4.2.11); (6) NAD synthetase [deamido-NAD–L-glutamine amidoligase (AMP), EC 6.3.5.1]; (7) NAD kinase (ATP–NAD 2′-phosphotransferase, EC 2.7.1.23); (8) microsomal NAD glycohydrolase (EC 3.2.2.5). ⟷ represents transport across the nuclear membrane, ◇→ allosteric effectors, ⟹ inhibitors. Microsomal enzymes are indicated by an asterisk. X indicates a pyridine base.

tissue DNA. A connexion with DNA synthesis has also been observed in partially hepatectomized rats by Myers (1962), who noted that small changes in NMN adenylyltransferase activity were correlated with the mean content of DNA per nucleus.

The reason for the connexion that we have

demonstrated between NMN adenylyltransferase activity and DNA synthesis is unknown. NAD itself may have a role in DNA synthesis in bacteria, where it appears that relatively short single-stranded polynucleotides are produced as intermediates (Sakabe & Okazaki, 1966; Okazaki, Okazaki, Sakabe, Sugimoto & Sugino, 1968; Oishi, 1968). Polynucleotide ligase, the enzyme that catalyses the joining of single-stranded interruptions in DNA, is probably required for linking up these fragments (Sugimoto, Okazaki & Okazaki, 1968; Oishi, 1968). The bacterial enzyme requires NAD as a cofactor (Zimmerman, Little, Oshinsky & Gellert, 1967; Olivera, Hall & Lehman, 1968). However the cofactor for the mammalian enzymes so far investigated, namely those of myeloid and lymphoid tissue of rabbit, is ATP (Lindahl & Edelman, 1968).

It is known that there is a positive correlation between the activity of NMN adenylyltransferase in a tissue and its content of NAD (see review by Morton, 1961). Irrespective of any function that NAD may have in the synthesis of DNA, it seems that there is a negative correlation between the concentration of nicotinamide nucleotides and mitosis (Morton, 1961). As a corollary to this observation, low concentrations of nicotinamide coenzymes are a characteristic of rapidly dividing tissues (Morton, 1958; Caiger, Morton, Filsell & Jarrett, 1962).

2n nuclei in the phase of DNA synthesis (S phase) lie between the 2n and 4nP peaks in the zonal rotor. (The chemical events in the phases of the mammalian cell cycle are discussed by Petersen, Tobey & Anderson, 1969). As they go from the S into the G2 phase the nuclei will be found further into the trailing edge of the 4n zone. From a consideration of Figs. 1 and 3 it will be seen that the development from S phase to G2 phase is accompanied by a decrease in the specific activity of NMN adenylyltransferase and an increase in that of poly (ADP-ribose) polymerase. Both changes will depress the concentrations of NAD at the time immediately preceding mitosis. However, although our experiments indicate a mechanism by which the NAD concentration is lowered, which would be advantageous for mitosis, the interpretation of the patterns of enzyme distribution is complicated by the fact that none of the zonal fractions is homogeneous. Also it must be remembered that the measurements *in vitro* of NMN adenylyltransferase activity (Siebert, 1968) indicate that this activity is 10–100 times that needed to account for the rate of NAD synthesis. This follows from a consideration of the estimates of the turnover time of hepatic NAD (Greenbaum & Pinder, 1968) and the NAD content of the tissue (Caiger *et al.* 1962). This is also evident from experiments in which rats were injected with large doses of nicotinamide (Myers, 1962).

The nuclear enzyme catalysing the polymerization of NAD to form poly (ADP-ribose) resembles the nuclear NAD glycohydrolase (Nakazawa *et al.* 1968; Nishizuka *et al.* 1968*a*), which also acts as a transglycosylase transferring ADP-ribose to certain pyridine derivatives (Nishizuka *et al.* 1968*a*). The microsomal glycohydrolase, although able to carry out the exchange reaction, lacks poly (ADP-ribose) polymerase activity. The nuclear enzyme requires chromatin (Nakazawa *et al.* 1968; Nishizuka *et al.* 1967) or certain polyanions for activity (Chambon *et al.* 1966). The product, poly (ADP-ribose), is claimed to be tightly bound to histone (Nishizuka, Ueda, Honjo & Hayaishi, 1968*b*; Otake, Miwa, Fujimura & Sugimura, 1969). ADP-ribose itself is not a substrate (Chambon *et al.* 1966), and it is probable that an enzyme–ADP-ribose intermediate is formed (cf. Zatman, Kaplan & Colowick, 1953) that is susceptible to attack by a water molecule, nicotinamide or an analogue, or by transfer to histone to begin the polymer chain.

It seems well established that NMN adenylyltransferase is located in the nucleolus (Baltus, 1956; Siebert *et al.* 1966; Kaufmann, Traub & Teitz, 1968), whereas the intranuclear location of poly (ADP-ribose) polymerase remains to be elucidated. Both enzymes, however, appear to be associated with deoxyribonucleoprotein (Nishizuka *et al.* 1968*a*).

Although the highest specific activities of NMN adenylyltransferase and poly (ADP-ribose) polymerase per nucleus occur in different classes of nuclei, 2n and 4n nuclei do contain substantial amounts of both enzymes. Considering tetraploid nuclei in adult rats, and assuming that the capacity of NMN adenylyltransferase is ten times greater than that required, the rate of NAD synthesis *in vivo* would be about 2 μmoles of NAD/hr./10^9 nuclei. Since the highest measurements of poly (ADP-ribose) polymerase in the same nuclei indicate the utilization of 1·0–1·6 μmoles of NAD/hr./10^9 nuclei, the rate of polymer formation is of the same order as the rate of formation of NAD. However, this takes no account of the two routes available for NAD synthesis (Scheme 1) or the extent of them. Further, the computed activity of the NAD-synthesizing systems is based partly on the estimated turnover time of total nicotinamide nucleotides and takes no account of the possibility that NAD may be stored as poly (ADP-ribose). Doly & Mandel (1967) have produced evidence that poly (ADP-ribose) does exist *in vivo*. As deamido-NAD is not a substrate for poly (ADP-ribose) polymerase and has to be returned to the cytoplasm for amidation, it is possible that only NAD produced in the nucleus via the nicotinamide pathway is utilized in the polymerization step.

The regulation of nicotinamide coenzyme concentrations can be regarded as an important homoeo-

static mechanism. It is therefore possible that poly ADP-ribose serves as a reservoir of NAD. It has been claimed that poly (ADP-ribose) is not converted into NAD by the attack of nicotinamide (Nishizuka *et al.* 1967). However, if the reversal of the synthesis of the polymer could be achieved by nicotinate, the product would be deamido-NAD, which cannot be reutilized by the polymerase (Nishizuka *et al.* 1967). Hence it would have to enter the cytoplasm. This would mean that typically degradation and synthesis would follow different pathways. It is noteworthy that diphtheria toxin inhibits protein synthesis by blocking aminoacyltransferase type II through the transfer of an ADP-ribose residue from NAD to covalent linkage in the transferase. In this case inhibition is reversible by nicotinamide (Honjo, Nishizuka, Hayaishi & Kato, 1968).

The remarkable variation of the specific activities of the two enzymes studied in this investigation across the gradients of nuclei calls for comment. Assuming that nuclear enzymes turn over in the same manner as cytoplasmic, their specific activities could be varied by altering the balance between the rate of synthesis and that of degradation. These could be controlled by cytoplasmic factors and indeed by the concentration of NAD or its metabolites. A less likely alternative is that the variation of specific activities arises from regulation of the activity of preformed enzymes.

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