Biochem. J. (1968) **108**, 89
Printed in Great Britain

Activity of Nicotinamide–Adenine Dinucleotide Pyrophosphorylase in Liver Nuclei

EFFECTS OF PARTIAL HEPATECTOMY, HEPATOTOXINS AND DIETARY CHANGES

By F. STIRPE and E. DELLA CORTE
Istituto di Patologia Generale dell' Università di Bologna, 40126 Bologna, Italy

(*Received* 21 *November* 1967)

1. The activity of NAD pyrophosphorylase is lower in nuclei isolated from regenerating rat liver than in normal nuclei, and this is due to leakage of the enzyme from the nuclei during the isolation. 2. The NAD pyrophosphorylase activity is lower in liver nuclei from newborn rats, and from rats on a protein-free diet, but no leakage occurs in these cases. 3. Poisoning with α-amanitin brings about a transient enhancement of NAD pyrophosphorylase activity in mouse liver nuclei. 4. No changes of enzyme activity were observed after 72hr. starvation, administration of actinomycin D or infection with MHV 3 virus.

The NAD pyrophosphorylase (ATP–NMN adenylyltransferase, EC 2.7.7.1) of the liver is located in the cell nuclei (Hogeboom & Schneider, 1952). The activity of this enzyme is very low in foetal and neonatal rat liver, in mammary carcinoma (Branster & Morton, 1956) and in the liver of young and of tumour-bearing mice (Waravdekar & McConnell, 1964). It is lower than in normal liver in azo-dye-induced and transplantable hepatomas (Shimoyama, Yamaguchi & Gholson, 1967). A progressive decrease of the NAD pyrophosphorylase activity was observed in nuclei isolated from regenerating rat liver from 24 to 72hr. after partial hepatectomy, whereas no significant changes were caused by the administration of various toxic substances, even when liver nuclei were obviously damaged (Stirpe & Aldridge, 1961). The lack of effect of one of these poisons, dimethylnitrosamine, was confirmed by Christie, Bailie & Le Page (1962), who observed also a decreased activity of the enzyme in liver nuclei after heliotrine poisoning. The results obtained with regenerating liver were confirmed by the late Professor R. K. Morton (personal communication to Dr W. N. Aldridge) and by Jones & Waravdekar (1965) by a histochemical method, but no changes were observed in the enzyme activity determined in the whole homogenate from regenerating rat liver (Myers, 1962) or in nuclei isolated in non-aqueous media from 29hr.-regenerating rat liver (Siebert, 1963, footnote at p. 405, and personal communication).

In the present experiments the activity of NAD pyrophosphorylase during liver regeneration was reinvestigated to ascertain whether these discrepancies were due to differences between the techniques used. It was confirmed that nuclei isolated from regenerating liver have decreased NAD pyrophosphorylase activity, and it was observed that this was due to leakage of the enzyme from these nuclei during their isolation and purification. The study was extended to other conditions of liver damage, and of increased synthesis of liver protein and RNA. A decreased activity of NAD pyrophosphorylase without leakage of the enzyme from nuclei was observed in some cases.

EXPERIMENTAL

Materials. ATP, NMN and alcohol dehydrogenase were purchased from Sigma Chemical Co. (St Louis, Mo., U.S.A.) and DNA (salmon sperm) was from Mann Research Laboratories Inc. (New York, N.Y., U.S.A.). Actinomycin D was obtained from Merck, Sharp and Dohme Inc. (Rahway, N.J., U.S.A.), albitocin from Dr A. Lipton (Brisbane, Queensl., Australia) and α-amanitin (prepared by Professor T. Wieland) from Dr L. Fiume of this Institute. Other chemicals were of analytical grade. Solutions were prepared in glass-redistilled water. MHV3 virus was supplied by Dr G. Giusti (Naples, Italy) and was injected intraperitoneally into Swiss mice as soon as it was received.

Animals. Male rats of the Wistar Glaxo strain weighing 120–150g. and male Swiss mice weighing 20–30g. were used. Newborn rats were of both sexes and were killed within 12hr. of birth. All animals were bred in our animal room and were maintained on a modified Randoin & Causeret (1947) diet used in this Institute. For dietary experiments, the 20%-casein diet was the diet A described by Bonetti & Stirpe (1962) with 150mg. of α-tocopherol acetate/kg. The protein-free diet was derived from the above by replacing casein by starch and sucrose in equal parts. All fed animals received food until they were killed or treated with hepatotoxins.

Partial hepatectomy was performed, under ether anaesthesia, by the procedure of Higgins & Anderson (1931). The livers of control rats for these experiments were exposed and the abdomens closed as with the partially hepatectomized rats.

The animals injected with MHV3 virus were killed when obviously sick, the liver was homogenized with 10vol. of Hanks medium, the homogenate was centrifuged at about 1000*g* for 15min. and 0·25ml. of the supernatant diluted 1:10 was injected into new mice. After two or three passages the preparation was injected into the mice used for the experiments.

Biochemical determinations. Liver homogenates were prepared with a Potter homogenizer with Teflon pestle (A. Thomas Co., Philadelphia, Pa., U.S.A.) as described by Widnell & Tata (1964) and used for isolation of nuclei as practised by the same authors. Livers from 12–15 newborn rats and from five or six mice were pooled for each preparation. Samples from each homogenate and from the impure nuclear fraction and supernatant obtained with the first centrifugation were kept at −18° during final isolation of nuclei, and assayed at the same time as those for enzyme activity.

The activity of NAD pyrophosphorylase was measured as described by Stirpe & Aldridge (1961). The NAD formed was determined by the method of Klingenberg (1965). A control and a treated animal (or group of animals) were usually killed at the same time, and the experiments were run in parallel. DNA and protein were determined by the methods of Burton (1956) and of Gornall, Bardawill & David (1949) respectively.

RESULTS

The activity of NAD pyrophosphorylase in regenerating liver was assayed 72hr. after the partial hepatectomy, since maximum decrease was observed at this stage (Stirpe & Aldridge, 1961). The enzyme activity referred to nuclear protein was decreased to about the same extent (−45%) as reported by Stirpe & Aldridge (1961) (mμmoles of NAD formed/20min./mg. of protein: 125 in regenerating liver, and 71 in liver from sham-operated rats; results are from the same experiments reported in Table 1). The change was somewhat less (−35%) when the activity was expressed per mg. of DNA (Table 1), the difference being accounted for by a higher protein/DNA ratio in regenerating liver (6·11 versus 5·15 in control liver nuclei). There was almost no difference between control and regenerating liver when the activity was measured in the unfractionated homogenate. The NAD pyrophosphorylase activity was very low in both nuclei and homogenates from neonatal liver, though not as low as reported by Branster & Morton (1956).

An attempt was made to determine whether the enzyme was lost by the nuclei from regenerating liver during the isolation procedure. Fractionation of liver homogenate by the standard method showed that the enzyme activity of the 'nuclear fraction' (obtained by centrifuging at 800*g* for 10min., and highly impure) was the same in normal and regenerating liver.

The recovery of enzyme activity was not good after the two centrifugations required to isolate the nuclei, probably owing to the dilution of the enzyme (Table 2). However, the lack of difference in the specific activity of the crude nuclear fraction from regenerating or normal liver was confirmed. The difference appeared in the nuclei only after they were purified by centrifugation in high-density sucrose. Very little activity was found in the first supernatant, but some enzyme was present in the supernatant obtained after the centrifugation in high-density sucrose, the specific activity being higher in preparations from regenerating liver.

No changes were caused by 72hr. starvation or re-feeding after starvation (Table 3), but the

Table 1. *Activity of NAD pyrophosphorylase in the liver of partially hepatectomized and newborn rats*

The reaction mixture contained, in a final volume of 0·8ml.: 0·3ml. of 0·25M-glycylglycine buffer brought to pH 7·4 with KOH, 5μmoles of ATP, 3μmoles of NMN, 300μmoles of nicotinamide, 15μmoles of $MgCl_2$ and 0·2ml. of suspension of purified nuclei or of liver homogenate. The mixtures were incubated at 37° in air for 20min. in a Dubnoff shaker. Results are mean values ±S.E.M. of four experiments for each group.

	Enzyme activity (mμmoles of NAD formed/20min./mg. of DNA)	
Rats	Nuclei	Whole homogenate
Sham-operated, 72hr.	619±19	636±39*
Partially hepatectomized rats, 72hr.	402±33†	590±13
Normal, 2 months old	499±91	593±18
Newborn	151±25†	218±41

* Three cases only.

† Significantly different from sham-operated or adult rats ($P<0.001$).

Table 2. *Recovery of NAD pyrophosphorylase activity from various rat liver fractions*

Experimental conditions were as described in Table 1. Results are mean values of two experiments.

Fraction	Enzyme activity (mμmoles/20min./mg. of DNA)		Enzyme activity (% recovery from crude nuclear fraction)	
	Sham-operated	Hepatectomized	Sham-operated	Hepatectomized
Homogenate	*	510		
Crude nuclear fraction	448	432	100†	100†
First supernatant	Traces	Traces	—	—
Purified nuclei	413	345	50	32
Second supernatant	267	367	31	52

* One of the samples was lost. The activity of the other sample was 603 (the value for the sham-operated rat of the same experiment was 625).

† 75% of the activity of the whole homogenate was recovered in the crude nuclear fraction.

Table 3. *Effect of dietary changes on the activity of rat liver NAD pyrophosphorylase*

Experimental conditions were as described in Table 1. Results are mean values ±S.E.M., with the numbers of experiments in parentheses.

Treatment	Enzyme activity (mμmoles of NAD formed/20min./mg. of DNA)	
	Nuclei	Whole homogenate
Control (4)	499±91	593±18
Starved for 72hr. (2)	492	601
Starved for 72hr., re-fed for 24hr. (2)	606	576
20%-casein diet for 5 days (4)	531±32	625±38
Protein-free diet for 5 days (4)	350±19*	399±44*
Protein-free for 5 days, re-fed with 20%-casein diet for 24hr. (4)	524±41	489±67

* Significantly different from corresponding value for rats on the 20%-casein diet ($P<0.001$).

enzyme activity was significantly decreased in nuclei and homogenate from rats fed on a protein-free diet, this change being almost completely reversed by re-feeding on the 20%-casein diet.

The effect of liver damage by various hepatotoxins is reported in Table 4. The agents used were actinomycin D, which damages nuclear structure and inhibits RNA polymerase (see review by Reich & Goldberg, 1964), albitocin, a glycoside from *Albitia gummifera* (Lipton, 1963) that causes liver necrosis with rapid and severe morphological alterations in nuclei (Kerr & Pound, 1966), and α-amanitin, a poison from *Amanita phalloides* (Wieland, 1964) that causes liver necrosis in the mouse with early morphological alterations in the nuclei (Fiume & Laschi, 1965) and inhibition of RNA synthesis (Stirpe & Fiume, 1967). Infection with mouse hepatitis virus MHV3 was also used. Viral infection and actinomycin D did not cause any change in the NAD pyrophosphorylase activity. Albitocin caused a non-significant decrease of the enzyme activity that was less in the homogenate than in the nuclei. Poisoning with α-amanitin was followed by a transient rise of the enzyme activity both in nuclei and in the homogenate that was apparent 3hr. after the poisoning, reached a maximum at 6hr. and disappeared by 12hr.

DISCUSSION

The fact that pyrophosphorylase activity was decreased in nuclei, but not in the homogenate, from regenerating rat liver should be interpreted as a leakage of the enzyme from the nucleus. Ginzburg-Tietz, Kaufmann & Traub (1964) reported a similar observation in ascites cells infected *in vitro* with various viruses. Since they had shown that the enzyme is bound to nuclear ribosomes (Traub, Kaufmann & Ginzburg-Tietz, 1964), and since in virus-infected cells they recovered the extranuclear enzyme in the ribosomal fraction, they concluded that the presence of NAD pyrophosphorylase outside the nucleus was due to passage of ribosomes from the nucleus to the cytoplasm. The experiments reported in Table 2 indicate that nuclei from regenerating liver lose

Table 4. *Activity of NAD pyrophosphorylase in the liver of poisoned rats and mice*

The toxic substances were dissolved in 0·9% NaCl solution and given by intraperitoneal injection at the time before killing as indicated for each experiment, at the following dosages: actinomycin D, 100 μg./100g. body wt.; albitocin, 4mg./100g.; α-amanitin, 50 μg./100g.; MHV3 virus, 0·25ml. of liver supernatant/mouse (see the Experimental section). Control animals were injected with an equal volume of 0·9% NaCl. Other details were as described in Table 3. N.D., Not determined.

	Expt. no.	Treatment	Enzyme activity (mμmoles of NAD formed/20min./mg. of DNA)	
			Nuclei	Homogenate
Rats				
	1	Controls (2)	521	N.D.
		Actinomycin D, 3hr. (2)	487	
	2	Controls (6)	521 ± 27	527 ± 42
		Albitocin, 4hr. (8)	428 ± 41	481 ± 35
Mice				
	3	Normal (4)	329 ± 25	385 ± 41
		Injected with MHV3 virus, 24hr. (4)	350 ± 19	399 ± 11
	4	Controls (6)	401 ± 25	N.D.
		α-Amanitin, 1hr. (4)	341 ± 40	
	5	Controls (6)	328 ± 25†	331 ± 24†
		α-Amanitin, 3hr. (6)	399†	400 ± 26†
	6	Controls (6)	349 ± 26*	N.D.
		α-Amanitin, 6hr. (7)	472 ± 15*	
	7	Controls (3)	440 ± 3	
		α-Amanitin, 12hr. (2)	468	
		α-Amanitin, 24hr. (2)	451	

* $P<0{\cdot}001$. † $0{\cdot}05>P>0{\cdot}10$.

their enzyme activity during the isolation procedure, the greatest loss occurring during the centrifugation in high-density sucrose. We have no data to assess whether free enzyme or enzyme-carrying ribosomes came out from these nuclei. Regardless of how the enzyme leaks out from nuclei, leakage of all nuclear proteins does not seem to occur, since the protein/DNA ratio is not decreased and is actually higher in nuclei from regenerating liver. It is noteworthy that Stenger & Confer (1966) found abnormal 'pores' in the nuclear membrane during liver regeneration. The leakage of NAD pyrophosphorylase does not seem to be related with the cell proliferation or with the increased synthesis of RNA and protein, since it does not occur in other conditions where these are enhanced, e.g. neonatal liver, infection with a RNA virus and re-feeding after starvation or protein deprivation.

These results demonstrate also that methods that are suitable for isolating nuclei from normal liver may give different results when nuclei are prepared from liver in conditions other than normal. A comparison of present results with those obtained by Stirpe & Aldridge (1961) indicates that, in this respect, the methods using high- or low-density sucrose are comparable. Since there was no difference between the crude nuclear preparations obtained after the first centrifugation with both methods, it is probable that with low-density sucrose the enzyme is lost during the repeated washings necessary to purify the nuclei.

The NAD pyrophosphorylase activity is decreased during deprivation of dietary protein, and is very low in the liver of newborn rats. This confirms the results of Branster & Morton (1956), but since rat liver at birth contains some haemopoietic tissue the difference from adult liver may not be due to a different enzyme concentration in the hepatocytes.

The severe nuclear changes caused by α-amanitin were followed by increased NAD pyrophosphorylase activity. Siebert *et al.* (1966) reported that the activity of this enzyme is 50% higher after disruption of nuclei by ultrasonic treatment, and consequently it is reasonable to suppose that the enzyme is masked or bound in a less active form by some nuclear structure(s), and that destruction or damage of the latter activates or unmasks the enzyme activity.

We are indebted to Professor E. Bonetti for his interest and advice, to Dr W. N. Aldridge for criticism and suggestions, and to Dr L. Fiume, to Dr G. Giusti, to Dr A. Lipton and to Merck, Sharp and Dohme Inc. for the gift of substances. This work was supported by a grant from the Consiglio Nazionale delle Ricerche, Rome.

REFERENCES

Bonetti, E. & Stirpe, F. (1962). *J. Nutr.* **77**, 179.

Branster, M. V. & Morton, R. K. (1956). *Biochem. J.* **63**, 640.

Burton, K. (1956). *Biochem. J.* **62**, 315.

Christie, G. S., Bailie, M. J. & Le Page, R. N. (1962). *Biochem. J.* **84**, 364.

Fiume, L. & Laschi, R. (1965). *Sperimentale*, **115**, 288.

Ginzburg-Tietz, Y., Kaufmann, E. & Traub, A. (1964). *Exp. Cell Res.* **34**, 384.

Gornall, A. G., Bardawill, C. J. & David, M. M. (1949). *J. biol. Chem.* **177**, 751.

Higgins, G. & Anderson, R. M. (1931). *Arch. Path.* **12**, 186.

Hogeboom, G. H. & Schneider, W. C. (1952). *J. biol. Chem.* **197**, 611.

Jones, W. A. & Waravdekar, V. S. (1965). *Lab. Invest.* **14**, 397.

Kerr, J. F. R. & Pound, A. W. (1966). *Aust. J. exp. Biol. med. Sci.* **44**, 197.

Klingenberg, M. (1965). In *Methods of Enzymatic Analysis*, p. 428. Ed. by Bergmeyer, H. U. New York and London: Academic Press Inc.

Lipton, A. (1963). *J. Pharm., Lond.*, **15**, 816.

Myers, D. K. (1962). *Canad. J. Biochem. Physiol.* **40**, 619.

Randoin, L. & Causeret, J. (1947). *Bull. Soc. sci. Hyg. aliment., Paris*, **35**, 16.

Reich, E. & Goldberg, I. H. (1964). *Progr. Nucleic Acid Res.* **3**, 184.

Shimoyama, M., Yamaguchi, J. & Gholson, R. K. (1967). *Cancer Res.* **27**, 578.

Siebert, G. (1963). *Exp. Cell Res.* Suppl. 9, p. 389.

Siebert, G., Villalobos, J., jun., Ro, T. S., Steele, W. J., Lindenmayer, G., Adams, H. & Busch, H. (1966). *J. biol. Chem.* **241**, 71.

Stenger, R. J. & Confer, B. B. (1966). *Exp. molec. Path.* **5**, 455.

Stirpe, F. & Aldridge, W. N. (1961). *Biochem. J.* **80**, 481.

Stirpe, F. & Fiume, L. (1967). *Biochem. J.* **105**, 779.

Traub, A., Kaufmann, E. & Ginzburg-Tietz, Y. (1964). *Exp. Cell Res.* **34**, 371.

Waravdekar, V. S. & McConnell, D. (1964). *Exp. Cell Res.* **34**, 24.

Widnell, C. C. & Tata, J. R. (1964). *Biochem. J.* **92**, 313.

Wieland, T. (1964). *Pure appl. Chem.* **9**, 145.