Biochem. J. (1966) 98, 546

The Concentration and Biosynthesis of Nicotinamide Nucleotides in the Livers of Rats Treated with Carcinogens

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(*Received* 30 *June* 1965)

1. The oxidoreduction state and concentration of both NAD and NADP as well as the maximum potential activities of NMN adenylyltransferase and NAD^+ kinase have been measured in the livers of rats treated for 14–28 days with 4-dimethylamino-3′-methylazobenzene, 4-dimethylamino-4′-fluoroazobenzene, α-naphthyl isothiocyanate or ethionine and in primary hepatomas induced by 4-dimethylamino-3′-methylazobenzene. 2. The total NAD and total NADP both decreased in the livers of rats treated with either azo-dyes or α-naphthyl isothiocyanate but not in those treated with ethionine. The activities of NMN adenylyltransferase and NAD^+ kinase did not alter appreciably after such treatments. 3. In the primary hepatomas the concentrations of both NAD and NADP fell drastically and the activities of NMN adenylyltransferase and NAD^+ kinase fell to about 50% of the control activities. 4. No correlation could be established between the concentrations of the nucleotides and the activities of the enzymes synthesizing them. It appears, however, that a relationship exists between the NAD content of the tissue and the amount of NADP present. 5. The results are discussed with respect to the control of NAD and NADP synthesis by ATP. At the concentrations of NAD normally present in the cell it is suggested that NAD may be a rate-limiting substrate in NADP synthesis.

Morton (1958) suggested that an inverse relationship exists between the rate of cell division in a tissue and the NAD content of that tissue, a generalization that has an obvious bearing on studies of tissues, such as tumours and foetal cells, where the rate of division is high. On this basis, one of the prerequisites for the initiation of rapid cell division in the precancerous state would be a decrease in the concentration of the nucleotide. Although there have been many studies of the concentration of NAD in tumour material, there have been relatively few in the early stages of carcinogenesis. Notable among the latter are the results of Jedeikin, Thomas & Weinhouse (1956), which showed a tendency for the NAD concentration in the precancerous liver to decrease with increasing time of treatment with the carcinogen 3′MeDAB,† and also those of Nodes & Reid (1964), who found that azo-dye carcinogenesis produced little change in NAD concentration in the early stages but tended to lower the NADP content. The decline of NAD content is even more marked in well-developed tumours and has been adequately documented for a number of tissues and a variety of carcinogens (Jedeikin & Weinhouse, 1955; Glock & McLean, 1957; Briggs, 1960; Wintzerith, Klein, Mandel & Mandel, 1961; Missale & Colajacomo, 1963; Nodes & Reid, 1964). The NADP content of hepatomas is also much lower than that of normal liver (Glock & McLean, 1957; Briggs, 1960; see Reid, 1962).

In the light of these observations it appeared worth while to study the oxidized and reduced forms of NAD and NADP in both the precancerous phase, after feeding with a number of agents with different potencies in respect to their ability to produce malignant change, and in primary hepatomas produced by one of these agents. The activities of two of the enzymes involved in the synthesis of these nucleotides, namely NMN adenylyltransferase (EC 2.7.7.1) and NAD^+ kinase

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† Abbreviations: 3′MeDAB, 4-dimethylamino-3′-methylazobenzene; 4′FDAB, 4-dimethylamino-4′-fluoroazobenzene.

(EC 2.7.1.23), have also been measured in view of the above-mentioned changes in concentration and the observation by Quastel & Zatman (1953) that the NAD^+-glycohydrolase activity of tumours was within the range found in normal tissue and hence could not have contributed to the depletion of these nucleotides. Measurements on NMN adenylyl-tranferase are pertinent in view of the results of Branster & Morton (1956), who found that this enzyme was less active in mammary tumours of mice than in the normal gland, and of the suggestion by Morton (1961) of a possible role for NMN adenylyltransferase as a regulator of tissue NAD content and of cell division.

METHODS

Animals. Unless otherwise stated, male albino rats, aged about 7 weeks and with initial body weights of about 200g., were used.

Treatment of rats with azo-dyes

Precancerous livers. The precancerous state was induced by the feeding of two azo-dyes (3′MeDAB and 4′FDAB) that are known to be hepatocarcinogens. The azo-dyes were supplied to the rats in the diet, which contained 20% of protein and 0·075% of azo-dye, for periods varying from 8 to 42 days. The diet was given to the experimental animals in weighed amounts and control rats were given the same weight of a similar diet except that it contained no carcinogen. On the day before they were killed, the rats were put into individual cages and given the 20%-protein diet, without dye, in restricted amount such that the rats were being starved at the time they were killed.

Treatment of rats with α-naphthyl isothiocyanate. To provide a comparison, some rats were treated with α-naphthyl isothiocyanate, which is said to be non-carcinogenic but which produces cell proliferation of the bile duct (Grant & Rees, 1958). This was also supplied in the same diet and at the same concentration as the azo-dyes, and the treatment of rats before they were killed was as described for those given azo-dyes.

Hepatomas. The rats were given a diet containing 20% of protein and 0·075% of 3′MeDAB for about 100 days. They were then given the stock diet *ad lib.* until hepatomas developed, usually between 7 and 12 months from the beginning of feeding with the azo-dye. Controls for these rats were taken as those of the same age that had been fed on the 20%-protein stock diet throughout the time.

Treatment of rats with ethionine

Female albino rats, body weight approx. 150g., were given a diet compounded of powdered rat cube diet 41B (Bruce & Parkes, 1949) and ethionine in aqueous solution so as to produce a paste in which the DL-ethionine concentration was 0·25%. This diet was fed *ad lib.* to the treated animals, while control animals were pair-fed with these experimental animals by using a similar diet but containing no ethionine.

Extraction and estimation of the nicotinamide nucleotides

Extraction procedures. The oxidized forms of the nucleotides were extracted in 0·1N-HCl and the reduced forms in 0·1N-NaOH according to the method of Glock & McLean (1955). A separate extraction in 0·02N-H_2SO_4–0·1M-Na_2SO_4, by the method of Burch, Lowry & Von Dippe (1963), was made by using rapidly frozen tissue as described by these authors, so that the 'extra' NADP reported by them could be measured. All the nicotinamide nucleotides were assayed by the polarographic method of Greenbaum, Clark & McLean (1965*a*).

Estimation of enzyme activities. NMN adenylyltransferase and NAD^+ kinase were estimated as described by Greenbaum, Clark & McLean (1965*c*).

Chemicals

3′MeDAB, 4′FDAB and α-naphthyl isothiocyanate were obtained from L. Light and Co. Ltd., Colnbrook, Bucks. All other chemicals used were as described by Greenbaum *et al.* (1965*a*,*c*) or were of AnalaR grade.

Expression of results and statistical treatment

Reid (1962) has examined critically the various bases on which results obtained from tumour tissues may be expressed and has concluded that the least objectionable parameter is per g. of tissue. For this reason, all the results given below are expressed either as μg. of nucleotide/g. wet wt. of tissue or as μg. of NAD or NADP formed/g. wet wt. of tissue/hr. For the statistical evaluation of results the 'paired-*t* test' has been used.

RESULTS

Precancerous livers produced by azo-dyes

Concentration of the nicotinamide–adenine dinucleotides. Rats were treated with either 3′MeDAB or 4′FDAB for times varying between 8 and 42 days and the concentration of nicotinamide nucleotides in the liver was examined. There appeared to be no discernible trend over this time with either of the carcinogens, and accordingly only data for the largest groups, i.e. those treated for 14–28 days, have been included in Table 1. The NAD^+ concentration was decreased by both carcinogens. NADH was not greatly affected by 3′MeDAB, but 4′FDAB decreased the concentration to about 45% of that in normal liver. 4′FDAB also caused a significant fall in the $NADP^+$ concentration, although 3′MeDAB failed to do so. Both carcinogens decreased NADPH concentration. It is noteworthy that α-naphthyl isothiocyanate caused a substantial rise in the concentration of $NADP^+$. The large variations among animals were not statistically significant, but nevertheless some of the highest concentrations of $NADP^+$ reported in liver were found in certain of these animals. The

range of values was 7–122μg./g. with four values greater than the average for the control group. Our results show rather larger falls in the concentration of the nucleotides for both azo-dyes than those given in the literature (Jedeikin *et al.* 1956; Kotnis, Narurkar & Sahasrabudhe, 1962; see Reid, 1962).

Activities of NMN adenylyltransferase and of NAD+ kinase. The effects of the same compounds on the activities of NMN adenylyltransferase and of NAD+ kinase are shown in Table 2. In Table 2 are also included the sums of NAD+ + NADH concentrations and NADP+ + NADPH concentrations for comparison with the enzyme activities. The activity of NMN adenylyltransferase is significantly decreased by 4'FDAB but 3'MeDAB has little effect. This difference is in accord with the greater effect of the fluoro compound in decreasing the amount of NAD (Table 2, column 4). Neither carcinogen decreased the activity of the NAD+ kinase, which is somewhat surprising in view of the fact that the same dose of carcinogen for the same length of time decreased the amount of NADP in the tissue.

Hepatomas produced by azo-dyes

Concentration of the nicotinamide–adenine dinucleotides. Only hepatomas derived from rats fed with 3'MeDAB for long periods were used in this investigation and the results for these are shown in Table 3. The concentrations of all four forms of the nucleotide are dramatically decreased. The decreases for these are: NAD+, 70%; NADH, 80%; NADP+, 65%; NADPH, 85%; ($\sum$NAD, 74%; $\sum$NADP, 85%). These findings confirm published data on nucleotide concentrations in tumours (Glock & McLean, 1957; Briggs, 1960; Wintzerith *et al.* 1961; see Reid, 1962). Glock & McLean (1957) noted that, although the concentrations of NAD and NADP were decreased, there was no difference in the ratio of the oxidized to reduced forms in either case. The present results confirm this for NAD but, as there was a greater proportional loss of the reduced form for NADP, there was a shift in the oxidized/reduced ratio for this coenzyme. Nodes & Reid (1964) have also found an increased NADP+/NADPH ratio.

Activities of NMN adenylyltransferase and NAD+ kinase. The effect of long-term treatment with 3'MeDAB on the activity of these two enzymes is shown in Table 4, which, again, includes the sums of NAD+ + NADH concentrations and NADP+ + NADPH concentrations for comparison. The activities of both these enzymes fall to about 50% of the levels in normal liver. A similar fall in the activity of NMN adenylyltransferase was recorded by Branster & Morton (1956) in mammary tumours of mice and by Atkinson, Jackson & Morton (1961) in nuclei isolated from hepatomas of pig liver.

Table 1. *Oxidoreduction state and concentration of the nicotinamide nucleotides in the livers of rats treated with azo-dyes or with α-naphthyl isothiocyanate*

The nicotinamide nucleotides were extracted and assayed by the method of Greenbaum *et al.* (1965*a*). The results are expressed as means ± S.E.M. Fisher's *P* in parentheses represents the probability of difference between the means. Values of Fisher's *P* greater than 0·1 are shown as N.S., i.e. not statistically significant. The diet containing the compounds at a concentration of 0·75% was fed to the rats for 14–28 days.

Compound given	No. of animals	Body wt. (g.)	Liver wt. (g.)	Concn. (μg./g. wet wt.) NAD+	NADH	NAD+/NADH ratio	NADP+	NADPH	NADPH/NADP+ ratio
None	7	297 ± 26	11·9 ± 0·2	380·0 ± 41	120·0 ± 21	3·79 ± 0·71	20·6 ± 3·8	267·0 ± 28	14·2 ± 1·4
3'MeDAB	8	278 ± 16	12·5 ± 0·73	246·0 ± 38 (0·016)	95·3 ± 13·2 (0·1)	3·43 ± 0·8 (N.S.)	17·7 ± 3·3 (N.S.)	176·5 ± 14·2 (0·002)	15·2 ± 3·3 (N.S.)
4'FDAB	8	269 ± 13	10·3 ± 1·0	230·0 ± 23 (0·001)	53·6 ± 7·1 (0·0002)	5·76 ± 0·99 (0·09)	15·0 ± 2·1 (0·04)	153·5 ± 23 (0·004)	13·0 ± 3·0 (N.S.)
α-Naphthyl isothiocyanate	6	269 ± 13·6	13·8 ± 1·0	272·5 ± 42 (0·05)	114·6 ± 16·9 (N.S.)	3·07 ± 0·66 (N.S.)	45·7 ± 16·2 (N.S.)	166·0 ± 23·8 (0·008)	8·69 ± 3·59 (N.S.)

Table 2. *Total cellular concentrations of NAD and NADP and the activities of NMN adenylyltransferase and NAD⁺ kinase in the livers of rats treated with azo-dyes or with α-naphthyl isothiocyanate*

The nicotinamide nucleotides and enzymes were assayed by the methods of Greenbaum *et al.* (1965*a,c*). The results are expressed as means ± S.E.M. Other conditions were as given in Table 1.

Compound given	No. of animals	Body wt. (g.)	Liver wt. (g.)	ΣNAD (μg./g.)	NMN adenylyltransferase (μg. of NAD formed/g./hr.)	ΣNADP (μg./g.)	NAD^+ kinase (μg. of NADP formed/g./hr.)
None	7	297±26·0	11·9±0·2	506±55	3755±368	287±31	1991±175
3′MeDAB	8	278±16·0	12·5±0·73	370±16 (0·001)	3467±506 (N.S.)	221±13 (0·001)	1865±182 (N.S.)
4′FDAB	8	269±13·0	10·3±1·0	301±12 (0·001)	2885±405 (0·02)	161±25 (0·004)	2020±229 (N.S.)
α-Naphthyl isothiocyanate	6	269±13·6	13·8±1·0	370±41 (0·051)	3364±350 (N.S.)	208±26 (0·04)	1983±121 (N.S.)

Table 3. *Oxidoreduction state and concentration of the nicotinamide nucleotides in rat hepatomas induced by 3′MeDAB*

The nicotinamide nucleotides were extracted and assayed by the method of Greenbaum *et al.* (1965*a*). The results are expressed as means ± S.E.M. *P* values are given in parentheses (see Table 1).

	No. of animals	Body wt. (g.)	Liver wt. (g.)	Concn. (μg./g. wet wt.) NAD^+	NADH	$NAD^+/NADH$ ratio	$NADP^+$	NADPH	$NADPH/NADP^+$ ratio
Control	6	718±42	15·7±0·75	422±67	154±13·2	2·69±0·3	36·0±9·3	262·0±45·7	10·15±2·8
Hepatomas	6	772±23	—	127±51 (0·002)	31±7·7 (< 0·001)	3·74±0·89 (N.S.)	12·2±1·6 (< 0·001)	34·1±8·9 (< 0·001)	4·73±1·6 (0·02)

Table 4. *Total cellular concentrations of NAD and NADP and the activities of the NMN adenyltransferase and NAD^+ kinase in rat hepatomas induced by 3′MeDAB*

The nicotinamide nucleotides and enzymes were assayed by the methods of Greenbaum *et al.* (1965*a*,*c*). The results are expressed as means ± S.E.M. *P* values are given in parentheses (see Table 1).

	No. of animals	Body wt. (g.)	Liver wt. (g.)	ΣNAD (μg./g.)	NMN adenylyl-transferase (μg. of NAD formed/g./hr.)	ΣNADP (μg./g.)	NAD^+ kinase (μg. of NADP formed/g./hr.)
Control	6	718 ± 42	15·7 ± 0·75	576 ± 78	3955 ± 191	297·0 ± 46	1963 ± 338
Hepatomas	6	772 ± 23	—	151 ± 52	1949 ± 171	44·3 ± 10·4	963 ± 165
				(< 0·001)	(< 0·001)	(< 0·001)	(< 0·001)

Table 5. *Oxidoreduction state and concentration of the nicotinamide nucleotides in the livers of rats given ethionine for 2 weeks*

Nucleotides were extracted and assayed as described by Greenbaum *et al.* (1965*a*). The values are given as means ± S.E.M. *P* values are given in parentheses (see Table 1).

				Concn. (μg./g. wet wt.)					
	No. of animals	Body wt. (g.)	Liver wt. (g.)	NAD^+	NADH	NAD^+/NADH ratio	$NADP^+$	NADPH	NADPH/$NADP^+$ ratio
Control	6	162 ± 7·8	8·40 ± 1·0	313 ± 13	83·9 ± 10·1	4·12 ± 0·71	19·3 ± 0·8	138 ± 16·0	7·63 ± 1·4
Ethionine-treated	6	140 ± 10·3	6·66 ± 0·7	276 ± 19	65·4 ± 4·8	4·92 ± 0·86	25·8 ± 4·7	127 ± 6·5	5·2 ± 2·1
				(N.S.)	(0·03)	(N.S.)	(N.S.)	(N.S.)	(N.S.)

Shimoyama, Kori, Usuki, Lan & Gholson (1965) also found that the activity of this enzyme is decreased in hepatomas derived from a 3′MeDAB-induced primary hepatoma.

Hepatomas produced by ethionine

Long-term treatment of rats with ethionine also produces hepatomas. The effects of this agent on the concentrations of nicotinamide nucleotides and on the activities of NMN adenylyltransferase and NAD^+ kinase in the precancerous phase were therefore studied.

Concentration of the nicotinamide–adenine dinucleotides. The concentrations of the oxidized and reduced forms of NAD and NADP in the livers of rats treated with ethionine for 2 weeks are shown in Table 5. At this short time-interval, ethionine had little effect on the concentration of any of the nicotinamide nucleotides; only that of NADH was lower in the ethionine-treated group.

Activities of NMN adenylyltransferase and of NAD^+ kinase. The feeding of ethionine for 2 weeks decreased the activity of NMN adenylyltransferase significantly but had no effect on the activity of NAD^+ kinase (Table 6).

Effects of α-naphthyl isothiocyanate

α-Naphthyl isothiocyanate differs from the other agents used, in that long-term feeding does not produce a hepatoma, but does produce a cell proliferation of the bile duct which is neither destructive nor invasive, in contrast with the hepatomas produced by the other agents used above.

Concentration of the nicotinamide–adenine dinucleotides. The effects of this agent on the nicotinamide nucleotides are shown in Table 1. α-Naphthyl isothiocyanate significantly decreases the amount of NAD^+ but has no effect on the amount of NADH. It increased the $NADP^+$ content by a factor of over 2 but, so great is the variability of response of $NADP^+$ to this agent, the increase was not statistically significant. The NADPH concentration is lowered significantly to about 65% of the control.

Activities of NMN adenylyltransferase and of NAD^+ kinase. The effects of α-naphthyl isothiocyanate on the activities of these two enzymes are shown in Table 2. Neither enzyme is significantly decreased in activity in the livers of rats treated with α-naphthyl isothiocyanate.

Changes in the 'extra, acid-labile' form of $NADP^+$

In view of the findings of Burch *et al.* (1963) on the existence of a form of $NADP^+$ that is acid-labile and not found in the usual type of acid extract used for assay of nicotinamide nucleotides, this 'extra' form was also estimated in the livers of

Table 6. *Total cellular concentrations of NAD and NADP and the potential activities of the NMN adenylyltransferase and NAD^+ kinase in the livers of rats given ethionine for 2 weeks*

The nicotinamide nucleotides and enzymes were assayed by the methods of Greenbaum *et al.* (1965*a*,*c*). The results are expressed as means ± S.E.M. *P* values are given in parentheses (see Table 1).

	No. of animals	Body wt. (g.)	Liver wt. (g.)	ΣNAD (μg./g.)	NMN adenylyltransferase (μg. of NAD formed/g./hr.)	ΣNADP (μg./g.)	NAD^+ kinase (μg. of NADP formed/g./hr.)
Control	6	162 ± 7·8	8·4 ± 1·0	397 ± 16	3885 ± 375	158 ± 16	2565 ± 330
Ethionine-treated	6	140 ± 10·3	6·66 ± 0·7	342 ± 22	3223 ± 212	153 ± 10	3087 ± 387
				(0·074)	(0·05)	(N.S.)	(N.S.)

Table 7. *Concentration of two forms of* $NADP^+$ *(differing with respect to their stability in* 0·1 N-*hydrochloric acid) in the livers of rats treated with azo-dyes, α-naphthyl isothiocyanate or ethionine*

The normal $NADP^+$ (*a*) was extracted and assayed by the method of Greenbaum *et al.* (1965*a*). Acid-labile $NADP^+$ (*b*) was extracted by the method of Burch *et al.* (1963), and estimated as above. The results are expressed as means ± S.E.M. *P* values are given in parentheses (see Table 1).

Compound given	No. of animals	Body wt. (g.)	Liver wt. (g.)	$NADP^+$ extracted in 0·1 N-HCl (μg./g.) (*a*)	$NADP^+$ extracted in 0·02 N-H_2SO_4–0·1 M-Na_2SO_4 (μg./g.) (*b*)	Quotient (*b*/*a*)	'Extra' $NADP^+$ (μg./g.) (*b*−*a*)
None	7	297 ± 26	11·9 ± 0·2	20·6 ± 3·8	56·0 ± 6·9	2·72	35·5 ± 5·9
3′MeDAB	8	278 ± 16	12·5 ± 0·73	17·7 ± 3·3 (N.S.)	32·6 ± 5·9 (0·01)	1·84	17·95 ± 6·7 (0·03)
4′FDAB	8	269 ± 13	10·3 ± 1·0	15·0 ± 2·1 (0·04)	33·3 ± 3·7 (0·002)	2·21	16·5 ± 4·5 (0·004)
α-Naphthyl isothiocyanate	6	269 ± 13·6	13·8 ± 1·0	45·7 ± 16·2 (N.S.)	32·3 ± 4·8 (0·004)	0·71	None
None	6	162 ± 8	8·4 ± 1·0	19·3 ± 0·8	40·4 ± 3·2	2·09	20·0 ± 4·0
Ethionine	6	140 ± 10	6·7 ± 0·7	25·8 ± 4·7 (N.S.)	54·7 ± 7·4 (0·056)	2·12	28·9 ± 4·9 (0·02)

Table 8. *Concentrations of two forms of* $NADP^+$ *(differing with respect to their stability in* 0·1 N-*hydrochloric acid) in rat hepatomas induced by 3′MeDAB*

(*a*) Normal $NADP^+$; (*b*) acid-labile $NADP^+$ (see Table 7). The results are expressed as means ± S.E.M. *P* values are given in parentheses (see Table 1).

	No. of animals	Body wt. (g.)	Liver wt. (g.)	$NADP^+$ extracted in 0·1 N-HCl (μg./g.) (*a*)	$NADP^+$ extracted in 0·02 N-H_2SO_4–0·1 M-Na_2SO_4 (μg./g.) (*b*)	Quotient (*b*/*a*)	'Extra' NADP (μg./g.) (*b*−*a*)
Controls	6	718 ± 42	15·7 ± 0·75	36·0 ± 9·3	55·8 ± 8·9	1·55	20·1 ± 5·95
Hepatomas	6	722 ± 23	—	12·35 ± 1·6 (0·001)	8·31 ± 2·79 (0·001)	0·67	−2·33 ± 1·57 (0·001)

rats that had been treated for 14–28 days with one of the four agents used above and in hepatomas. The results of the assay of this form of $NADP^+$ are shown in Tables 7 and 8. Also included in Tables 7 and 8 are the results of assays of the normal form of $NADP^+$, which is present in 0·1 N-acid extracts. Short-term treatment with either of the two azo-dyes or with α-naphthyl isothiocyanate significantly decreased the concentration of $NADP^+$ that is extracted in weak acid and also the 'extra' $NADP^+$ (Table 7, columns 6 and 8). α-Naphthyl isothiocyanate was particularly active with respect to the 'extra' $NADP^+$, there being no 'extra' $NADP^+$ present at all in tissues treated with this agent. Ethionine had the reverse effect, in that it increased both the $NADP^+$ extractable in weak acid and the 'extra' $NADP^+$. In hepatomas the $NADP^+$ extracted in weak acid is actually less than that extracted in 0·1 N-hydrochloric acid.

Effect of the concentration of NAD^+ on the synthesis of NADP by NAD^+ kinase

To investigate this effect the assay system for NAD^+ kinase (Greenbaum *et al.* 1965*c*) was used except that the amount of NAD^+ was varied from 0·44mg. to 13·2mg. in 1ml. of reaction mixture. The NADPH formed at each concentration of NAD^+ was extracted and assayed as above. The results for this experiment are shown in Fig. 1.

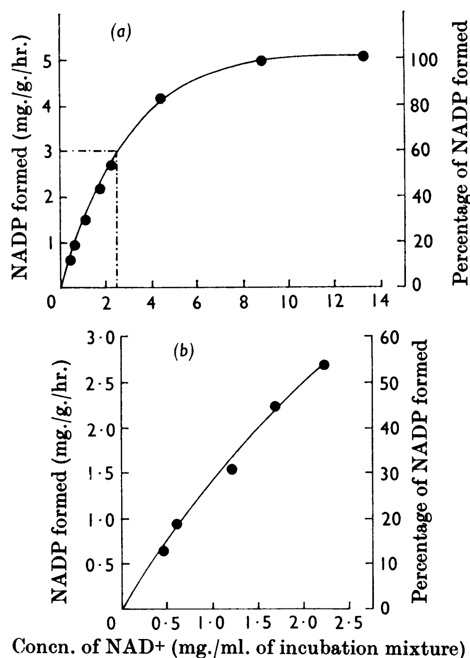

Fig. 1. Effect of NAD^+ concentration on the activity of rat-liver NAD^+ kinase. The liver was homogenized (1:10) in 0·15 M-KCl–0·16 mM-$KHCO_3$ and centrifuged for 135000*g*-min. The supernatant was used as the enzyme source as described by Greenbaum *et al.* (1965*c*). Fig. 1(*b*) is an expanded version of the area marked in Fig. 1(*a*).

DISCUSSION

One of the more interesting manifestations of the advent and development of malignancy in hepatic cells is the loss of nicotinamide nucleotides, in particular of NADP. In a fully developed hepatoma the NADP concentration is less than one-fifth of that of a normal liver. The loss of NADP is readily investigated since its synthesis requires only three components, all readily measurable. These are the two substrates, NAD^+ and ATP, and the enzyme NAD^+ kinase. In the present investigation both NAD^+ kinase and NAD^+ have been measured. Table 9 summarizes our findings and shows the concentrations of the nicotinamide nucleotides and the activities of the enzymes concerned in their synthesis, expressed as percentages of the control values. It is apparent that no correlation exists between the measured activity of NAD^+ kinase and the concentration of the nucleotide. 4′FDAB, for instance, causes a loss of over 40% of the NADP in a precancerous liver, but has no effect on the activity of NAD^+ kinase. Similarly, with 3′MeDAB and α-naphthyl isothiocyanate there were falls in NADP concentration with no corresponding change in NAD^+-kinase activity. Although it is true that the profound loss of NADP that occurs in hepatomas is accompanied by a severe decrease in NAD^+-kinase activity, it is not certain that there is a cause-and-effect relationship here. It is probable that by the time a hepatoma has developed widespread secondary changes have occurred in the liver and the loss of NAD^+-kinase activity may merely reflect a generalized depletion of some enzyme activities.

In contrast with the lack of correspondence between enzyme activity and NADP concentration, there appears to be a reasonably close parallelism between the concentrations of NAD and NADP (Table 9, columns 1 and 3). The relationships between these two can be evaluated by reference to Fig. 1, which shows the relationship between the activity of NAD^+ kinase and the amount of NAD present. Since the enzyme is measured as the maximum potential activity with saturating concentration of substrate, it follows that a truer measure of intracellular activity would be the enzyme activity at the NAD concentration actually found in the cell. From Fig. 1 it is possible to express this activity as a percentage of the maximum rate. Thus from the measured NAD content of the cell and the measured maximum potential activity of NAD^+ kinase an estimate can be formed of the actual rate

Table 9. *Concentrations of coenzymes and activities of enzymes expressed as percentages of control values*

The values for the coenzymes and enzymes are calculated from Tables 2, 4 and 6. The values in **bold** type are significantly different from the control value.

Compound given	$\sum$NAD	NMN adenylyltransferase	$\sum$NADP	NAD+ kinase
14–28 days				
3'MeDAB	**73**	92	**77**	94
4'FDAB	**59**	**77**	**56**	101
Ethionine	86	**83**	97	100
α-Naphthyl isothiocyanate	73	89	72	120
Hepatomas				
3'MeDAB	**26**	**49**	15	**49**

Table 10. *Comparison between the observed concentrations of NADP in livers of rats treated with 3'MeDAB, 4'FDAB, α-naphthyl isothiocyanate or ethionine, and the concentration calculated from the tissue NAD content*

$\sum$NAD and the 'observed' $\sum$NADP are taken from Tables 2, 4 and 6. NAD+-kinase activities are taken from Tables 2, 4 and 6.

Experimental condition	$\sum$NAD (μg./g.)	NAD+ kinase (μg. of NADP/g./hr.)		$\sum$NADP (μg./g.)	
		(maximum potential activity)	(corrected to NAD content of tissue $=A$)*	Observed	Calc.†
1. Control group	506	1990	318	287	—
3'MeDAB-treated	370	1865	232	221	210
4'FDAB-treated	300	2020	194	161	175
α-Naphthyl isothiocyanate-treated	370	1983	246	208	222
2. Control group	400	3087	402	158	—
Ethionine-treated	340	2565	308	153	121
3. Control group	575	1963	344	300	—
3'MeDAB hepatomas	150	963	46	44	40

* NAD+-kinase activities corrected to activities at the cellular concentration of NAD are calculated from Fig. 1.

† Calculated from:

$$\frac{A \text{ for experimental group}}{A \text{ for control group}} \times \text{NADP content of control group}$$

of NAD+-kinase activity. Let this be called A. Assuming that the concentration of NADP in a cell is in equilibrium with the rate of its formation by NAD+ kinase, it is possible to write:

$$\text{NADP content of experimental animal} = \frac{A \text{ for experimental animal}}{A \text{ for control animal}} \times \text{NADP content of control animal}$$

The results of such a calculation are shown in Table 10. There is a sufficient degree of correspondence between the expected concentration of NADP, calculated from the NAD content, and the actual concentration of NADP to suggest that the cellular content of NAD may regulate the formation of NADP. However, it is unlikely that this form of control extends over a wide range. In particular, it is unlikely that the NAD concentration controls the NADP concentration when the NAD concentration is supranormal. This is suggested by the fact that when the cellular NAD concentration is increased to supranormal values by injection of nicotinamide there is only a small increase in the concentration of NADP (Kaplan, Goldin, Humphreys, Ciotti & Stolzenbach, 1956; Wintzerith *et al.* 1961; Gordon & Reichlin, 1962; Greengard, Quinn & Reid, 1964). It is thus possible that at low cellular concentrations of NAD it is this component which is rate-limiting for NADP synthesis, but at high concentrations of NAD it is clearly not the

rate-limiting compound. This suggests that the third component of the NADP-synthesizing system, ATP, may well become so. Evidence in favour of this view can be derived from the work of Dietrich & Yero (1964), who found that the injection of nicotinamide into mice led to a rise in the hepatic NAD concentration but a fall in the hepatic ATP concentration. Thus injection of or feeding with nicotinamide leads to increased amounts of NAD accompanied by lower ATP concentrations, and under these conditions NADP synthesis is not greatly stimulated (Kaplan *et al.* 1956; Wintzerith *et al.* 1961; Gordon & Reichlin, 1962; Greengard, *et al.* 1964), which suggests a competition for the available ATP by the enzymes synthesizing either NAD or NADP. This possibility is discussed further below. There is also evidence from the work of Stekol, Bedrak, Mody, Burnette & Somerville (1963) that the availability of ATP can influence the rate of NAD synthesis after feeding with nicotinamide.

As a result of the above considerations, it seemed important to investigate the possible role of ATP as a regulator of NADP synthesis. To study the effect of a lowered ATP concentration, advantage was taken of the observation of Shull (1962) that injection of ethionine into rats led to a decrease of the hepatic ATP concentration within 2hr. that persisted for up to 6hr. In such ethionine-treated rats it was found that the NAD concentration was only slightly lowered 5hr. after treatment, whereas the NADP concentration was lowered by about 40% and the NAD^+-kinase activity was unchanged (Greenbaum, Clark & McLean, 1964). In this experiment, neither the very small fall in NAD concentration nor the virtually unchanged activity of NAD^+ kinase could account for the decline in the NADP concentration, and the most likely cause was the lowered ATP concentration resulting from the ethionine injection. To explain the differential effects of ATP on the reactions leading to the synthesis of NAD and NADP, both of which are ATP-requiring, Greenbaum *et al.* (1964) considered the different K_m values for these two reactions. For half maximum activity, NAD^+ kinase requires a concentration of ATP that is approx. 150 times as great as is required by NMN adenylyltransferase. Thus, when ATP is limiting, the limitation will press more hardly on NADP synthesis than on NAD synthesis. This difference could account for the failure to find appreciable NADP synthesis in nicotinamide-treated rats and in ethionine-treated rats. It is also noteworthy that after injection of ethionine the concentrations of both NAD and NADP fall together, but that NAD recovers earlier, returning to normal by 5hr. after the injection, whereas NADP remains depressed (J. B. Clark & S. Pinder, unpublished work). The NAD recovery occurs at a time when the concentration of ATP is still below normal.

The precancerous liver, as well as the fully developed hepatoma, both contain subnormal amounts of NAD. All carcinogens decreased the concentration of this coenzyme and the greatest effect was produced by the most potent of the carcinogens, 4′FDAB (Table 9). There is very little relationship between the activity of NMN adenylyltransferase and the concentration of NAD in these treated livers except, perhaps, that 4′FDAB, which produces the greatest fall in NAD concentration, also has the greatest effect on NMN adenyltransferase. This lack of correspondence between enzyme and product is not surprising in view of the results of Shimoyama *et al.* (1965), which show that in hepatomas the probable rate-limiting step is the formation of nicotinic acid ribonucleotide.

The biological significance of the form of NADP that is extractable in weak acid (Burch *et al.* 1963) is obscure, but it may be noted that all the agents, with the exception of ethionine, decreased the concentration of this form in the livers of treated rats. Neubert, Schulz & Hoehne (1964) suggested that this acid-labile NADP is a haem-catalysed reoxidation of NADPH, which is the predominant form of NADP in the cell, and consider that the 'extra', acid-labile, NADP would be related to the $NADPH/NADP^+$ quotient, being high when this quotient is high. In fact, we have found considerable changes in the amount of acid-labile NADP in the precancerous livers of 3′MeDAB- and 4′FDAB-treated rats even though there is no appreciable change in the $NADPH/NADP^+$ quotient. However, for α-naphthyl isothiocyanate and in the hepatomas the suggested relationship is found, i.e. there is a considerable fall in the $NADPH/NADP^+$ quotient and the acid-labile NADP virtually disappears. However, previous studies of the content of oxidized and reduced forms of NADP in the livers of rats subjected to a number of treatments (Greenbaum, Clark & McLean, 1965*b*) also failed to show the relationship suggested by Neubert *et al.* (1964). Further studies on the acid-labile form of NADP are needed before its biological source and role can be assessed.

We thank Dr E. Reid for providing some of the tumour material. We are grateful to the Medical Research Council for a Research Grant to J. B. C. The work has been supported, in part, by a grant to The Middlesex Hospital Medical School from the British Empire Cancer Campaign.

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