Oxidized and Reduced Nicotinamide-Adenine Dinucleotide Phosphate in Tissue Suspensions of Rat Liver

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1. The concentrations of NADP and NADPH₂ in homogenates of rat liver (expressed as $\mu\text{g./g.}$ wet wt. of tissue homogenized) were compared with values obtained from intact samples of liver taken from the same female rat. With 0.25 M-sucrose alone as the suspending medium, or in combination with tris buffer or 0.01–0.1 M-nicotinamide, considerable decreases in the sum of the NADP + NADPH₂ concentrations were occasionally observed during 30 min. storage of homogenates at 0°. However, addition of 0.5 M-nicotinamide + 5 mM-tris buffer to 0.25 M-sucrose for use as a suspending medium maintained the sum of the NADP + NADPH₂ concentrations in homogenates at the level found in intact tissue for at least 30 min. at 0°.
2. The effects of freezing intact tissue and homogenates in liquid nitrogen before the extraction of NADP and NADPH₂ were studied. Freezing alone appears to convert a significant amount (approx. 30%) of liver NADPH₂ into an equivalent amount of NADP in intact tissue. This is discussed in terms of the 'bound NADP' reported by Burch, Lowry & Von Dippe (1963).
3. The intracellular distributions of NADP and NADPH₂ in intracellular fractions of rat liver were studied by using a modified centrifuging scheme that allows extraction of the isolated fractions to be performed within 45 min. of killing the animal. Approx. 50% of the total NADP + NADPH₂ was found in the large-particle fractions and the remaining 50% was mostly in the soluble fraction of the cell.
4. Further investigations are reported on the nature of 'bound NADP' in rat liver. Most of this material appears associated with the 'nuclear' (containing nuclei, debris, erythrocytes etc.) or large-mitochondrial fractions, or both, obtained by low-speed centrifuging of rat-liver homogenates.
5. Although in some experiments the variations produced in the concentration of NADPH₂ present in large-particle fractions were followed by similar changes in that of 'bound NADP', in other cases no such direct relationship was obtained. Addition of phenazine methosulphate, for example, consistently lowered the concentration of NADPH₂ yet raised the concentration of 'bound NADP' in rat-liver mitochondrial fractions.

Several hepatotoxic agents when administered to rats produce very early changes in the liver concentrations of NADP and NADPH₂ as measured in intact liver samples (Slater, Sträuli & Sawyer, 1964; Slater & Sawyer, 1966*a,b,c*). In attempting to elucidate the mechanisms responsible for these early changes it is necessary to perform experiments on liver homogenates and intracellular fractions. Since the nucleotide changes occurring in the early stages of the types of liver injury being studied are only some 20% of the initial intact liver values, it is important that significant losses should not arise as a consequence of the preparation of homogenates and intracellular fractions.

Estimations of NADP and NADPH₂ in homogenates stored for 30 min. at 0°, whether in 0.25 M-

sucrose or in sucrose supplemented with 0.05 M-nicotinamide (Jacobson & Kaplan, 1957*b*), gave values for the sum of the NADP + NADPH₂ concentrations that were considerably lower than did determinations carried out on intact liver samples from the same animal. This led to an investigation of conditions required to maintain the sum of the NADP + NADPH₂ concentrations in liver homogenates during a period of approx. 30 min. at 0° at the value normally present in intact liver samples. The arbitrary period of 30 min. was chosen since this is approximately the time required for the separation of intracellular fractions (see the Methods section). The intracellular localization of NADP and NADPH₂ is also considered, and some data on the acid-labile 'bound NADP' (Burch,

Lowry & Von Dippe, 1963) are provided. A brief account of some of this work has been published elsewhere (Slater, 1965a).

METHODS

General procedures. The rats used were albino females of the Wistar strain, body weight approx. 130 g.; they were killed by cervical dislocation and, when required, samples of whole liver were immediately taken for the assay of the nicotinamide-adenine dinucleotides by the method of Slater, Sawyer & Sträuli (1964). Samples of liver for homogenate or mitochondrial preparations, or both, were likewise taken immediately *post mortem* and dropped into ice-cold medium, chopped finely with scissors and homogenized in a Potter-Elvehjem-type homogenizer with a nylon piston, diameter clearance 0.02 in. All tissue suspensions were stored in ice until assay unless stated otherwise in the legends to the Tables. For experiments involving frozen intact tissue, small samples of liver were taken immediately after killing the rat and dropped into liquid N₂; after several minutes pieces totalling approx. 0.4 g. were quickly weighed and then subjected without further thawing to the routine hot-alkali and cold-acid extractions used in the estimation procedure for NADPH₂ and NADP respectively. In the experiments described in Table 2, some samples of frozen intact tissue were used to prepare homogenates (called here 'frozen-tissue homogenates'). In these instances, small pieces of frozen tissue were added to the appropriate homogenizing medium at 0° (usually 0.25 M-sucrose-0.5 M-nicotinamide-5 mM-tris) and, after a partial thawing, homogenization was performed as described above for unfrozen tissue. It is important to note that acid and alkaline extractions of frozen intact tissue were carried out without previous thawing, whereas extractions of homogenates prepared from frozen tissue had necessarily been preceded by thawing and usually by a subsequent storage of 30 min. at 0° (see the legend to Table 2).

In some experiments mentioned in Table 8 a single-stage extraction for both NADP and NADPH₂ was performed on tissue suspensions by using the procedure of Spirtes & Eichel (1955).

'Bound NADP' (Burch *et al.* 1963) was estimated in intact liver samples and in homogenates by first freezing the tissue in liquid N₂ and then thawing in the presence of an excess of ice-cold 0.02 N-H₂SO₄-0.1 M-Na₂SO₄-16% (v/v) ethanol (Neubert, Schulz & Hoehne, 1964); usually 10 ml. of acid medium was added/100 mg. wet wt. of tissue. After thawing, the acid suspension (pH 2.8) was warmed for 20 min. at 60° and then centrifuged and neutralized; 'total NADP' was estimated in the extract by the normal colorimetric procedure (Slater *et al.* 1964); NADP estimated after such a weak acid extraction is here called 'total NADP'. NADP estimated by the routine strong acid extraction of unfrozen samples is simply termed 'NADP'; the difference between 'total NADP' and 'NADP' is the 'bound NADP' that is acid-labile.

Intracellular fractionation procedures. Mitochondria for experiments involving subsequent incubation with ADP, isocitrate and malate (see Table 7) were prepared as follows. A 1:10 suspension of liver was made in ice-cold 0.25 M-sucrose; this was centrifuged for 10 min. at 600g and the pellet of nuclei, erythrocytes and debris discarded. The supernatant was centrifuged for 10 min. at 15000g and the

mitochondrial pellet was resuspended in 0.25 M-sucrose containing 33 mM-nicotinamide and 1.9 mM-disodium ADP (see Purvis, 1958); other details are given in the legend to Table 7.

Mitochondrial suspensions for other purposes (Table 8) were separated by the centrifuging scheme given above but the final suspending medium was ice-cold 0.25 M-sucrose-0.5 M-nicotinamide-5 mM-tris, pH 7.4.

The intracellular distribution of NADP and NADPH₂ was studied by using two different centrifuging procedures. The first ('slow') procedure (A), shown in Scheme 1, is a commonly used sequence of centrifugings based on the procedures described by Schneider & Hogeboom (1950). The second ('rapid') procedure (B), shown in Scheme 1, was developed in the present investigation so that nucleotide analysis of all fractions could be commenced 30-40 min. after killing the rat. In procedure B no provision is made for separation of the debris fraction D (containing nuclei, erythrocytes and other components; see Hogeboom, Schneider & Pallade, 1948; Schneider & Hogeboom, 1950) from the mitochondrial fraction. With procedure A the average time between killing the rat and having all fractions available for nucleotide extraction was approx. 2½ hr.; with procedure B the time involved was approx. 45 min. The significance of this difference in speed of assay is discussed below.

Cytochrome oxidase in homogenates and intracellular fractions was measured by the procedures described by Slater & Planterose (1960).

Variants on the routine conditions and additions to the suspending medium are described at the appropriate position in the Results section.

RESULTS

Table 1 presents results on the concentrations of NADP and NADPH₂ in rat-liver suspensions compared with the values obtained with intact liver samples from the same animal. The experiments reported in each case are typical of several performed with each additive; a range of concentrations was studied in most instances. In many cases the homogenates were assayed for NADP and NADPH₂ both immediately after preparation and 30 min. later after storing in ice. Assays carried out immediately after preparing the homogenates are called 'time 0' values in Table 1.

Suspensions prepared in 0.25 M-sucrose alone, or in 0.25 M-sucrose-5 mM-buffer (tris or phosphate), pH 7.4, had lower concentrations of NADP + NADPH₂ than had the equivalent samples of intact liver; this difference was more pronounced after the homogenates had been stored for 30 min. at 0° [Expts. (a), (b) and (c) in Table 1]. In six experiments in which the sums of the NADP + NADPH₂ concentrations in intact pieces of liver and in 0.25 M-sucrose homogenates stored for 30 min. at 0° were compared, the homogenate sum was 68 ± 3% of the value for intact liver. In attempts to maintain the sum of the NADP + NADPH₂ concentrations in sucrose homogenates

phosphate, nicotinamide) of NAD(P) glycohydrolase and of NAD(P)H₂ nucleotidohydrolases, and inhibitors of metal-catalysed and free-radical

processes [EDTA, promethazine (Phenergan) and 4,4,4-trifluoro-1-(thien-2-yl)butane-1,3-dione]; NADPH₂ is known to be unstable in the presence of free-radical reactions in solution (Scaife, 1963). Various NADP-linked substrates were also tried in case the nucleotide destruction that occurred was primarily of the oxidized form. Although the relevant data are not included in Table 1, the total nucleotide concentration (NADP + NADPH₂) in homogenates was not consistently affected by the inclusion of EDTA (0.2 mM) or promethazine (0.07–1.7 mM) in the 0.25 M-sucrose suspending medium. The total nucleotide content was not

affected by storing homogenates for 30 min. at 0° under anaerobic conditions in a Thunberg tube compared with normal storage under aerobic conditions.

The addition of nicotinamide (final concn. 0.01–0.1 M) to the 0.25 M-sucrose medium was often beneficial in maintaining the concentration of NADP + NADPH₂ in homogenates at values comparable with that in liver samples, but the results were not consistently reproducible in this respect and occasionally serious discrepancies were observed. In five out of ten experiments with 0.25 M-sucrose homogenates supplemented with 5 mM-tris,

Table 1. *Effects of various treatments on the concentrations of NADP and NADPH₂ in rat-liver homogenates*

All homogenates were prepared in ice-cold 0.25 M-sucrose with and without other additions; in the Table, H stands for a 0.25 M-sucrose homogenate. In many instances nicotinamide was included in the suspending medium; this is abbreviated NAA. The addition of 5 ml. of 0.1 M-tris or -phosphate buffer to 100 ml. of suspending medium is abbreviated as tris or phosphate respectively. Other abbreviations used are: G6P, glucose 6-phosphate; TFTB, 4,4,4-trifluoro-1-(thien-2-yl)butane-1,3-dione. In Expts. (c), (g) and (i) the tissue was frozen in liquid N₂ before homogenizing or extraction. In Expt. (e) the values marked with an asterisk are values for NADPH₂ only. Nucleotide values are given as µg./whole liver/100 g. body wt.

Expt.	Tissue sample	Additive	Time of storage at 0° (min.)	NADP + NADPH ₂	NADPH ₂ /NADP ratio
(a)	Intact	—	—	1221	5.4
	H	—	30	970	3.8
	H	PP _i (0.1 M)	30	656	3.3
	H	Atebrin (5 mM)	30	912	6.3
(b)	Intact	—	—	1209	10.3
	H-tris	—	0	936	11.3
	H-tris	—	30	754	24.1
(c)	Intact	—	—	995	6.0
	H-phosphate	—	30	665	2.5
	H-phosphate	Vitamin C (1.14 mM)	30	675	5.3
	H-phosphate	AMP (2.55 mM)	30	638	2.5
	H-phosphate	Azide (0.1 M)	30	710	1.2
(d)	Intact	—	—	1372	4.8
	H-0.05 M-NAA-tris	—	0	1308	6.1
	H-0.05 M-NAA-tris	—	30	1294	6.9
	H-0.05 M-NAA-tris	—	60	1167	8.5
(e)	Intact	—	—	930*	—
	H-0.1 M-NAA-tris	—	0	755*	—
	H-0.1 M-NAA-tris	—	30	267*	—
(f)	Intact	—	—	900	5.4
	H-0.5 M-NAA-phosphate	—	30	1081	3.4
	H-0.5 M-NAA-phosphate	G6P (1.65 mM)	30	1013	4.6
	H-0.5 M-NAA-phosphate	Isocitrate (1.7 mM)	30	1019	6.1
(g)	H-0.5 M-NAA-tris	—	30	1173	3.0
	H-0.5 M-NAA-tris	TFTB (10.3 mM)	30	1080	0.2
	H-0.5 M-NAA-tris	Vitamin C (1.03 mM)	30	1107	4.3
(h)	H-0.5 M-NAA-tris	—	30	1210	2.3
	H-0.5 M-sucrose-tris	—	30	823	3.0
	H-0.5 M-acetamide-tris	—	30	1040	4.2
(i)	Intact	—	—	870	3.7
	H	—	30	670	3.5
	H-0.5 M-acetamide-tris	—	30	842	2.6
	H-0.1 M-azide-tris	—	30	500	2.8

pH 7.4, and nicotinamide (final concn. 0.02–0.1 M), and stored for 30 min. at 0°, the sums of the NADP + NADPH₂ concentrations in the homogenates were 10–33% lower than the sum found in whole liver samples from the same rats. Similar large decreases in the sum of the NADP + NADPH₂ concentrations were not observed in four experiments with liver homogenates from male rats. The inclusion of 0.5 M-nicotinamide with 0.25 M-sucrose–5 mM-tris, pH 7.4, however, was consistently effective in maintaining homogenate nucleotide concentrations at the same level as found in intact tissue for at least 30 min., and this medium was subsequently used as a routine in the preparation of homogenates. No attempt was made to study the effects of concentrations of nicotinamide in the range 0.1–0.5 M.

Addition to the 0.25 M-sucrose–0.5 M-nicotinamide–tris medium of substrates for NADP-linked enzymes (e.g. glucose 6-phosphate, isocitrate) and of vitamin C increased the value of the NADPH₂/NADP concentration ratio towards that obtained with intact liver samples [Expts. (f) and (g) in Table 1]. Addition of the non-haem iron chelator 4,4,4-trifluoro-1-(thien-2-yl)butane-1,3-dione to liver suspensions resulted in an extensive oxidation of NADPH₂ with a resultant decrease in the NADPH₂/NADP concentration ratio [Expt. (g) in Table 1].

In Table 2 the effect of freezing samples of intact liver by immersion in liquid nitrogen is shown. Some NADPH₂ is oxidized to NADP, leaving the sum of the NADP + NADPH₂ concentrations unaltered. With homogenates, however, a different behaviour was observed. After freezing there was a much larger increase ($P < 0.02$) in NADP than the

decrease found in NADPH₂. Consequently, after freezing, there was a tendency ($P < 0.02$) for the sum of the NADP + NADPH₂ concentrations to be greater in homogenates prepared from frozen tissue compared with those prepared from fresh tissue. This tendency for a net appearance of NADP + NADPH₂ after freezing is similar to the data reported by Burch *et al.* (1963) for intact liver samples. It must be emphasized again that in the experiments considered in Table 2 the suspensions prepared from frozen tissue were subjected to the strong (N-perchloric acid) acid extraction procedure and not the weak (0.02 N-sulphuric acid–sodium sulphate–ethanol) acid procedure normally used for demonstrating 'bound NADP'.

Table 2 also indicates that by using 0.25 M-sucrose–0.5 M-nicotinamide–tris medium for preparing liver suspensions from both fresh or frozen material the sum of the NADP + NADPH₂ concentrations in the suspension 30 min. after preparation and storing on ice was not significantly different from the value obtained with corresponding fresh or frozen intact liver samples. In fact, direct comparisons between intact liver samples assayed immediately and homogenates prepared with liver tissue from the same rats and stored for 30 min. at 0° showed that with fresh tissue the sum of the NADP + NADPH₂ concentrations in the homogenate was $96 \pm 6\%$ of the corresponding intact liver values (mean of five separate experiments). With frozen tissue, the sum of the NADP + NADPH₂ concentrations in the homogenate was $100 \pm 3\%$ of the frozen intact liver values (mean of six separate experiments).

Tables 3 and 4 give the results obtained for the

Table 2. *Effect of freezing tissue samples and homogenates in liquid nitrogen before extraction with N-perchloric acid for NADP assay or with 0.1 N-sodium hydroxide for the estimation of NADPH₂*

Values are given as $\mu\text{g./whole liver/100 g. body wt.} \pm \text{s.e.m.}$ with the numbers of rats used in parentheses. For intact samples no thawing of the frozen tissue was allowed before adding to the acid or alkaline extraction medium. With frozen-tissue homogenates the tissue was first frozen and then thawed during homogenizing and kept at 0° for 30 min.; homogenates were prepared as 1:10 suspensions in 0.25 M-sucrose–0.5 M-nicotinamide–5 mM-tris, pH 7.4. In each case assays on fresh and frozen material were done at the same time on samples from the same rat. For other details see the Methods section. The mean values were compared by using a paired *t* test; N.S., not significant.

Sample	NADP	NADPH ₂	NADP + NADPH ₂
Intact			
Fresh	114 ± 9 (5)	897 ± 64 (8)	1067 ± 106 (5)
Frozen	228 ± 22 ($P < 0.005$)	803 ± 75 ($P < 0.02$)	1077 ± 129 (N.S.)
Difference between mean values	+ 114	– 94	+ 10
Homogenate			
Fresh	212 ± 39 (5)	778 ± 137 (5)	990 ± 127 (5)
Frozen	457 ± 69 ($P < 0.01$)	689 ± 99 (N.S.)	1156 ± 92 ($P < 0.02$)
Difference between mean values	+ 245	– 89	+ 166

distribution of NADP and NADPH₂ in various intracellular fractions. In Table 3 the typical 'slow' procedure A (Scheme 1) was used, and by the end of the isolation procedure a substantial loss of NADP + NADPH₂ had occurred. In fact the recovery of NADP + NADPH₂ in the three fractions isolated was only 53% of the initial value for intact whole liver. Table 4 presents results obtained by using the modified 'rapid' procedure B (Scheme 1). Recovery in the isolated fractions is satisfactory (98–107%) compared with the whole homogenate value. All extractions on the separate fractions, as also for the results in Table 3, were done at about the same time (± 5 min.) and so are directly comparable. Approx. 50% of the sum of NADP + NADPH₂ was found in the composite large-particle fraction and approx. 50% in the soluble fraction (Table 4). The estimation of cytochrome oxidase in the various fractions obtained by the 'rapid' procedure B (Scheme 1) showed that less than 5% of the overall total homogenate activity remained in the U + S fraction after a single spinning at 15000g for 15 min. This is similar to the value

obtained with 0.25M-sucrose alone as suspending medium, and indicates that most of the mitochondria sediment in the D + M fraction under the conditions used.

Estimations of 'bound NADP' were also performed on intracellular fractions separated by the 'rapid' procedure B (Scheme 1). The results (Table 4) show that 80% of the total 'bound NADP' was in the composite 'mitochondrial + nuclear' fraction, 13% was in the microsomal fraction, and a zero or even negative value was consistently obtained with the high-speed supernatant fraction. This last finding meant that NADP estimated by the freezing and weak acid extraction procedure was less than the NADP estimated by the routine N-perchloric acid extraction process.

In Table 5 are collected results bearing on the concentration of 'bound NADP' in rat-liver samples. Similar values were obtained for intact liver samples and for homogenates; no change occurred in the value found in homogenates after a 30 min. storage at 0°. Several attempts were made to induce the appearance of 'bound NADP' in

Table 3. *Intracellular distribution of NADP and NADPH₂ in rat liver by using the standard 'slow' procedure A for isolating the intracellular fractions*

The time taken to carry out the isolation procedure was 165 min. in this experiment with a female rat, body wt. 118 g. The nucleotide values are given as $\mu\text{g.}/\text{whole liver}/100\text{ g. body wt.}$ and as percentages of the corresponding homogenate value. For other details see the Methods section. The homogenates and intracellular fractions were estimated together 165 min. after killing the rat. In this particular experiment, fractions D and M were assayed together. For abbreviations see Scheme 1.

Fraction	NADP		NADPH ₂		NADP + NADPH ₂		NADPH ₂ /NADP ratio
	($\mu\text{g.}$)	(%)	($\mu\text{g.}$)	(%)	($\mu\text{g.}$)	(%)	
Intact liver	107		1155		1262		10.6
Homogenate (H)	265	(100)	615	(100)	880	(100)	2.3
D + M	124	47	158	26	282	32	1.3
U	28	11	6	1	34	4	0.2
S	119	44	237	38	356	40	2.0
Recovery		102		65		76	

Table 4. *Intracellular distribution of NADP, 'bound NADP' and NADPH₂ in rat liver by using the modified 'rapid' procedure B for isolating intracellular fractions*

Mean values $\pm$ S.E.M. are given as percentages of the homogenate values, which are also expressed as $\mu\text{g.}$ of nucleotide/whole liver/100 g. body wt. For other details see the Methods section. For abbreviations see Scheme 1.

Fraction	NADP		'Bound NADP'		NADPH ₂		NADP + NADPH ₂	
	($\mu\text{g.}$)	(%)	($\mu\text{g.}$)	(%)	($\mu\text{g.}$)	(%)	($\mu\text{g.}$)	(%)
Homogenate (H)	358	(100)	243	(100)	853	853	1212	(100)
D + M		44 ± 8		80		54 ± 4		50 ± 3
U		6 ± 2		13		2 ± 2		3 ± 1
S		48 ± 9		-21		51 ± 5		49 ± 5
Recovery		98 ± 12		—		107 ± 6		102 ± 3

high-speed supernatant fractions by adding washed rat-erythrocyte suspensions. However, despite the presence of considerable amounts of NADPH₂ no 'bound NADP' was detectable before or after the addition of erythrocytes. No 'bound NADP' could be detected in washed erythrocytes. Similarly, the addition of blood to a mitochondrial fraction did not increase the amount of 'bound NADP' detected (Table 6).

Incubation of mitochondria with ADP and nicotinamide (Purvis, 1958) produced an almost complete oxidation of NADPH₂ to NADP, and subsequent incubation with isocitrate and malate (Henley & Laughrey, 1965) partially restored the

NADPH₂/NADP concentration ratio to normal (Table 7). Under these conditions the changes in 'bound NADP' followed very closely the pattern of change exhibited by NADPH₂.

Phenazine methosulphate rapidly and non-enzymically oxidizes NADPH₂ to NADP (Stollar,

Table 5. *Concentrations of NADP, NADPH₂ and 'bound NADP' in intact samples of rat liver and in homogenates*

Homogenates were prepared in 0.25 M-sucrose-0.5 M-nicotinamide-5 mM-tris, pH 7.4. The values for NADP and NADPH₂ are given for comparison and are taken from Slater *et al.* (1964). Nucleotide values are as $\mu\text{g./g.}$ wet wt. of liver. The numbers of rats used are in parentheses. The homogenate was assayed (i) as quickly as possible, which proved to be approx. 1 min. after killing the rat, and (ii) after storing in ice for 30 min.

Normal rat liver	'Bound NADP'	NADP	NADPH ₂
Intact samples	77 $\pm$ 8 (7)	27 $\pm$ 2 (10)	190 $\pm$ 6 (10)
Homogenate			
(1 min.)	69 $\pm$ 11 (10)		
(30 min.)	71 $\pm$ 7 (6)		

Table 7. *Effect of incubating rat-liver mitochondria with various additives on the concentrations of NADP, 'bound NADP' and NADPH₂*

In each case a mitochondrial fraction was prepared as described in the Methods section. A 40 ml. sample of this suspension was immediately mixed with 2.4 ml. of 1.7 M-sucrose, giving a final sucrose concentration of 0.33 M: (i) 12 ml. was immediately withdrawn, mixed with 5 ml. of tris buffer, pH 7.4 (0.1 M), and 5 ml. portions were used for assay of the nucleotides; (ii) 12 ml. of mitochondrial suspension + 5 ml. of tris buffer were incubated for 7 min. at 30°, after which 5 ml. portions were used for assay; (iii) 12 ml. of mitochondrial suspension + 5 ml. of tris buffer were incubated for 7 min. at 30°, after which 2.5 ml. of 13 mM-isocitrate in tris buffer and 2.5 ml. of 0.26 M-malate in tris buffer were added; a further incubation of 5 min. at 30° was carried out, after which 5 ml. portions were withdrawn for assay. The results given are means $\pm$ S.E.M. of four separate experiments. The means are expressed as percentages of the values obtained for freshly prepared mitochondria [stage (i) above]. For other details see the Methods section.

	'Bound NADP'	NADPH ₂	NADP + NADPH ₂
(i) Control	100	100	100
(ii) ADP	26 $\pm$ 11	17 $\pm$ 3	100 $\pm$ 6
(iii) ADP + isocitrate + malate	49 $\pm$ 15	42 $\pm$ 11	95 $\pm$ 6

Table 6. *Effects of blood on 'bound NADP' in rat-liver intracellular fractions*

A 1:10 homogenate of rat liver in 0.25 M-sucrose-0.5 M-nicotinamide-5 mM-tris was prepared. A mitochondrial fraction was isolated by the 'slow' procedure A (Scheme 1) to give fraction M1. The supernatant fraction S was prepared by centrifuging the homogenate at 105 000g for 20 min. In Expt. (a) 2 ml. of blood was collected and haemolysed with 2 ml. of water; 2 ml. of the clear haemolysate was mixed with 15 ml. of mitochondrial fraction to constitute the 'M + blood' fraction. In Expt. (b) 0.5 ml. of heparinized blood was mixed with 4.5 ml. of 0.25 M-sucrose. The erythrocytes were spun down at 600g for 5 min. and washed twice with 0.25 M-sucrose with the same centrifuging conditions. The erythrocytes were finally suspended in 0.25 M-sucrose to give a volume of 5 ml. The composite fraction 'S + erythrocytes' was prepared by mixing 5 ml. of supernatant with 0.1 ml. of erythrocyte suspension; 0.1 ml. of erythrocyte suspension was also assayed separately for nucleotide content. Assays in Expts. (a) and (b) were performed immediately all the fractions were prepared. Values are given as $\mu\text{g.}$ of nucleotide/g. wet wt. of liver. For other details see the Methods section.

Expt.	Fraction	NADP	'Total NADP'	'Bound NADP'	NADPH ₂
(a)	M	21	41	20	—
	M + blood	45	54	9	—
(b)	S	82	82	0	186
	Erythrocytes	0*	0*	0*	0*
	S + erythrocytes	82	77	-5	200

* Too low to be measured.

Table 8. *Effect of incubation with phenazine methosulphate on the concentrations of NADP, 'bound NADP' and NADPH₂ in rat-liver homogenates or mitochondrial suspensions*

Nucleotide values are expressed as $\mu\text{g./g. wet wt.}$ of original liver tissue. Expt. (a): a 1:10 homogenate (60 ml.) in 0.25 M-sucrose-0.5 M-nicotinamide-5 mM-tris, pH 7.4, was centrifuged as described in the Methods section to isolate a mitochondrial fraction. This was resuspended in the sucrose medium (final vol. 30 ml.) and part (14.5 ml.) was incubated for 1 min. at 37° with 0.5 ml. of water, after which nucleotide extractions were performed. A further part (14.5 ml.) of the mitochondrial suspension was mixed with 0.5 ml. of phenazine methosulphate (20 mg./5 ml. of water) and incubated for 1 min. at 37°, and nucleotide extractions were then performed. Expt. (b): a 1:20 homogenate was prepared in the sucrose medium described in Expt. (a). Two 20 ml. portions were incubated for 30 min. at 0°; 2 mg. of phenazine methosulphate was added to one of the portions at the beginning of the incubation. Extractions of NADP, 'bound NADP' and NADPH₂ were then performed. Expt. (c): a 1:8 homogenate was prepared in the sucrose medium described in Expt. (a). One portion (20 ml.) was mixed with 1.3 ml. of water and another portion (20 ml.) with 1.3 ml. of phenazine methosulphate (20 mg./5 ml. of water). After incubation for 1 min. at 37° NADP was extracted normally with N-HClO_4 , but the sum NADP+NADPH₂ was determined jointly by using the extraction procedure of Spirtes & Eichel (1955); NADPH₂ was calculated by difference. Expt. (d): a mitochondrial fraction (1 ml. $\equiv$ 0.135 g. of original tissue) was prepared as described in Expt. (a). One portion (15 ml.) was incubated with 0.5 ml. of water and another with 0.5 ml. of phenazine methosulphate (25 mg./10 ml. of water). After incubation for 1 min. at 37°, NADP and NADP+NADPH₂ extractions were performed as in Expt. (c). For other details see the Methods section.

Expt.	Incubation time (min.)	Addition	NADP	'Bound NADP'	NADPH ₂	NADP+NADPH ₂	NADP+'bound NADP'+NADPH ₂
(a)	1	Control	7	12	52	59	71
		Phenazine	16	37	6	22	59
(b)	30	Control	24	80	174	198	278
		Phenazine	155	72	36	191	263
(c)	1	Control	35	59	154	187	246
		Phenazine	151	97	40	191	288
(d)	1	Control	4	12	36	40	56
		Phenazine	8	26	40	48	74

1960). Table 8 shows that after a brief period of incubation with this compound there is an increase in the 'bound NADP' concentration coinciding with a decreased concentration of NADPH₂. With longer incubation times although the 'bound NADP' concentration decreases slightly this is much less than the very substantial decrease that occurs in NADPH₂ concentration. The results in Table 8 are corrected for the breakdown of NADPH₂ that occurs in the presence of 0.1 N-alkali and phenazine methosulphate (or its alkali-degradation products; see Ramage & Landquist, 1959) owing to oxidation of NADPH₂ followed by alkaline degradation of NADP. The rate of destruction of NADPH₂ (and NADH₂) in the presence of phenazine methosulphate is very much less at pH 7.4. The results found by using the Spirtes & Eichel (1955) extraction procedure at pH 7.4 were in agreement with the results obtained by using the routine 0.1 N-sodium hydroxide extraction (Table 8).

DISCUSSION

In a detailed study, using male rats, Jacobson & Kaplan (1957b) showed that after the addition of

0.05 M-nicotinamide to the 0.25 M-sucrose suspending medium the concentrations of the nicotinamide-adenine dinucleotides in rat-liver homogenates were maintained for 30 min. at 0° at levels similar to those found in intact liver samples. Their results indicate that the breakdown of the nicotinamide-adenine dinucleotides that normally occurs in unsupplemented sucrose homogenates [e.g. see Expt. (a) in Table 1] is largely due to the activity of NAD(P) glycohydrolase, an enzyme that is strongly inhibited by low concentrations of nicotinamide (Mann & Quastel, 1941; Handler & Klein, 1942; McIlwain & Rodnight, 1949; Kaplan, 1955) and that is extremely active in rat-liver suspensions (Greenbaum, Clark & McLean, 1964; Slater & Sawyer, 1966c).

In the present study, with female rats, the inclusion of nicotinamide (final concn. 0.02–0.1 M) in the homogenizing medium often maintained the sum of the NADP+NADPH₂ concentrations near to the value obtained with intact liver samples from the same rats, but there were instances where it did not. Therefore it seems likely, at least in homogenates prepared from female rat liver, that although the major breakdown of NADP is mediated through a nicotinamide-sensitive stage,

presumably NAD(P) glycohydrolase, other factors may contribute to the decreased nucleotide content. This is also suggested by the results obtained with intracellular fractions (Table 4). The pattern of distribution of the nucleotides among the several fractions obtained by the 'rapid' procedure B (Scheme 1) is similar to that with procedures taking much longer to perform and where substantial loss of nucleotides occurs (see Table 2; also Jacobson & Kaplan, 1957*b*; Glock & McLean, 1956). This suggests that the breakdown occurring in such 'slow' procedures is proportionately divided among the fractions. This again tends to rule out NAD(P) glycohydrolase as the major route of breakdown occurring in 0.25M-sucrose homogenates since that enzyme is largely concentrated in the endoplasmic reticulum in rat liver, with virtually no activity in the mitochondria (Jacobson & Kaplan, 1957*c*; Waravdekar & Griffin, 1964), where approx. 50% of the total NADP + NADPH₂ is localized (Table 4).

Other mechanisms that might contribute to the decrease observed in NADP + NADPH₂ in suspensions must therefore be considered. NAD(P)H₂ nucleotidohydrolase (Jacobson & Kaplan, 1957*a*) is unlikely to be significantly involved since inhibitors of this enzyme (AMP, fluoride, pyrophosphate) did not maintain the concentration of NADP + NADPH₂ in tissue suspensions at the value present in intact liver (Expts. *a* and *c* in Table 1). NAD(P) nucleotidohydrolase (Kornberg, 1955) is also unlikely to be of major importance since its activity in rat liver is very low (Jacobson & Kaplan, 1957*c*; Greenbaum, Clark & McLean, 1965), and it has a higher Michaelis constant for NADP than NAD ($K_m^{\text{NAD}} 1.5 \times 10^{-4}\text{M}$; $K_m^{\text{NADP}} 3 \times 10^{-3}\text{M}$; Kornberg, 1955).

The inclusion of 0.5M-nicotinamide-5mM-tris in the 0.25M-sucrose suspending medium consistently maintained the sum of the NADP + NADPH₂ concentrations at the level found in intact samples of liver taken from the same rat. It is possible that such a very high concentration of nicotinamide is effective through inhibiting the oxidation of NADPH₂ (Jacobson & Kaplan, 1957*b*), and this might explain the occasional effectiveness of promethazine when added to a sucrose suspending medium. Phenothiazines of the promethazine type are known to inhibit the oxidation of NAD(P)H₂ in several systems *in vitro* (Dawkins, Judah & Rees, 1960; Slater, 1965*b*). A further possibility was thought to be dependent simply on the osmotic strength of the medium used, but this is unlikely since substitution of 0.5M-sucrose for 0.5M-nicotinamide was not effective in maintaining the concentration of NADP + NADPH₂ in stored suspensions. It is noteworthy, however, that 0.5M-nicotinamide can be effectively replaced by 0.5M-acetamide [Expts. (*h*) and (*i*) in Table 1].

The results of Tables 2, 4, 6, 7 and 8 are relevant to the nature of the 'bound NADP' first reported by Burch *et al.* (1963). Although workers in several Laboratories have confirmed the original findings, attempts to clarify the nature and biochemical role of 'bound NADP' have not been entirely successful. Neubert *et al.* (1964) have suggested that a major part of the acid-labile NADP in rat-liver mitochondria arises from a haem-catalysed oxidation of NADPH₂ during the weak (0.02N-sulphuric acid) acid extraction stage that is used to demonstrate the existence of this 'bound NADP'. The results of Heldt *et al.* (1965) also favour the conclusion that 'bound NADP' is an artifact arising during weak acid extraction. The findings of the present investigation give only partial support to such an explanation. Table 2, for example, shows that the process of freezing alone accounts for a substantial part of the 'bound NADP' detected under the conditions used here. In fact, freezing in liquid nitrogen appears to convert approx. 100 μg . of NADPH₂/whole liver/100g. body wt. into an equivalent quantity of NADP. Since the entire amount of 'bound NADP' detectable in intact liver samples after freezing in liquid nitrogen is approx. 300 μg ./whole liver/100g. body wt., this infers that about one-third is the result of freezing alone. However, even allowing for such an inter-conversion, additional NADP (presumably 'bound NADP') is still demonstrable in liver homogenates prepared from frozen tissue (Table 3) where the strong (N-perchloric acid) acid extraction procedure of NADP was used after thawing. Under such conditions it is unlikely that significant haem-catalysed oxidation of NADPH₂ to NADP occurs.

If 'bound NADP' were largely due to conversion of NADPH₂ into NADP during acid extraction then it could be expected (Neubert *et al.* 1964) that prior depletion of NADPH₂ in liver samples before the acid extraction stage would decrease the amount of 'bound NADP' subsequently found. This argument has been tested in several ways. First, a single dose of carbon tetrachloride causes substantial depletion in NADPH₂ in the livers of rats 1hr. after administration (Slater *et al.* 1964), but the amount of 'bound NADP' found at this time is increased (Slater *et al.* 1964). Secondly, oxidation of NADPH₂ by brief incubation of homogenates or mitochondrial suspensions with phenazine methosulphate (Stollar, 1960) led to increases in 'bound NADP' but NADPH₂ concentrations were greatly decreased (Table 8). Thirdly, it was not possible to induce the appearance of 'bound NADP' in high-speed supernatant fractions by the addition of washed erythrocytes despite the presence of considerable quantities of NADPH₂ (Table 6). In contrast, the results in Table 7 show a close correspondence

between the concentrations of 'bound NADP' and of NADPH₂ in the large-particle fraction.

The above results suggest that there is no obligatory stoichiometric relationship between 'bound NADP' and the total amount of NADPH₂ either in liver tissue or (Table 8) in mitochondria. This suggestion is supported by the variation in the NADPH₂/bound NADP concentration ratio observed among different tissues (Burch *et al.* 1963), being approx. 1.0 for liver and 1.7 for kidney.

The data presented by Heldt *et al.* (1965) and by Neubert *et al.* (1964) are convincing that 'bound NADP' mainly arises during the acid extraction, presumably from the oxidation of NADPH₂. However, the present results suggest that perhaps 'bound NADP' is not simply an artifact of little importance. It appears that 'bound NADP' arises almost exclusively in the large-particle fraction of rat-liver homogenates (Table 4). Assuming that 'bound NADP' is derived from the oxidation of NADPH₂, this last result shows that not all of the NADPH₂ present in the liver cell is equally susceptible to oxidation to yield the bound form under the acid conditions of the extraction procedure used (N-perchloric acid). Perhaps NADPH₂ in a particular compartment only of the liver cell may be readily convertible into the bound form. The large-particle fraction studied here, which was found to contain the bulk of the 'bound NADP' present in the whole homogenate (Table 4), is a grossly heterogeneous fraction containing erythrocytes, nuclei, debris, mitochondria and a considerable amount of ribosome-rich material (Howell, Loeb & Tomkins, 1964). However, Burch, Lowry, Hunter, Bradley & Lerner (1965) have reported that little 'bound NADP' could be detected in a mitochondrial fraction, presumably containing large amounts of bound haem compounds. Thus the precise intracellular site(s) of 'bound NADP' is at present not known, but the above discussion suggests either that a particular haem group is required for the conversion of NADPH₂ into 'bound NADP' or that 'bound NADP' represents a fraction of liver NADPH₂ that is undergoing some particular metabolic activity. If the latter possibility is true then 'bound NADP' may be of importance in reflecting the overall amount of this fraction of NADPH₂ despite the probability that it is itself an artifact of the extraction procedure.

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