uronic amide and L-gulonic amide are not converted into L-ascorbic acid by the microsomes.

4. Reduced glutathione and some metal-binding agents enhance the microsomal synthesis of L-ascorbic acid by stimulating the synthesis and not indirectly by protecting the synthesized ascorbic acid.

5. L-Gulonolactone and L-*xylo*hexulonolactone have been identified as the intermediates in the conversion of D-glucuronolactone into L-ascorbic acid in the presence of cyanide by the soluble enzyme preparations from the liver of rat and goat.

6. Both D-glucuronolactone reductase and L-gulonolactone oxidase are absent from the tissues of the primates, guinea pig, Indian fruit bat and red-vented bulbul—species which cannot synthesize ascorbic acid.

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Liver Damage in Acute Heliotrine Poisoning

1. THE INTRACELLULAR DISTRIBUTION OF PYRIDINE NUCLEOTIDES

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Heliotrine belongs to the group of pyrrolizidine alkaloids of which many members are selectively hepatotoxic when ingested in the diet or administered by injection (Bull, 1955; Bull, Dick, Keast & Edgar, 1956; Schoental & Magee, 1957, 1959; Bull & Dick, 1959). The purified alkaloid can produce extensive liver-cell necrosis in rats within 24 hr. (Bull, Dick & McKenzie, 1958; Christie, 1958*a*), is rapidly metabolized in the rat, and the liver appears to be the main and possibly the only site of its metabolism (A. T. Dann, personal communication).

Administration of a dose of heliotrine which might be expected to kill 70% of rats (LD_{70}) within 72 hr. (Bull *et al.* 1958) was found to result, after an interval of 16–20 hr., in a loss of the capacity of homogenate and isolated mitochondrial preparations of liver to oxidize certain substrates (Christie, 1958*b*). The enzyme systems affected were all pyridine nucleotide-dependent, and could

be re-activated by the addition of diphosphopyridine nucleotide to the incubation medium. The time of onset of respiratory loss was found on histological examination to coincide with the development of extensive and severe liver-cell damage (Christie, 1958*a*). It was therefore proposed that the requirement for pyridine nucleotide that developed was an index of disorganization of the tricarboxylic acid cycle and thus of energy generation *in vivo*, and might be due to actual leakage of the pyridine nucleotide, as in 'aging' of mitochondria, to inactivation of the nucleotide *in situ*, to a derangement of coenzyme synthesis, or to an inaccessibility of coenzyme to the affected enzymes.

Studies *in vitro* by Gallagher (1958, 1960*a*, *b*) and Gallagher & Koch (1959) have demonstrated an inhibition by pyrrolizidine alkaloids of enzyme systems that require coenzyme I; partial prevention of the inhibition was obtained by adding the coenzyme.

Christie, Le Page & Bailie (1961) showed that the diphosphopyridine nucleotide content of liver fell progressively from the twelfth hour after administration of a single large dose of heliotrine to rats. In the present work the amounts of oxidized and reduced diphosphopyridine nucleotide, and of oxidized and reduced triphosphopyridine nucleotide, in the liver homogenate and homogenate fractions after treatment with heliotrine have been directly determined.

MATERIALS AND METHODS

Animals. Adult male hooded Wistar rats weighing 270–330 g. were used (Bull *et al.* 1958; Christie, 1958*a*). The diet consisted of Poultry Growers' pellets, fresh green vegetables and water. Heliotrine (320 mg./kg. body wt., in aqueous solution neutralized to pH 7·3 with HCl) was injected intraperitoneally under light ether anaesthesia. Control rats received injections of 0·9% NaCl soln.

Reagents. Inorganic reagents were of analytical grade; solutions were made in distilled de-ionized water. Cytochrome *c* was prepared by the method of Keilin & Hartree (1937) and dialysed against distilled de-ionized water. Oxidized and reduced pyridine nucleotides, isocitric dehydrogenase, alcohol dehydrogenase, isocitric acid, α-oxoglutaric acid and sodium L-glutamate were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.; AMP and ATP were from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.; EDTA, tris and acetaldehyde were laboratory reagents from British Drug Houses Ltd.; sodium citrate, sucrose and hexane were A.R. from British Drug Houses Ltd.; nicotinamide and sodium pyruvate were obtained from L. Light and Co. Ltd., Colnbrook, Bucks.

Solutions required for pyridine nucleotide estimation. These were as described by Bassham, Birt, Hems & Loening (1959) except that isocitric dehydrogenase was used instead of glucose 6-phosphate dehydrogenase. The concentration of an aqueous solution of this enzyme was adjusted so that 10 μl. reduced 10 μm-moles of TPN^+ at pH 8·2 (tris–HCl buffer) in a total volume of 0·5 ml. in 10 min.

Tissue preparations. Rats were killed by stunning and exsanguination, the livers were rapidly removed and chilled and weighed; samples were then transferred to ice-cold homogenizing medium. Samples for histological examination were fixed in 10% (v/v) formalin buffered to neutral pH with sodium acetate. Except where indicated in the text homogenates were 10% (w/v) and were made at 2° in a Potter–Elvehjem all-glass power-driven homogenizer with a loosely fitting pestle. Homogenates for respiration experiments and for the separation of cell particles were made with 0·25 M-sucrose–25 mM-nicotinamide in distilled de-ionized water at 2° as the suspending medium. Homogenates for pyridine nucleotide estimations and for the preparation of the supernatant fraction were made with a medium containing 0·155 M-NaCl and 50 mM-nicotinamide in distilled de-ionized water at 2°. Sedimented cell particles were washed and finally resuspended in the medium of Chappell & Perry (1954) to which had been added 25 mM-nicotinamide (modified Chappell & Perry medium). Differential centrifuging was carried out by the method of Schneider (1948) in a Spinco Model L refrigerated centrifuge; attention was given to obtaining a quantitative recovery of the cell fractions. Tissue slices were cut at 2° with a Stadie–Riggs microtome, weighed on a torsion balance and transferred to chilled flasks.

Analytical methods

Manometric estimations. Estimations of aerobic oxidation by homogenates and mitochondria were carried out in a standard medium in which the final concentrations of reagents in the Warburg flasks were: AMP, mM; KCl, 25 mM; $MgSO_4$, 6·6 mM; cytochrome *c*, 27 μM; sodium phosphate buffer, pH 7·3, 16 mM. Substrates were added to give a final concentration of 0·01 M; 0·5 ml. of homogenate or of mitochondrial suspension was used and the total volume of the incubation mixture in each Warburg flask was 2 ml. The quantity of DPN^+ required for each experiment was weighed shortly before use, and made up to 10 mM in aqueous 0·4 M-nicotinamide. These precautions were taken to eliminate the possibility of non-enzymic hydrolysis of DPN^+, which may be rapid at neutral or even at acid pH. A volume of 0·2 ml. of the DPN^+–nicotinamide mixture was added to each flask immediately before addition of the tissue preparation. The gas phase was air and the temperature 37°. Tissue slices were incubated in Krebs–Ringer phosphate solution (Umbreit, Burris & Stauffer, 1959) at 37° in a gas phase of oxygen.

Nitrogen. This was estimated colorimetrically after Kjeldahl digestion of samples containing 15–30 μg. of N. The Nessler reagent was prepared as described by King & Wootton (1956). The N values of the suspending media were determined with each group of samples, and subtracted from the total N.

Estimation of pyridine nucleotides

Extraction procedure. The procedure used for all fractions was that described by Bassham *et al.* (1959) for homogenates.

Analytical procedure. The method used for the determination of pyridine nucleotides, both in the oxidized and reduced states, was essentially that of Bassham *et al.* (1959),

with the following modifications. Isocitric dehydrogenase was used instead of glucose 6-phosphate dehydrogenase; 10 μl. of 0·2 M-$MgSO_4$ was therefore added to the preparations whose final volumes were thus 0·53 ml. (acid extract) and 0·56 ml. (alkaline extract). The step involving acid-treatment of the alkaline extract (solution B of Bassham *et al.* 1959) was omitted. Instead of allowing the tubes to stand at room temperature during enzyme treatment, they were incubated at 30° for 30 min. A sample (75 μl.) of the enzyme-treated extracts was pipetted into each fluorimeter tube, and the additions of standard acid or alkali were adjusted accordingly. Each of the standard tubes contained 50 μl. of 20 μM-DPN^+ solution plus 25 μl. of tris–HCl buffer, pH 7·3.

Measurement of fluorescence. The fluorescence developed in samples containing pyridine nucleotides was measured in a Locarte single-sided fluorimeter. The primary filter was Type LF1, with a transmission band between 254 and 400 mμ; the secondary filters were Ilford 622 and Locarte Type LF6 (transmitting wavelengths above 470 mμ). The instrument was checked before and during use against a solution containing 0·1 mg. of quinine sulphate/l. of 0·1 N-H_2SO_4.

RESULTS

Normal amounts of pyridine nucleotides in rat-liver homogenates and homogenate fractions. The values obtained for DPN^+, DPNH, TPN^+ and TPNH in preparations made under standard conditions from normal rats are shown in Table 1. We found that DPN^+ was present in excess of DPNH, and TPNH in excess of TPN^+; in each case the ratio was about 3:1.

A recovery of 78 % of the pyridine nucleotide of the whole homogenate was obtained in the fractions; 65 % was in the supernatant and 28 % in the mitochondria.

Pyridine nucleotide in livers of control and heliotrine-treated rats. More satisfactory comparison of the data from control and treated animals was obtained when the amounts of the liver pyridine nucleotides were expressed relative to 100 g. body wt. of rat rather than to g. wet wt. of liver (Christie, 1958*b*). This allowed for the possibility that a considerable loss of liver substance might eventually occur in a treated rat, although the residual liver might still show normal pyridine nucleotide values/g. wet wt. Thomson, Heagy, Hutchison & Davidson (1953) showed that it was 'necessary to relate the results of tissue analysis' to a standard of reference that 'should not change during the experiment'; they obtained satisfactory results by relating data to total liver weight in fasting, protein deficiency, thiamine deficiency and thioacetamide poisoning. In the present instance, total liver weight has been standardized relative to 100 g. body wt.; this was considered valid because heliotrine did not produce serious alteration of body wt. (such as by induction of oedema or dehydration) during the experimental period.

Table 1. *Pyridine nucleotide content of normal rat-liver homogenate and of homogenate fractions*

Results are mean values, in μm-moles (±S.E.M.), relative to the units indicated in the second column. Pyridine nucleotides were estimated by the method described in the text.

Fraction	Pyridine nucleotides (μm-moles)	No. of rats	DPN^+	DPNH	TPN^+	TPNH	DPN	TPN	DPN^+ + TPN^+	DPNH + TPNH	Total pyridine nucleotides
Homogenate	/g. wet wt.	19	738	238	141	447	976	588	879	685	1564
Mitochondria	/g. wet wt.	11	118	38	47	134	156	181	165	172	337
	/mg. of N	11	12·7	4·1	5·1	14·3	16·8	19·4	17·7	18·5	36·2
	/mg. of protein (N × 6·25)	11	2·02	0·66	0·81	2·29	2·68	3·10	2·83	2·95	5·79
Homogenate	/100 g. body wt.	19	2524	814	483	1527	3337	2010	3006	2341	5347
			(52)	(62)	(28)	(60)	(87)	(32)	(54)	(59)	(69)
Supernatant	/100 g. body wt.	4	1735	265	147	582	2000	729	1882	847	2729
			(75)	(9)	(10)	(79)	(82)	(79)	(67)	(87)	(141)
Mitochondria	/100 g. body wt.	11	405	132	163	459	537	622	568	591	1159
			(17)	(17)	(17)	(24)	(21)	(18)	(30)	(29)	(18)
Microsomes	/100 g. body wt.	2	27	0	23	15	27	38	50	15	65
			(11)	(0)	(2)	(4)	(11)	(5)	(10)	(4)	(6)
Nuclei	/100 g. body wt.	1	—	124	—	118	124	118	—	242	242
Total pyridine nucleotides recovered in fractions	/100 g. body wt.	—	2167	521	333	1174	2688	1507	2500	1695	4195
Recovery (% of homogenates)	/100 g. body wt.	—	85·8	64·0	68·9	76·8	80·5	74·9	83·1	72·4	78·4

Table 2. *Pyridine nucleotide content of liver homogenates from normal and heliotrine-treated rats*

Results are mean values in μm-moles of liver pyridine nucleotides/100 g. body wt. (±S.E.M.).

Duration of treatment (hr.)	No. of rats	DPN^+	DPNH	TPN^+	TPNH	DPN	TPN	$DPN^+ + TPN^+$	DPNH + TPNH	Total pyridine nucleotides
0	19	2524 (52)	814 (62)	483 (28)	1527 (60)	3337 (87)	2010 (32)	3006 (54)	2341 (59)	5347 (69)
12	1	2266	888	463	1419	3154	1882	2729	2307	5036
17	7	1827 (48)	899 (31)	350 (23)	1066 (74)	2726 (73)	1416 (73)	2177 (84)	1965 (85)	4142 (117)
22	3	1813 (92)	698 (85)	256 (28)	974 (25)	2510 (177)	1229 (50)	2067 (65)	1672 (160)	3739 (225)
25	4	1685 (86)	1095 (116)	386 (65)	816 (145)	2780 (164)	1201 (119)	2071 (56)	1910 (178)	3981 (232)
30	1	1515	1425	405	583	2940	988	1920	2008	3928

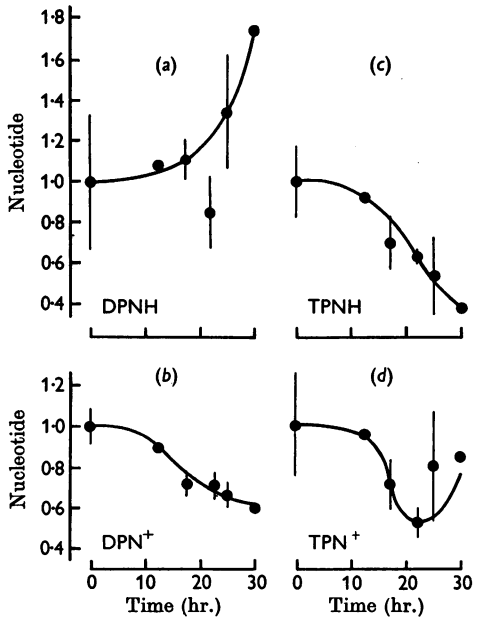

Fig. 1. Change of amount of liver DPNH (*a*) and DPN^+ (*b*) and of TPNH (*c*) and TPN^+ (*d*) during the 30 hr. period after injection of heliotrine (320 mg./kg. body wt.) into rats at 0 hr. Amounts of nucleotide (ordinate) are expressed as the ratio of the mean values from the treated rats to those from the control rats and were calculated from the data of Table 2. Vertical lines show ±S.D. population.

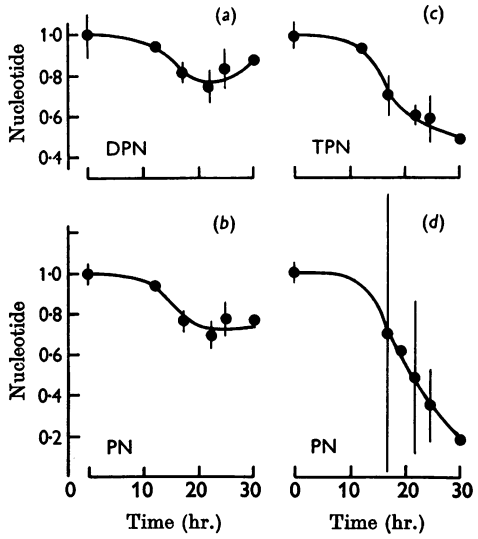

Fig. 2. Change of amount of liver DPN (*a*), TPN (*c*) and total pyridine nucleotide (*b*), and of total pyridine nucleotide of isolated mitochondria (*d*), during the 30 hr. period after injecting heliotrine (320 mg./kg. body wt.) into rats at 0 hr. The nucleotide amounts (ordinates) are expressed as the ratio of the mean values from the treated rats to those from the control rats and were calculated from the data of Tables 2 and 5. Vertical lines show ±S.D. population.

Pyridine nucleotide values of liver homogenates from 16 rats injected with heliotrine at various times before killing, and from 19 control rats, are given in Table 2.

With the treated rats the following trends were discernible by 17 hr.: total TPN (TPN^+ + TPNH) was considerably lower than total DPN (DPN^+ + DPNH); amounts of the nucleotides in the reduced state moved in opposite directions (the mean value of DPNH rose whereas that of TPNH fell rapidly); mean values of the oxidized nucleotides both fell, DPN^+ more quickly than TPN^+.

In the present experiments, the critical period for partial or complete loss of pyridine nucleotide-linked respiration of the liver homogenate (10% in 0·25M-sucrose; Christie, 1958*b*) was between 20 and 25 hr. after heliotrine administration. The greatly decreased oxygen uptake could often be raised considerably, even up to control values, by an addition of 1 μmole of DPN^+/ml. of incubation medium.

During this 20–25 hr. period the trends in amounts of pyridine nucleotide established before 20 hr. developed further (Table 2, Figs. 1 and 2): total pyridine nucleotide and DPN^+ were about two-thirds of the control value; DPNH showed a systematic upward trend, resulting in an elevation above mean control, although not outside the upper control limit until 30 hr.; TPNH fell to a greater extent than any other form. As the N of homogenates from treated animals was within control limits over this period, the fall of pyridine nucleotide of the order of 1600 μm-moles/100 g. body wt. below the control amount was accepted as a real decrease of liver-cell pyridine nucleotide in the treated animals.

Pyridine nucleotide in the supernatant fraction. In the rats treated with heliotrine, the trends observed in the supernatant fraction (Table 3) resembled those of the whole homogenate: total pyridine nucleotide and DPN^+ had fallen to approximately two-thirds of the control by 23 hr.; total TPN was lower than total DPN; TPNH showed a greater relative fall than any other form, but DPNH was still at the control value. The supernatant values accounted for 866 μm-moles of the total of 1608 μm-moles of pyridine nucleotide (/100 g. body wt. of rat) lost by the liver at this time (Table 6).

Pyridine nucleotide of the microsomal and nuclear fractions. Total microsomal pyridine nucleotide was not decreased in preparations from heliotrine-treated rats (18 and 24 hr.) but the DPN^+ fell and a corresponding rise of DPNH occurred (Table 4).

A considerable decrease in DPNH and TPNH below the control value was observed in the nuclear fraction from a heliotrine-treated rat (19 hr.) (Table 4).

Pyridine nucleotide in the liver-mitochondria fraction. In mitochondrial preparations of liver from heliotrine-treated rats the mean total pyridine nucleotide content was decreased to 70% of the control level at 17 hr. and became progressively lower as the interval between heliotrine injection and killing of the rat increased, until at 30 hr. about 75% of the total pyridine nucleotides had been lost (Table 5). Losses of DPN and TPN in the oxidized state and in the reduced state were detected, however; TPNH was lost in greater amounts, and after less treatment, than the other forms (Table 5).

Results in Table 5 from treated rats show the great degree of variability (as indicated by the large S.E.M.) between different preparations from different rats that had been treated for the same length of time; for example, a preparation from one rat might show very small losses and a second very great losses of pyridine nucleotides from the isolated mitochondria (Fig. 2).

Furthermore, the treated mitochondria showed greater relative losses of pyridine nucleotides (Fig. 2) than were observed in the homogenate and supernatant preparations and also differed in the

Table 3. *Pyridine nucleotide content of the supernatant fraction of liver homogenate of normal and heliotrine-treated rats*

Results are mean values in μm-moles of liver pyridine nucleotides/100 g. body wt. (±S.E.M.).

Duration of treatment (hr.)	No. of rats	DPN^+	DPNH	TPN^+	TPNH	DPN	TPN	$DPN^+ + TPN^+$	DPNH + TPNH	Total pyridine nucleotides
0	4	1735 (75)	265 (9)	147 (10)	582 (79)	2000 (82)	729 (79)	1882 (67)	847 (87)	2729 (141)
16¼	1	1531	423	270	468	1954	738	1801	891	2692
23	3	1150 (124)	265 (39)	95 (16)	354 (46)	1414 (124)	449 (60)	1245 (139)	618 (39)	1863 (174)

Table 4. *Pyridine nucleotide content of the microsomal and nuclear fractions of liver homogenate of normal and heliotrine-treated rats*

Results are mean values in μm-moles of liver pyridine nucleotides/100 g. body wt. (±S.E.M.).

Fraction	Duration of treatment (hr.)	No. of rats	DPN^+	DPNH	TPN^+	TPNH	DPN	TPN	$DPN^+ + TPN^+$	DPNH + TPNH	Total pyridine nucleotides
Microsomal	0	2	27 (11)	0 (0)	23 (1)	15 (4)	27 (11)	38 (5)	50 (10)	15 (4)	65 (6)
	19	1	12	9	25	18	21	42	37	26	63
	24	1	16	22	18	16	38	34	34	38	72
Nuclear	0	1	—	124	—	118	—	—	—	242	—
	19	1	—	62	—	55	—	—	—	117	—

Table 5. *Pyridine nucleotide contents of isolated liver mitochondria from normal and heliotrine-treated rats*

Results are mean values in μm-moles of liver pyridine nucleotides/100 g. body wt. (±S.E.M.).

Duration of treatment (hr.)	No. of rats	DPN^+	DPNH	TPN^+	TPNH	DPN	TPN	$DPN^+ + TPN^+$	DPNH + TPNH	Total pyridine nucleotides
0	11	405 (17)	132 (17)	163 (17)	459 (24)	537 (21)	622 (18)	568 (30)	591 (29)	1159 (18)
17	3	358 (107)	84 (31)	199 (42)	176 (58)	442 (143)	375 (77)	557 (147)	260 (80)	817 (460)
19	2	303 (72)	70 (57)	112 (26)	231 (163)	373 (115)	343 (169)	415 (46)	301 (252)	716 (297)
21½	2	198 (81)	102 (69)	85 (10)	184 (153)	300 (150)	269 (158)	283 (86)	286 (221)	569 (307)
24½	3	171 (109)	29 (2)	149 (51)	67 (23)	200 (54)	216 (68)	320 (105)	96 (23)	416 (121)
30	1	113	38	72	71	151	143	185	109	294

Table 6. *Decreases in content of pyridine nucleotide of homogenate and homogenate fractions of liver from heliotrine-treated rats*

'Observed' and 'expected' losses are expressed in μm-moles of liver pyridine nucleotides/100 g. body wt. 'Observed' losses were calculated from data in Tables 2, 3 and 5 by subtracting treated mean total values from the corresponding control mean total values. 'Expected' losses for the fractions were determined from control mean total values by assuming a 30% decrease in content of pyridine nucleotides, because of the 30% decrease of the homogenate value observed after treatment for 22–23 hr.

Fraction	Duration of treatment (hr.)	Pyridine nucleotides 'Observed' loss	Pyridine nucleotides 'Expected' loss
Homogenate	22	1608	1608
Supernatant (*S*)	23	866	819
Mitochondria (*M*)	21½	590	348
S + *M*	—	1456	1167

pattern of the losses. The N values of the mitochondrial suspensions used for pyridine nucleotide estimation (8·6 mg. of mitochondrial N/g. wet wt. of liver at 21 hr.) were only slightly lower than those of the simultaneously prepared controls (9·2 mg.). Thus true loss of mitochondrial pyridine nucleotides had taken place rather than destruction of the particles.

The observed losses of liver pyridine nucleotides detected in homogenates and in the mitochondrial and supernatant fractions of heliotrine-treated rats (20–25 hr.) are indicated in Table 6.

In the homogenate (22 hr.) the mean pyridine nucleotide loss was 1608 μm-moles (Table 6), which was a 30% decrease in control value. However, the amounts in the component fractions were not affected to the same relative extent as the whole homogenate, the supernatant being lowered by 866 μm-moles, which was slightly more than 30% (819 μm-moles), and the mitochondria by 590 μm-moles, which was considerably more than 30% (348 μm-moles), of the respective control values. It therefore seemed possible that losses of pyridine nucleotides might have occurred from the mitochondria during their isolation.

Losses of pyridine nucleotides during isolation of liver mitochondria. In view of the indications in the preceding section of this paper which suggested that losses of mitochondrial pyridine nucleotides occurred *in vitro*, an investigation was made of the amounts of pyridine nucleotides in liver mitochondria of control and heliotrine-treated rats after each of the successive washings in modified Chappell & Perry medium required to remove sucrose during the procedure of isolating mitochondria for pyridine nucleotide estimation.

The procedure was as follows: liver homogenates (in sucrose–nicotinamide medium) were rapidly prepared from control and heliotrine-treated rats (22 hr.) at a concentration of 50%, w/v (to minimize dilution of the supernatant fraction); the initial sedimentation of mitochondria was completed within 15 min. of killing the rats, priority being given to speed of preparation rather than to obtaining a quantitative recovery. The particles were resuspended in modified Chappell & Perry medium; samples of these suspensions were analysed for pyridine nucleotide content (unwashed preparation, Table 7), and the remainder of each suspension was sedimented and resuspended in fresh medium (constituting the once-washed preparation). After two further washings in the medium the particles were finally suspended in the same medium (to constitute the thrice-washed preparation).

Results (Table 7) indicate that a progressive loss of pyridine nucleotides occurred from the liver mitochondria of the heliotrine-treated rats during washing, whereas the total pyridine nucleotide content of the control mitochondria was unaffected.

Table 7. *Losses of pyridine nucleotides from liver mitochondria on repeated washing*

Values are given for pyridine nucleotide (μm-moles) and N (mg.) of liver mitochondria/100 g. body wt. of rat. The mitochondria were isolated rapidly from liver homogenates (50%, w/v, in 0·25 M-sucrose) from two control and two heliotrine-treated (22 hr.) rats, and samples analysed after resuspension in modified Chappell & Perry medium (unwashed), after one washing and after three washings in medium. The pyridine nucleotide/N ratio is shown. Details of the method are given in the text.

Preparation	No. of washings	DPN^+	DPNH	TPN^+	TPNH	DPN	TPN	DPN^+ + TPN^+	DPNH + TPNH	Total	N of preparation (mg.)	Pyridine nucleotides (μm-moles/mg. of N)
Control	0	45	645	17	430	690	447	62	1075	1137	39·5	28·8
Treated	0	63	387	17	218	450	235	80	605	685	29·4	23·3
Control	1	219	466	53	346	685	399	272	812	1084	28·4	38·2
Treated	1	199	84	102	53	283	155	301	137	438	20·0	21·9
Control	3	467	201	256	215	668	470	722	416	1138	28·0	40·6
Treated	3	100	15	71	19	115	89	170	34	204	19·8	10·3

In the unwashed control preparation (Table 7) 94·5% of the total pyridine nucleotide was in the reduced state, but during washing there was a progressive oxidation of the reduced nucleotides such that in the thrice-washed control preparation only 36·6% of the total pyridine nucleotide was in the reduced state. Birt & Bartley (1960) observed a similar oxidation of pyridine nucleotides during sedimentation of mitochondria.

Since the control total pyridine nucleotide content did not change from the unwashed to the thrice-washed mitochondrial preparation, we concluded that any interference with fluorescence measurements by traces of sucrose remaining in the unwashed preparation must have been minimal (cf. Bassham *et al.* 1959).

Recovery of pyridine nucleotides from mitochondrial washings. Because pyridine nucleotides were lost from the treated mitochondria during washing, it was important to determine whether the losses from the particles could be recovered in the pooled washings. The same procedure was used as is described in the previous section; liver mitochondria from control and heliotrine-treated (22 hr.) rats were rapidly isolated and pyridine nucleotides were estimated (*a*) in samples of mitochondria that had been washed once and three times respectively in modified Chappell & Perry medium, (*b*) in the pooled supernatant fluid of the second and third mitochondrial washings (Table 8). The pyridine nucleotide lost from the treated mitochondria was quantitatively recovered in the washings (Table 8), although alterations in oxidation state, and possibly conversion of TPN into DPN, occurred during manipulation.

Pyridine nucleotide and respiration of mitochondria from control and heliotrine-treated rats. A study was made of the respiration of mitochondria from livers of control and heliotrine-treated rats in relation to their pyridine nucleotide content. In these experiments each homogenate (10%, w/v, in sucrose–nicotinamide) was divided into two parts: the first part was used to prepare a mitochondrial suspension (once-washed and resuspended in sucrose–nicotinamide medium) for manometric measurements of oxygen uptake; the second part was used to prepare mitochondria (washed three times in modified Chappell & Perry medium and resuspended in the same medium) for analysis of pyridine nucleotide content.

Table 9 shows the rate of respiration with three substrates of mitochondria from control and heliotrine-treated rats, both in the presence and absence of added mM-DPN^+, together with their pyridine nucleotide content. The DPN^+-reversible failure of oxygen uptake shown (with the three DPN-linked dehydrogenase systems tested) by liver mitochondria from heliotrine-treated rats (19 hr. or

Table 8. *Recovery of pyridine nucleotides lost from liver mitochondria on repeated washing*

Values are given for pyridine nucleotide (μm-moles) and N (mg.)/100 g. body wt. of rat, of mitochondria rapidly prepared from homogenates (50%, w/v, in 0·25M-sucrose) of liver from two control and two heliotrine-treated (22 hr.) rats. Estimations were made on mitochondria suspended in modified Chappell & Perry medium, after the first and third washes, and on the pooled washings from the second and third washes. The pyridine nucleotide/N ratio is also shown. Details of the method are given in the text.

Preparation	No. of washings	DPN^+	DPNH	TPN^+	TPNH	DPN	TPN	DPN^+ + TPN^+	DPNH + TPNH	Total	N of preparation (mg.)	Pyridine nucleotides (μm-moles/ mg. of N)
Control	1	152	192	33	360	344	393	185	552	737	41·7	17·7
	3	152	142	62	384	294	446	214	526	740	41·7	17·7
Pooled washings		20	2	4	17	22	21	24	19	43	1·4	—
Treated	1	99	39	60	67	138	127	159	106	265	30·5	8·7
	3	51	28	17	20	79	37	68	48	116	25·8	4·5
Pooled washings		77	44	16	34	121	50	93	78	171	0·5	—

Table 9. *Rate of respiration and pyridine nucleotide content of liver mitochondria from normal and heliotrine-treated rats*

Mean values (±S.E.M.) of mitochondrial oxygen uptake (μl./hr./liver/g. body wt.) and pyridine nucleotides (μm-moles/liver/100 g. body wt.). The effect on respiration rate of an addition of DPN^+ (mM) to the medium is shown for each substrate. Methods of preparation and estimation were as described in the text.

Duration of treatment (hr.)	No. of rats	Respiration rate (μl. of O_2)						Pyridine nucleotides (μm-moles)				
		Citrate		α-Oxoglutarate		L-Glutamate						
		$-DPN^+$	$+DPN^+$	$-DPN^+$	$+DPN^+$	$-DPN^+$	$+DPN^+$	DPN^+	DPNH	TPN^+	TPNH	Total
0	5	84 (6)	106 (4)	77 (3)	87 (5)	74 (2)	91 (1)	429 (23)	140 (12)	142 (15)	461 (18)	1172 (19)
17	1	95	107	61	64	67	74	481	125	118	291	1015
19	1	10	86	4	60	8	73	231	13	138	36	418
21	2	15 (0·1)	79 (10)	7 (3)	67 (17)	9 (3)	41 (10)	198 (81)	102 (69)	85 (10)	184 (153)	569 (307)
25	1	26	69	26	27	—	—	61	33	54	27	175
30	1	12	67	24	34	—	—	113	38	102	41	294

Table 10. *Rate of respiration of liver slices and homogenates from normal and heliotrine-treated rats*

Mean values (±S.E.M.) of oxygen uptake of tissue slices (expressed as μl. of $O_2/\frac{1}{2}$ hr./10 mg. dry wt. of liver) and of homogenates (10%, w/v, in 0·25M-sucrose) (expressed as μl. of O_2/hr./liver/g. body wt.). The effect on respiration of an addition of mM-DPN^+ to the medium is shown. The substrate was α-oxoglutarate. The methods are described in the text.

Duration of treatment (hr.)	No. of rats	Tissue-slice respiration			Homogenate respiration		
		No substrate	Substrate	Substrate and DPN^+	No substrate	Substrate	Substrate and DPN^+
0	4	55 (4)	59 (6)	68 (4)	49 (14)	179 (12)	236 (11)
$23\frac{1}{2}$	1	50	65	48	73	187	226
28	1	46	57	62	53	35	183
$28\frac{1}{2}$	1	—	—	—	14	13	97
$31\frac{1}{2}$	1	34	25	39	20	12	33

later) coincided with a decrease of the total mitochondrial DPN to about 50% or less of the control value (Table 9).

Respiration of liver-slice and liver-homogenate preparations from control and heliotrine-treated rats. The oxygen uptake of liver-slice and liver-homogenate (10%, w/v, in 0·25M-sucrose) preparations from control and heliotrine-treated rats was estimated simultaneously (with α-oxoglutarate as substrate), and estimations were made both with and without the addition of mM-DPN^+ to the incubation medium. In the preparations made after heliotrine treatment of the rat for 28 hr. no oxygen uptake was observed with the homogenates in the absence of DPN^+; the slices, however, still respired at 28 hr. (Table 10). In the $31\frac{1}{2}$ hr. preparation, the homogenate showed little re-activation by the added DPN^+ and respiration of slice preparations was greatly reduced (Table 10).

DISCUSSION

Analytical procedure. The analytical procedure of Bassham *et al.* (1959) was found to be reliable, and its high sensitivity permitted measurements with pathological material in which small amounts of pyridine nucleotide were encountered. The most variable step was the development of fluorescence, but as the intensity of the standard was affected as well as that of the unknown samples, the consequences were not serious (cf. Bassham *et al.* 1959).

Normal pyridine nucleotide values. Many reports of the amounts of pyridine nucleotides in normal rat liver are now available (Table 11) (Kaplan, 1960; Klingenberg & Bücher, 1960). Strict comparisons between the different results are not valid owing to variations in animal strain, age, sex, nutrition and living conditions, in addition to any differences in extraction and analytical procedures, and in the units in which the data were expressed. However, we consider that the amounts of DPN^+ and DPNH obtained in the present work (Table 11) are in good agreement with other published findings, but that our values for TPN^+ and TPNH are somewhat higher. A high content of TPN was also evident in the supernatant fraction, so that this trend was considered a characteristic of the rat strain used.

In general, with all the liver preparations on which estimations were made, the values for total pyridine nucleotide showed least variation, values for total DPN and total TPN showed more variation and those for the individual nucleotides showed most variation.

The present values with mitochondria isolated from normal liver were similar to those of Birt & Bartley (1960), allowing for differences in the method of preparation of the particles and in rat strain; they agree with the data of Jacobson & Kaplan (1957) in showing a higher total for TPN than for DPN. We also confirmed the observation of Birt & Bartley (1960) that progressive oxidation of nucleotides occurs during repeated sedimentation of mitochondria. In one instance, in which the particles were sedimented four times, a change from an initial oxidized/reduced ratio of 1/17·2 to a final ratio of 1·7/1 was observed (Table 7). Variation in the extent to which such oxidation occurred must have contributed to the variations encountered in the amounts of pyridine nucleotide in the oxidized and reduced states in different mitochondrial preparations. Most of the pyridine nucleotide detected in a rapidly prepared, unwashed, mitochondrial preparation (Table 7) was in the reduced state.

Animal variation in response to the toxic effects of heliotrine. Sensitivity of rats to the toxin varied in part in a random manner. In addition, a consistent trend in the direction of either an increase or a decrease in the time required to produce a certain degree of biochemical or cytological damage (Christie, 1958*a*, *b*) was sometimes observed; it is therefore desirable to make a histological study of samples of the livers used for biochemical investigations. Bull & Dick (1960) found, with male adult rats of the same strain and stock as used in our

Table 11. *Concentrations of endogenous pyridine nucleotides in whole rat liver*

Pyridine nucleotide values are expressed as μg./g. wet wt. of liver.

DPN+	DPNH	TPN+	TPNH	DPN	TPN	DPN+ + TPN+	DPNH + TPNH	Total	Remarks	Authors
490	158	105	333	648	438	595	491	1086	—	This paper
441	105	47	214	546	261	488	319	807	Quoted directly	Jacobson & Kaplan (1957)
359	145	53	47	504	100	412	192	604	Calc. from values given as m-moles/kg. of liver	Lowry, Roberts & Kapphahn (1957)
358·8	202·8	15·6	202·8	561·6	218·4	374·4	405·6	780	Quoted directly	Glock & McLean (1955)
505	192	6·5	227·5	697	234	511·5	419·5	931	Calc. mean values from two sets of values given	Holzer, Busch & Kröger (1958)
518	345	—	—	863	—	—	—	—	Quoted directly	Spirtes & Eichel (1954)
322	102	19	187	424	206	341	289	630	Calc. from values given as μm-moles/g. fresh wt.	Bassham *et al.* (1959)
204	147	48	194	351	242	252	341	593		

experiments, that some individuals were highly susceptible to the toxic effects of heliotrine. Susceptibility has also been reported to vary with age (Ratnoff & Mirick, 1949; Bull & Dick, 1960; Schoental, 1959).

Pyridine nucleotides of liver homogenates in heliotrine poisoning. The progressive fall of homogenate pyridine nucleotide shown by the data presented could have been due to accelerated loss of pyridine nucleotide by leakage or destruction, or to reduction of synthesis, or to both factors, so that the liver cells were unable to compensate for both normal and abnormal losses.

A reduced rate of synthesis of pyridine nucleotide in the liver of the rat in heliotrine poisoning has been demonstrated by Christie & Le Page (1962); but the same work showed that abnormal losses also occurred.

There are several possible ways in which such abnormal losses of coenzymes might occur. At the time when the loss began, liver cells that show only a minor degree of damage might lose pyridine nucleotides by passage of these through the cell membrane into the spaces of Disse, or directly into the blood flowing through the adjacent sinusoids, and thence into the general circulation. Consideration of our biochemical data and histological evidence from material used in these experiments, together with information from previous histological studies (Christie, 1958*a*; Bull, Dick & McKenzie, 1958), has led us to the conclusion that the abnormal losses of pyridine nucleotide mainly occurred by this process.

At a later stage in the course of the toxic process, a further contribution to the decrease of liver coenzyme might be made by autolytic disruption of, and general release of contents into the blood by, severely damaged liver cells, which are present in considerable numbers at this time. As such cells may already have lost most of their pyridine nucleotides, the amount released into the blood by this means may not be great.

Assuming that the pyridine nucleotides that are lost from liver cells pass into the blood stream and are not lost from, or destroyed in, the blood too rapidly (Hauss & Leppelmann, 1958), it should be possible to demonstrate in heliotrine poisoning a rise in serum pyridine nucleotide during the period when the rate of loss from the liver is greatest; studies in progress in our Laboratory indicate that this is the case.

Evidence that substances from the liver enter the blood in experimental acute liver damage in rats has been reported by Gallagher & Rees (1960), who detected a rise in the serum contents of substances absorbing at 260 mμ (Siekevitz & Potter, 1955) 9 hr. after carbon tetrachloride administration. Gallagher (1961) found similarly raised serum

E_{260} values in rats poisoned with lasiocarpine. Rees & Sinha (1960) demonstrated changing serum activities of enzymes characteristic of liver, in rats, during the course of liver poisoning by carbon tetrachloride or thioacetamide.

Some loss of liver pyridine nucleotide in heliotrine poisoning might be expected to occur as a result of enzymic destruction, for example by 'pyridine nucleotidases' (Kaufman & Kaplan, 1959) in extensively damaged cells (Gallagher, 1960*c*; Emmelot, Bos & Reyers, 1960). A small part of the losses observed in the present experiments may have resulted from this process.

Each of the four forms of pyridine nucleotide estimated in homogenate preparations showed a characteristic rate and direction of change in amount. The rise in DPNH coincided with a fall of DPN^+ (Fig. 1), which suggests that conversion into the reduced state may have been occurring (Birt & Bartley, 1960); however, we found no indication of a similar conversion of TPN^+ into TPNH. If the main role of diphosphopyridine nucleotide is as DPN^+ in hydrogen transport in oxidative pathways, and that of the triphosphopyridine nucleotide is as TPNH in reductive synthesis, then the difference in their behaviour (rise of DPNH and fall of TPNH) may be reconciled on the grounds that the toxic process disturbs both of these metabolic processes. In addition, some conversion of TPN into DPN may also have occurred (Fig. 2) (Birt & Bartley, 1960). Thus the processes leading to decrease of liver pyridine nucleotide in heliotrine poisoning are complex, and the result cannot be adequately explained as a consequence of 'selective leakage' or 'altered permeability' of the cell membrane alone. As well as passage of pyridine nucleotides out of the cell and impairment of their synthesis, increased destruction, interconversion and physiological disturbances affecting pyridine nucleotides at multiple points within the cell may also occur before gross disruption of cell structure.

Pyridine nucleotide of liver mitochondria in heliotrine poisoning. We have found that mitochondria isolated from the livers of rats treated with heliotrine for 20 hr. or more had lost about 30% of their pyridine nucleotide content, presumably *in vivo*, and that during a washing procedure further losses of pyridine nucleotides occurred *in vitro* (even though the medium contained ATP and EDTA), with little accompanying change in nitrogen value; furthermore, the pyridine nucleotides that were lost during washing were recovered in the pooled washings. The decrease in content *in vitro* thus appeared to have been due to an actual passage of pyridine nucleotide from the mitochondria into the surrounding fluid rather than to destruction *in situ*.

The small amounts of pyridine nucleotides in isolated liver mitochondria from heliotrine-treated rats (20–25 hr.) were therefore somewhat artificial in that the particles had experienced losses of pyridine nucleotides *in vitro* as well as *in vivo* before being analysed for pyridine nucleotide content. It is apparent, however, that with increasing duration of heliotrine treatment, progressively greater losses of pyridine nucleotides occurred from the mitochondria, and therefore the trends remain of importance. It is probable that the amount lost *in vivo* progressively increased, and that the tendency to lose pyridine nucleotides *in vitro* and therefore the amount lost *in vitro* correspondingly increased as the duration of treatment lengthened. The great variation in the observed amounts of pyridine nucleotides in mitochondria from different rats treated for the same length of time must largely have been a result of variation in the extent of the losses *in vitro*, in addition to variation in the sensitivity of rats to the toxin. It is also probable that the virtual depletion of the mitochondrial fraction in pyridine nucleotides, relative to the losses detected in the supernatant fraction and whole homogenate, was a consequence of the additional losses occurring *in vitro* during the isolation procedure. Results in Table 7 show a relatively high pyridine nucleotide content for mitochondria that had been isolated rapidly from a 50% homogenate of liver from a heliotrine-treated rat (22 hr.); comparison with the values found in the supernatant fraction and the whole homogenate at this time suggests that *in vivo* the pyridine nucleotide losses from the mitochondria may be of the same order as those of the surrounding cytoplasm.

Effect of pyridine nucleotide losses in vitro *on respiration.* As a result of the losses of pyridine nucleotides *in vitro* incurred during isolation of liver mitochondria from treated animals for study of respiration rate, gross reduction of activity of many DPN-dependent respiratory enzymes was observed at a much earlier time than would have been the case if such losses of pyridine nucleotides *in vitro* had not occurred. It would be difficult on the basis of such experiments to specify a particular time at which respiration would fail *in vivo*; however, it is reasonable to assume that such a stage may eventually be reached, and that these experiments therefore serve a purpose in demonstrating, at an earlier time, a defect that may eventually lead to serious consequences.

In our studies of DPN-dependent oxidative enzyme systems we have generally made parallel observations on mitochondrial and homogenate (10%, w/v, in 0·25 M-sucrose) preparations; reduction of oxygen uptake was usually evident with the mitochondrial preparations from animals killed at a slightly earlier time than was the case with the

homogenates, but sometimes the defect was apparent in both after a minimum duration of treatment. Thus merely making a homogenate must be sufficient to precipitate the respiratory effect with liver from rats treated for the requisite period (usually about 20 hr.). One may therefore conclude that the factor responsible is the dilution of the fluid surrounding the mitochondria in respect of pyridine nucleotide, which is incurred in making and testing the homogenate, and which, in the presence of a mitochondrial defect permitting loss of pyridine nucleotides, is sufficient to lower the intramitochondrial concentration to a value below that critical for the activity of certain respiratory enzymes.

Recknagel & Anthony (1959) and Share & Recknagel (1959), working with livers of rats treated with carbon tetrachloride, suspected that loss of mitochondrial pyridine nucleotides and consequent inactivation of pyridine nucleotide-dependent dehydrogenases might have occurred during the preparation of enzymes and assay of enzyme activity, particularly during the equilibration period, before taking readings of oxygen uptake (see also Gallagher, 1960*c*).

If dilution were the factor precipitating the reduction of activity of various DPN-linked dehydrogenases in homogenate preparations (10%, w/v, in 0·25M-sucrose) made from livers of treated animals, one might expect that more concentrated homogenates (such as 50%, w/v, in 0·25M-sucrose) made from the same livers might show normal activity. Furthermore, when the pyridine nucleotide concentration of the fluid surrounding the mitochondria has not been diluted, as with liver slices, the activity of these systems might be expected to be normal. These predictions have been verified in the present work. Emmelot *et al.* (1960) found that liver mitochondria from rats treated with dimethylnitrosamine (18–20 hr.) showed a decreased oxidation rate, but that slice preparations were not inhibited.

Gross reduction of the activity of various DPN-linked oxidases in homogenate and mitochondrial preparations, and complete or partial reactivation by addition of DPN^+ to the incubation medium, has been observed with several experimental liver poisons: carbon tetrachloride (Christie & Judah, 1954; Recknagel & Littera, 1960); dimethylnitrosamine (Bailie & Christie, 1959; Emmelot *et al.* 1960); heliotrine (Christie, 1958*b*; Gallagher, 1960*b*). The mitochondrial change is therefore not specific to a particular liver toxin, but, in fact, shows many resemblances to the 'aging' effect that is produced by damage *in vitro* to mitochondria from normal liver (Ernster & Lindberg, 1958).

The concentration (mM) of DPN^+ in the medium surrounding the mitochondria, which was shown in the present experiments to be effective in reversing the loss of activity of DPN-linked systems, was of the same order as that found necessary (Hunter, Malison, Bridgers, Schutz & Atchison, 1959) for the restoration of oxidation in phosphate-treated mitochondria.

Sequence of mitochondrial changes in heliotrine poisoning. We may now review the sequence of changes observed in the mitochondria from livers of rats treated with heliotrine (320 mg./kg. body wt.).

(*a*) 0–12 hr. No changes have been detected by biochemical methods, but histological studies showed that the staining properties were altered (Christie, 1958*a*).

(*b*) 12–17 hr. The oxidation rate of isolated mitochondria was normal; pyridine nucleotides were not lost *in vitro* during washing procedures, but an accelerated 'aging' effect has been observed (Christie, 1958*b*).

(*c*) 17–25 hr. Mitochondria from rats killed during this period after heliotrine treatment had already lost a considerable portion of their pyridine nucleotides on isolation, both from losses *in vivo* and *in vitro*, which became greater as the period of treatment of the rat was lengthened. Activity of DPN-dependent enzymes was moderately or greatly decreased, according to the extent of the decrease of coenzyme content, but could be restored by mM-DPN^+.

(*d*) 25–30 hr. Mitochondria were almost depleted of pyridine nucleotides on isolation; respiration rate was generally greatly reduced and showed little reactivation with mM-DPN.

(*e*) 30–36 hr. After 30 hr. liver-slice respiration rate was reduced, and histological preparations showed irreversible damage of almost all liver cells. Death often occurred after treatment for 32–36 hr.

Significance of the mitochondrial lesion. We consider that the mitochondrial lesion produced by heliotrine plays an important role in the toxic process by leading to disorganization of energy generation *in vivo*. The onset of the respiratory effect *in vivo*, however, may not be so early or so clear-cut as was observed in experiments with homogenates and isolated mitochondria. Study of the mitochondrial defect may also be of value in elucidating the underlying cytotoxic effects of the alkaloid.

Nature of the mitochondrial lesion. Although we realize that the mitochondrial lesion is complex, that it may be interrelated with other changes in the cell and may begin before the onset of the changes of coenzyme content that we have investigated, we consider that study of the behaviour of the mitochondria at this time may assist in understanding the basic disturbance.

It appears to us that an observation of importance in this regard is that full respiratory activity

could be restored by mM-DPN^+ to mitochondrial preparations isolated from livers of rats killed at or shortly after the time when reduction of DPN-linked respiration in homogenates was first demonstrable, whereas only partial or negligible reactivation occurred if the rat were killed several hours after the expected time when depression of respiration was first demonstrable. In the latter case, losses of pyridine nucleotides during isolation were considerable, and the amounts in the mitochondria were low.

One possible explanation of failure of reactivation by DPN is that the enzymes may have leaked from the particles after loss of considerable amounts of the coenzyme had occurred. However, Siekevitz & Potter (1955), working with normal liver mitochondria that had been damaged after isolation, showed that many nucleotides, including DPN and TPN, were lost from the particles, but they did not record the loss of enzyme protein. Rees & Sinha (1960) identified enzymes characteristic of mitochondria in rat serum in acute carbon tetrachloride poisoning, and work in progress in our Laboratory indicates that this phenomenon occurs in heliotrine poisoning also. However, this finding does not necessarily mean that a leakage of mitochondrial enzymes readily occurs from damaged cells; the enzymes entering the serum could have been released from the small proportion of cells that can be observed histologically at all times after 6 hr. to be undergoing gross disorganization. Furthermore, we detected only slight losses of nitrogen in mitochondrial preparations subjected to considerable coenzyme losses by washing, and which showed irreversible failure of DPN-linked respiration. If the mitochondrial membrane had become sufficiently 'permeable' to allow release of DPN-linked enzymes soon after the loss of coenzyme, one might also expect release of other mitochondrial proteins in amounts sufficient to lower the mitochondrial nitrogen and to raise the nitrogen of the washings to a considerable extent, but such was not the case. Thus although it remains a possibility that losses of DPN-dependent enzymes may occur, the evidence available is against this being a convincing explanation for the irreversible phase of DPN-linked respiration.

It is also possible that an inhibition to the re-entry of DPN might develop. If the loss of coenzymes from mitochondria is due to increased permeability of the limiting membranes of the particles one might expect that re-entry should become easier rather than increasingly difficult, as the loss becomes easier. Although we cannot cite data to exclude this hypothesis, a unidirectional increase in membrane 'permeability' appears an unlikely concept.

Failure of reactivation could be explained even if the added DPN^+ readily entered the mitochondria, and if most of the enzyme protein were still present, provided that partial denaturation had rapidly ensued after removal of critical amounts of coenzyme. Shifrin & Kaplan (1960) have reviewed evidence showing that the pyridine nucleotide-dependent enzymes so far investigated actually bind their specific coenzyme, at specific sites, with a high affinity, and that the presence of bound coenzyme protects the enzyme from denaturation. If this evidence is admissible, the sequence of mitochondrial events in heliotrine poisoning might be that the toxin, either directly or as a result of intermediate processes, reduces the binding affinity of the enzymes for their pyridine nucleotide coenzymes; this allows pyridine nucleotides to be removed by a washing procedure which does not remove pyridine nucleotides from normal mitochondria. When the coenzyme content is greatly decreased, partial denaturation of the enzymes rapidly ensues, and restoration of activity by replacement of the coenzyme, in a concentration effective before denaturation, is no longer effective.

SUMMARY

1. The amounts of DPN^+, DPNH, TPN^+ and TPNH in liver homogenates and in the supernatant and mitochondrial fractions of homogenates have been estimated by a fluorimetric method in normal male rats, and in a series of rats during the first 30 hr. after injection of a LD_{70} dose of the selectively hepatotoxic pyrrolizidine alkaloid heliotrine.

2. In the homogenate and supernatant fractions from normal rat liver, DPN^+ was present in excess of DPNH, and TPNH in excess of TPN^+; in the homogenate the ratio was about 3:1. Homogenates from treated animals showed a progressive depression of this ratio. Depression of the total DPN and total TPN concentrations was evident at 17 hr. after treatment, and by 25 hr. was of the order of 30 % with each coenzyme.

3. In the isolated mitochondrial fraction the mean total pyridine nucleotide was decreased by 30 % at 17 hr. and by 75 % at 25 hr. Depression of the amounts of all four components was recorded, but TPNH was lost earlier, and in greater amounts, than the other forms. The variability of estimated amounts between preparations from rats treated for equal periods was greater with isolated mitochondria than with the homogenate and supernatant fraction.

4. With special preparations it was possible to establish: that mitochondria from the livers of treated rats lost pyridine nucleotides during the isolation process; that quantitative recovery of the lost nucleotides could be obtained in the washings;

that only slight loss of nitrogen accompanied the nucleotide loss; that the mitochondrial state permitting nucleotide loss was evident by 17 hr.; that the ease of loss increased the later the rat was killed. Progressive oxidation of the nucleotides occurred during repeated mitochondrial sedimentation and resuspension.

5. Respiratory activity of mitochondrial DPN-linked dehydrogenase systems was greatly impaired when the total mitochondrial DPN content was depressed by about 50% or more of the mean control value. With mitochondrial preparations from livers of heliotrine-treated rats killed at the time when this degree of depression of coenzyme content was first found in the standard preparations, respiration could be restored by the addition of mM-DPN^+ to the incubation medium. With mitochondrial preparations from rats killed at later times, losses of pyridine nucleotides during isolation were large, and restoration of respiration by DPN^+ addition was partial or negligible.

6. Respiratory activity of DPN-linked enzyme systems continued for a longer period in liver slices than in homogenate or isolated mitochondrial preparations, but eventually failed also.

7. The significance of these observations has been discussed.

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