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# Liver Damage in Acute Heliotrine Poisoning

## 2. THE EFFECT OF NICOTINAMIDE ON PYRIDINE NUCLEOTIDE CONCENTRATIONS*

BY G. S. CHRISTIE AND R. N. LE PAGE
*Department of Pathology, University of Melbourne, Melbourne, Australia*

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Earlier studies on the effects of heliotrine poisoning demonstrated an apparent deficiency of DPN in homogenate and mitochondrial preparations from the livers of heliotrine-poisoned (16–20 hr.) rats (Christie, 1958*b*). More recent work has shown that the concentrations of pyridine nucleotides were, in fact, decreased in homogenates and in the mitochondrial and soluble fractions of liver from rats treated with heliotrine for 17–20 hr. (Christie, Le Page & Bailie, 1961; Christie & Le Page, 1962). From these studies it was proposed that much of the decrease in the concentrations of pyridine nucleotides *in vivo* came about by passage of coenzymes out of liver cells when they were, as yet, only moderately damaged. Further losses could have occurred when cells were eventually disrupted, and some losses of nucleotide may have occurred from breakdown by enzymes such as pyridine nucleotidases. It was also suggested that heliotrine might reduce the capacity of the liver to synthesize pyridine nucleotides: thus the ability of the liver to maintain normal concentrations in the presence of increased losses would be decreased (Christie & Le Page, 1962).

In this paper we describe studies of the capacity of the heliotrine-poisoned-rat liver to synthesize pyridine nucleotide coenzymes *in vivo*. Nicotinamide administration to normal mice was shown by Kaplan, Goldin, Humphreys, Ciotti & Stolzenbach (1956*b*) to lead to a marked elevation of the total pyridine coenzyme concentration of the liver. The increase was largely accounted for by a tenfold increase in the DPN concentration. Further, the newly synthesized DPN was nearly all in the soluble fraction of mouse liver; there was no significant rise in the concentration of TPN (Kaplan *et al.* 1956*b*). Nicotinic acid, on the other hand, although favoured as a direct precursor of DPN (Preiss & Handler, 1957, 1958*a*, *b*), when administered in equivalent dosage, gave only a relatively small increase in DPN (Kaplan *et al.* 1956*b*; Kaplan, Goldin, Humphreys & Stolzenbach, 1957; Kaplan, 1960).

In view of these findings it is apparent that one possible way of testing whether heliotrine interferes with DPN synthesis in rat liver *in vivo* would be to study the 'response' to injections of nicotinamide shown by rats treated with heliotrine. Our experiments show that the ability to 'respond' to nicotinamide injections is reduced with increasing duration of heliotrine treatment.

* Part 1: Christie & Le Page (1962).

## MATERIALS AND METHODS

*Abbreviations.* Diphosphopyridine nucleotide: oxidized form, $DPN^+$, reduced form, DPNH, total, DPN; triphosphopyridine nucleotide: oxidized form, $TPN^+$, reduced form, TPNH, total, TPN.

*Animals.* Adult male hooded Wistar rats (270–330 g.) were used (Bull, Dick & McKenzie, 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 into rats under light ether anaesthesia. Nicotinamide (500 mg./kg. body wt. in aqueous solution) was also given by intraperitoneal injection, and untreated control rats received injections of 0·9% NaCl.

*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, isocitrate dehydrogenase, alcohol dehydrogenase, isocitric acid, α-oxoglutaric acid, sodium L-glutamate, L-malic acid and β-hydroxybutyric acid were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.; adenosine 5′-monophosphate (AMP) and adenosine 5′-triphosphate (ATP) were from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.; EDTA, tris and acetaldehyde were from British Drug Houses Ltd.; nicotinamide and sodium pyruvate were from L. Light and Co. Ltd., Colnbrook, Bucks. Ethanol was benzene-free. Crystalline heliotrine was kindly supplied by C.S.I.R.O., Animal Health Research Laboratory, Parkville, N. 2, Victoria.

*Methods.* Washing of glassware, and preparation of solutions for the fluorimetric estimation of pyridine nucleotides (Bassham, Birt, Hems & Loening, 1959), were as described by Christie & Le Page (1962). Tissue preparations, manometric measurements, N determinations and estimations of pyridine nucleotides by the fluorimetric procedure (Bassham *et al.* 1959) were carried out as described by Christie & Le Page (1962). A spectrophotometric method for estimation of $DPN^+$, by the use of alcohol dehydrogenase (Ciotti & Kaplan, 1957; Kaplan, Ciotti & Stolzenbach, 1956*a*), was also employed. In this procedure, the rat was killed by stunning and exsanguination, a weighed amount of liver (1–2 g.) was rapidly transferred to ice-cold 5%

trichloroacetic acid in a homogenizer tube, and disrupted by means of a power-driven pestle. After adjustment of the volume (10%, w/v) and centrifuging, the extract was neutralized with 2N-NaOH. The estimation of $DPN^+$ was carried out in 0·2M-glycine buffer, pH 9·6 (Kornberg, 1950), after addition of ethanol and alcohol dehydrogenase, by spectrophotometric measurement of the absorption of DPNH at 340 mμ and application of the millimolar extinction coefficient of 6·22 (Horecker & Kornberg, 1948; Kaplan, 1960). Suitable volumes of extract were chosen to give differences of extinction in the range 0·05–0·2; all estimations were carried out in duplicate, and at two concentrations.

Table 1. *Concentrations of $DPN^+$ in liver from control and heliotrine-treated rats*

Results are mean values (μm-moles of liver $DPN^+$/100 g. body wt.) ± S.E. Rats received intraperitoneal injections of heliotrine (320 mg./kg. body wt.) and were killed at the times indicated. Estimations of $DPN^+$ were made by the spectrophotometric method, details of which are given in the text.

| Duration of heliotrine treatment (hr.) | No. of rats | Concn. of $DPN^+$ |
|---|---|---|
| Nil | 6 | 1530 ± 71 |
| 13 | 2 | 1470 ± 124 |
| 20 | 3 | 1090 ± 75 |
| 26 | 2 | 960 ± 18 |

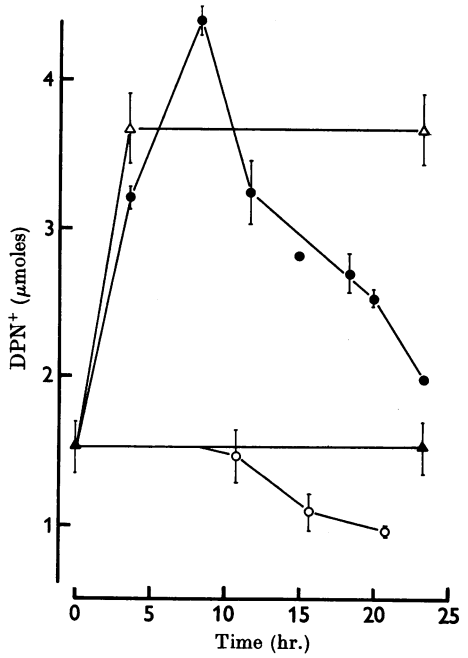


Fig. 1. Changes in liver $DPN^+$ concentration of rats treated with heliotrine and nicotinamide. $DPN^+$ values are expressed as μmoles/liver/100 g. body wt. of rat and were estimated by the spectrophotometric method (see text). Rats received intraperitoneal injections of heliotrine (320 mg./kg. body wt.) or nicotinamide (500 mg./kg. body wt.). Points show mean $DPN^+$ values: ▲, untreated controls; △, nicotinamide 4 hr. before killing; ○, heliotrine at 0 hr.; ●, heliotrine at 0 hr. and nicotinamide 4 hr. before killing. Vertical lines represent ± S.D. population.

## RESULTS

*Effect of heliotrine treatment on concentration of liver $DPN^+$.* Initial experiments were carried out to determine whether treatment of rats with heliotrine would produce any alteration in the concentration of $DPN^+$ in the liver; estimations were made by the spectrophotometric method. The data (Table 1, Fig. 1) show that, in rats killed 13 hr. after injection of heliotrine, the concentration of $DPN^+$ was still normal; at 20 hr. the mean value of $DPN^+$ was well below the control; after 26 hr. of treatment the decrease in the $DPN^+$ was highly significant (cf. Christie & Le Page, 1962).

*Effect of nicotinamide treatment on concentration of liver $DPN^+$.* The extent and duration of the rise in concentration of $DPN^+$ in whole rat liver after administration of nicotinamide (500 mg./kg. body wt.) was determined (Table 2, Fig. 1). The concentration of $DPN^+$ rose to a maximum value (between 2·4 and 2·75 times the normal) 4–5 hr. after the injection of nicotinamide; thereafter the concentration fell slowly, but was still above normal at 22 hr.

*Effect of heliotrine-plus-nicotinamide treatment on concentration of liver $DPN^+$.* The liver $DPN^+$ concentration was determined in a group of rats that had been treated for 4 hr. with nicotinamide

Table 2. *Effect of nicotinamide on concentration of $DPN^+$ in liver of normal rats*

Results are mean values (μm-moles of liver $DPN^+$/100 g. body wt.) ± S.E. Rats received intraperitoneal injections of nicotinamide at the doses indicated, and were killed at the times stated. Estimations of $DPN^+$ were made by the spectrophotometric method, details of which are given in the text.

| Duration of nicotinamide treatment (hr.) | Dose of nicotinamide (mg./kg. body wt.) | No. of rats | Concn. of $DPN^+$ |
|---|---|---|---|
| Nil | — | 6 | 1530 ± 71 |
| 2 | 500 | 1 | 2620 |
| 4 | 500 | 11 | 3670 ± 77 |
| 4½ | 500 | 1 | 3700 |
| 5 | 500 | 1 | 4140 |
| 6 | 500 | 1 | 3520 |
| 8 | 500 | 1 | 3960 |
| 11 | 500 | 2 | 4020 ± 274 |
| 12 | 500 | 1 | 3060 |
| 13 | 500 | 1 | 2510 |
| 18 | 500 | 3 | 3160 ± 240 |
| 22 | 500 | 1 | 2190 |
| 3½ | 750 | 2 | 5350 ± 668 |
| 5 | 1000 | 1 | 4320 |

(500 mg./kg. body wt.) and pretreated with heliotrine (320 mg./kg. body wt.) for various lengths of time. The data (Table 3, Fig. 1) show that the concentration of $DPN^+$ attained in 4 hr. in response to an injection of nicotinamide decreased as the duration of heliotrine pretreatment was increased; for example, with 18 hr. pretreatment the concentration of $DPN^+$ attained was significantly less than in controls that had received nicotinamide only, whereas with 24 hr. pretreatment the value for $DPN^+$ rose only slightly above that of untreated controls.

Table 3. *Concentrations of $DPN^+$ in liver of rats receiving nicotinamide plus heliotrine*

Results are mean values (μm-moles of liver $DPN^+$/100 g. body wt.) ± S.E. Rats received intraperitoneal injections of heliotrine (320 mg./kg. body wt.) at the times stated before killing, and of nicotinamide (500 mg./kg. body wt.) 4 hr. before killing. The liver-$DPN^+$ content of untreated rats was 1530 ± 71 μm-moles/100 g. body wt. $DPN^+$ was estimated by the spectrophotometric method, details of which are given in the text.

| Duration of heliotrine treatment (hr.) | No. of rats | Concn. of $DPN^+$ |
|---|---|---|
| Nil | 11 | 3670 ± 77 |
| 4 | 2 | 3210 ± 50 |
| 10 | 2 | 4400 ± 68 |
| 14 | 2 | 3250 ± 153 |
| 18 | 1 | 2820 |
| 22 | 2 | 2700 ± 92 |
| 24 | 2 | 2540 ± 35 |
| 28 | 2 | 1990 ± 0 |

*Effect of heliotrine-plus-nicotinamide treatment on concentrations of liver pyridine nucleotide.* In further studies, heliotrine and nicotinamide were simultaneously injected into rats and the concentrations of the oxidized and reduced forms of DPN and TPN were determined fluorimetrically at various times after injection.

In the control group of rats treated with heliotrine only (Table 4, group II) decreases in the concentrations of $DPN^+$, $TPN^+$ and TPNH, but not of DPNH, were apparent by 17½ hr., and were more advanced at 21½ hr. Similar findings have been reported by Christie & Le Page (1962).

In the control group of rats treated with nicotinamide only (Table 4, group III) no change in the concentration of any of the nucleotides except $DPN^+$ was found at the peak of the response (5 hr.). At later times progressively lower $DPN^+$ values were obtained, although even at 22 hr. the $DPN^+$ concentration was still significantly higher than in untreated controls. During the period when the concentration of $DPN^+$ was falling, a slight elevation of mean DPNH and TPNH values, and a fall in $TPN^+$ value, occurred. However, these changes were not of a significant degree, and probably reflected only altered equilibria between the nucleotides, resulting from the great rise in concentration of $DPN^+$.

Administration of heliotrine plus nicotinamide (Table 4, group IV; Fig. 2) did not produce any significant difference of liver-pyridine nucleotide concentrations from those of control rats receiving nicotinamide only (group III) at 17½ hr.; at 20 hr., however, the value for $DPN^+$ had fallen slightly;

Table 4. *Concentrations of pyridine nucleotide in liver homogenates from rats receiving nicotinamide plus heliotrine*

Results are mean values (μm-moles of liver pyridine nucleotide/100 g. body wt.) ± S.E. (in parentheses). Rats received injections of heliotrine (320 mg./kg. body wt.) and nicotinamide (500 mg./kg. body wt.) and were killed at the times stated. Pyridine nucleotide estimations were made by the fluorimetric method, details of which are given in the text.

| | Duration of treatment (hr.) | | | Homogenate pyridine nucleotides | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Group | Heliotrine | Nicotinamide | No. of rats | $DPN^+$ | DPNH | $TPN^+$ | TPNH | $DPN^+$ + DPNH | $TPN^+$ + TPNH | Total pyridine nucleotide |
| I | Nil | Nil | 23 | 2535 | 806 | 490 | 1542 | 3341 | 2032 | 5373 |
| | | | | (47) | (53) | (24) | (33) | (37) | (29) | (58) |
| II | 17½ | Nil | 8 | 1913 | 886 | 360 | 1083 | 2799 | 1443 | 4242 |
| | | | | (101) | (29) | (30) | (66) | (97) | (69) | (143) |
| | 21½ | Nil | 3 | 1813 | 698 | 256 | 974 | 2510 | 1229 | 3739 |
| | | | | (92) | (85) | (28) | (25) | (177) | (50) | (225) |
| III | Nil | 5 | 1 | 5917 | 629 | 655 | 1592 | 6546 | 2247 | 8793 |
| | Nil | 17½ | 2 | 5294 | 907 | 340 | 1674 | 6201 | 2014 | 8215 |
| | | | | (322) | (280) | (123) | (16) | (587) | (132) | (718) |
| | Nil | 22 | 1 | 3381 | 1080 | 334 | 1827 | 4461 | 2161 | 6622 |
| IV | 17½ | 17½ | 1 | 5826 | 1011 | 638 | 1597 | 6837 | 2235 | 9072 |
| | 20 | 20 | 1 | 4920 | 1508 | 357 | 1609 | 6428 | 1966 | 8394 |
| | 22 | 22 | 1 | 2175 | 627 | 372 | 479 | 2802 | 851 | 3653 |

a marked rise in the concentration of DPNH (found in control rats receiving either heliotrine alone or nicotinamide alone) had occurred at this time; the total DPN concentration had fallen only slightly (suggesting that actual loss of the nucleotide was still small). At 22 hr. the concentrations of both $DPN^+$ and DPNH were much below the corresponding values in nicotinamide-treated controls; in fact, all forms were actually at lower concentrations than in untreated rats; TPNH and total TPN were greatly depressed, and the total liver-pyridine nucleotide value (Fig. 2) was similar to that of the control rats receiving heliotrine alone for 21½ hr. (with the difference that more pyridine nucleotide was in the oxidized state, and less in the reduced state, after treatment with heliotrine plus nicotinamide).

*Effect of heliotrine-plus-nicotinamide treatment on concentrations of pyridine nucleotide in the soluble supernatant fraction.* Pyridine nucleotide concentrations in the soluble supernatant fraction of liver homogenates from rats killed after heliotrine-plus-nicotinamide treatment are shown in Table 5. In the control group treated with nicotinamide only (Table 5, group III), the concentrations of $DPN^+$ and DPNH were significantly raised but those of $TPN^+$ and TPNH were not altered. Comparison of values in the supernatant fraction after treatment with heliotrine plus nicotinamide (22 hr.) (Table 5, group IV) with those of the control group treated with nicotinamide only (Table 5, group III) shows that the concentration of total DPN was lower after heliotrine plus nicotinamide, in spite of a rise in the concentration of DPNH. However, the concentrations of both $DPN^+$ and DPNH (group IV) were significantly higher than those of preparations from untreated controls (group I). The concentration of total TPN (group IV) was below

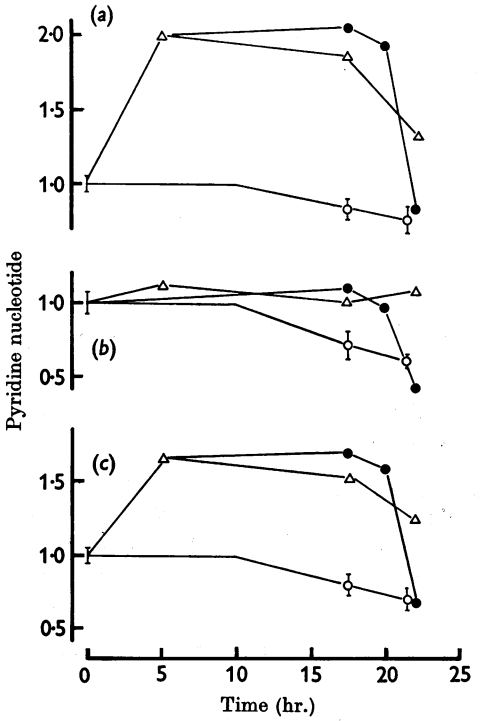


Fig. 2. Changes in the concentrations of liver pyridine nucleotides of rats treated with heliotrine and nicotinamide. Values are shown for (*a*) total DPN, (*b*) total TPN and (*c*) total pyridine nucleotide, and are expressed as the ratio of mean content in treated rats to mean content in untreated control rats; concentrations were estimated fluorimetrically (see text). Treated rats received heliotrine (320 mg./kg. body wt.) or nicotinamide (500 mg./kg. body wt.). Treatment of rats: ○, heliotrine at 0 hr.; △, nicotinamide at 0 hr.; ●, heliotrine plus nicotinamide at 0 hr. Vertical lines represent ± S.D. population.

Table 5. *Concentrations of pyridine nucleotide in the supernatant fraction of liver homogenates from rats receiving nicotinamide plus heliotrine*

Results are mean values (μm-moles of liver pyridine nucleotide/100 g. body wt.) ± S.E. (in parentheses). Rats received intraperitoneal injections of heliotrine (320 mg./kg. body wt.) and nicotinamide (500 mg./kg. body wt.) and were killed at the times stated. Pyridine nucleotides were estimated by a fluorimetric method, details of which are given in the text.

| | Duration of treatment (hr.) | | | Supernatant pyridine nucleotides | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Group | Heliotrine | Nicotinamide | No. of rats | $DPN^+$ | DPNH | $TPN^+$ | TPNH | $DPN^+$ + DPNH | $TPN^+$ + TPNH | Total pyridine nucleotide |
| I | Nil | Nil | 4 | 1735 (75) | 265 (9) | 147 (10) | 582 (79) | 2000 (82) | 729 (79) | 2729 (141) |
| II | 23 | Nil | 3 | 1150 (124) | 265 (39) | 95 (16) | 354 (46) | 1414 (124) | 449 (60) | 1863 (174) |
| III | Nil | 22 | 1 | 3501 | 342 | 138 | 656 | 3843 | 794 | 4637 |
| IV | 22 | 22 | 1 | 2206 | 542 | 163 | 170 | 2748 | 333 | 3081 |

that of untreated controls (group I), because of a considerable fall of TPNH, the predominant form; the $TPN^+$ was little altered. Total pyridine nucleotide content of the supernatant fraction after treatment with heliotrine plus nicotinamide (Table 5, group IV) was similar to that of untreated controls (group I), but considerably lower than that after treatment with nicotinamide only (group III). It was apparent that the findings with the supernatant were similar to those with the homogenate in that heliotrine treatment did not prevent a rise of the concentration of $DPN^+$ in response to nicotinamide (Tables 4 and 5, group IV). However, after treatment for 22 hr., the concentration of $DPN^+$ was much lower (group IV) than in controls treated for 22 hr. with nicotinamide only (Tables 4 and 5, group III).

*Effect of heliotrine-plus-nicotinamide treatment on concentrations of mitochondrial pyridine nucleotide.* Pyridine nucleotide concentrations in the liver mitochondrial fraction after heliotrine-plus-nicotinamide treatment of the rat are shown in Table 6. In mitochondrial preparations from controls treated with nicotinamide only (22 hr.) (Table 6, group III) the pyridine nucleotide concentrations were normal. After treatment with heliotrine plus nicotinamide for 20 hr. (group IV), the greatest change in the pyridine nucleotide values compared with untreated control values (group I) was a decrease in the concentration of TPNH. However, after treatment of the rat with both agents for 22 hr., the concentrations of all four nucleotides were much decreased. It was therefore apparent that administration of nicotinamide with heliotrine did not alter to any great extent the time of development of the mitochondrial changes that are produced by heliotrine, because the tendency to lose pyridine nucleotides during the isolation procedure was found to be present at about the same time as in controls treated with heliotrine only (Christie & Le Page, 1962).

*Effect of heliotrine-plus-nicotinamide treatment on concentrations of pyridine nucleotide and respiration rate.* The respiration rates of homogenates and

Table 6. *Concentrations of pyridine nucleotide in the mitochondrial fraction of liver homogenates from rats receiving heliotrine plus nicotinamide*

Results are mean values (μm-moles of liver pyridine nucleotide/100 g. body wt.) ± S.E. (in parentheses). Rats received intraperitoneal injections of heliotrine (320 mg./kg. body wt.) and nicotinamide (500 mg./kg. body wt.) and were killed at the times stated. Pyridine nucleotides were estimated by the fluorimetric method, details of which are given in the text.

| | Duration of treatment (hr.) | | | Mitochondrial pyridine nucleotides | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Group | Heliotrine | Nicotinamide | No. of rats | $DPN^+$ | DPNH | $TPN^+$ | TPNH | $DPN^+$ + DPNH | $TPN^+$ + TPNH | Total pyridine nucleotide |
| I | Nil | Nil | 12 | 409 (16) | 140 (17) | 163 (16) | 469 (24) | 550 (25) | 632 (21) | 1182 (31) |
| II | 19 | Nil | 2 | 303 (72) | 70 (57) | 112 (26) | 231 (163) | 373 (115) | 343 (169) | 716 (297) |
| | 21½ | Nil | 2 | 198 (81) | 102 (69) | 85 (10) | 184 (153) | 300 (150) | 269 (158) | 569 (307) |
| III | Nil | 22 | 1 | 381 | 98 | 179 | 522 | 479 | 701 | 1180 |
| IV | 20 | 20 | 1 | 328 | 111 | 123 | 234 | 439 | 357 | 796 |
| | 22 | 22 | 1 | 104 | 46 | 56 | 0 | 150 | 56 | 206 |

Table 7. *Respiration rate and pyridine nucleotide concentrations of liver homogenates from rats treated with heliotrine plus nicotinamide*

Results are mean values (μl. of $O_2$/hr./liver/g. body wt. for respiration rate, and μm-moles of pyridine nucleotide/100 g. body wt.). Rats received intraperitoneal injections of heliotrine (320 mg./kg. body wt.) and nicotinamide (500 mg./kg. body wt.) and were killed at the times stated. Pyridine nucleotides were determined by the fluorimetric method, details of which are given in the text.

| Duration of treatment (hr.) | | | Respiration rate | | Pyridine nucleotides | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Heliotrine | Nicotinamide | No. of rats | No substrate | Glutamate | $DPN^+$ | DPNH | $TPN^+$ | TPNH | Total pyridine nucleotide |
| Nil | Nil | 1 | 28 | 105 | 2591 | 953 | 481 | 1746 | 5771 |
| 20 | 20 | 1 | 30 | 91 | 4920 | 1507 | 357 | 1609 | 8393 |

Table 8. *Respiration rate and pyridine nucleotide concentrations of liver mitochondria from rats treated with heliotrine plus nicotinamide*

Effect on the respiration rate of addition of 1·5 mM-DPN$^+$ to the standard medium was studied. Other details were as shown for Table 7. Mean values ± S.E. (in parentheses) are given. +, DPN$^+$ added; −, no addition of DPN$^+$.

| | Duration of treatment (hr.) | | | | Respiration rate | | | | Pyridine nucleotides | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Group | Heliotrine | Nicotinamide | No. of rats | DPN$^+$ addition | Glutamate | α-Oxoglutarate | Citrate | Malate | DPN$^+$ | DPNH | TPN$^+$ | TPNH | Total pyridine nucleotide |
| I | Nil | Nil | 6 | − | 77 | 78 | 84 | . | 416 | 147 | 148 | 460 | 1171 |
| | | | | | (4) | (3) | | | (22) | (29) | (13) | (30) | (18) |
| | | | | + | 93 | 87 | 106 | . | . | . | . | . | . |
| | | | | | (4) | (4) | | | | | | | |
| II | 19 | Nil | 2 | − | 8 | 4 | 10 | . | 303 | 70 | 112 | 231 | 716 |
| | | | | | | | | | (72) | (57) | (26) | (163) | (297) |
| | | | | + | 73 | 60 | 86 | . | . | . | . | . | . |
| | 21½ | Nil | 2 | − | 9 | 7 | 15 | . | 198 | 102 | 85 | 184 | 569 |
| | | | | | (3) | (4) | (0·1) | | (81) | (69) | (10) | (153) | (307) |
| | | | | + | 41 | 67 | 79 | . | . | . | . | . | . |
| | | | | | (7) | (17) | (10) | | | | | | |
| III | Nil | 22 | 1 | − | 83 | . | 89 | 57 | 381 | 98 | 179 | 522 | 1180 |
| | | | | + | 99 | . | 105 | 59 | . | . | . | . | . |
| IV | 20 | 20 | 1 | − | 74 | 74 | . | . | 328 | 111 | 123 | 234 | 796 |
| | | | | + | 89 | 78 | . | . | . | . | . | . | . |
| | 22 | 22 | 1 | − | 6 | . | 14 | 3 | 104 | 46 | 56 | 0 | 206 |
| | | | | + | 43 | . | 45 | 21 | . | . | . | . | . |

Table 9. *Respiration rate of liver mitochondria and DPN$^+$ content of whole-liver preparations from rats treated with heliotrine plus nicotinamide*

Results are mean values (μl. of $O_2$/hr./liver/g. body wt. for respiration rate, and μm-moles of DPN$^+$ relative to 100 g. body wt.). Rats received injections of heliotrine (320 mg./kg. body wt.) plus nicotinamide (500 mg./kg. body wt.), except where indicated, and were killed at the times stated. Effect on the respiration rate of addition of 1·5 mM-DPN$^+$ to the standard incubation medium was studied. Pyridine nucleotides were estimated by the spectrophotometric method. Details of methods are given in the text.

| Duration of treatment (hr.) | | Mitochondrial respiration rate | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | α-Oxoglutarate | | Citrate | | β-Hydroxybutyrate | | |
| Heliotrine | Nicotinamide | Without DPN$^+$ | With DPN$^+$ | Without DPN$^+$ | With DPN$^+$ | Without DPN$^+$ | With DPN$^+$ | Liver DPN$^+$ |
| Nil | 18, 12† | 56 | 61 | 72 | 89 | 31 | 31 | 3065 |
| 19½* | Nil | 58 | 71 | 57 | 97 | 30 | 33 | 1635 |
| 19½* | 18, 12† | 21 | 88 | 23 | 81 | 15 | 28 | 3310 |
| Nil | 20, 13, 9 | 54 | 60 | 70 | 85 | — | — | 3615 |
| 19½ | Nil | 25 | 48 | 45 | 80 | — | — | 1495 |
| 19½ | 20, 13, 9 | 66 | 77 | 83 | 123 | — | — | 4535 |
| Nil | 23, 15 | 52 | 56 | 68 | 73 | — | — | 2910 |
| 22½ | Nil | 2 | 19 | 3 | 51 | — | — | 1075 |
| 22½ | 23, 15 | 1 | 79 | 3 | 39 | — | — | 2740 |

* Heliotrine: 400 mg./kg. body wt. † Nicotinamide: 250 mg./kg. body wt.

mitochondria from the livers of rats treated with heliotrine plus nicotinamide were studied in relation to the pyridine nucleotide content.

Treatment with heliotrine plus nicotinamide (20 hr.) did not significantly affect the capacity of rat-liver homogenates (10%, w/v, in 0·25M-sucrose) to oxidize glutamate (Table 7) or of isolated mitochondria to oxidize glutamate or α-oxoglutarate (Table 8, group IV). However, mitochondria isolated from the liver after treatment with both agents for 22 hr. showed a very low oxygen uptake with each substrate tested except when the medium contained added DPN$^+$, and even then the rate of respiration was only half that of control preparations. The pyridine nucleotide values of the mitochondrial preparations (Table 8) indicated that the decrease of respiratory activity could be correlated with a low concentration of DPN$^+$ and other pyridine nucleotide coenzymes in the mitochondria. Confirmatory data are shown (Table 9) from three experiments in which rats received multiple injections of nicotinamide, and in which the mitochondrial respiration rate and the homogenate DPN$^+$ concentrations were determined.

It was apparent that a high concentration of liver DPN$^+$ could be maintained for at least 22½ hr. (Table 9) after rats were treated with heliotrine plus nicotinamide, which contrasts with the considerable decrease in the concentration of DPN$^+$ found at 22½ hr. after treatment with heliotrine alone. However, the respiration rate of mitochondria isolated from the liver of the rats treated with nicotinamide plus heliotrine (22½ hr.) was as low as that of the preparation from the rats that had received heliotrine only. A decrease of the rate of oxidation of β-hydroxybutyrate, which could be reversed by DPN$^+$ addition to the incubation medium, was even observed with mitochondria from an animal killed 19½ hr. after heliotrine plus nicotinamide and which had received additional nicotinamide at 9 hr. and 13 hr. (Table 9). It was thus apparent that although a high concentration of DPN$^+$ could be produced in the livers of rats treated with heliotrine plus nicotinamide, the mitochondria from these livers still developed a DPN$^+$-reversible loss of respiratory activity on isolation.

*Changes in liver weight/body weight ratio.* Results are shown (Fig. 3) that demonstrate the changes in the liver wt./body wt. ratio which were observed in rats treated with heliotrine alone, nicotinamide alone and heliotrine plus nicotinamide. The observations on the group treated with heliotrine alone confirm those of Christie (1958*b*). A rapid fall in the ratio occurred in the initial period after the injection, but by 8 hr. the fall was arrested and thereafter a slow rise took place until 20 hr., when the rise became accelerated and very high concentrations were eventually reached. A fall in the liver wt./body wt. ratio also occurred soon after an injection of nicotinamide, and was followed by a gradual return to normal values, which were reached in 22–24 hr. from the outset. The fall exceeded that found after heliotrine treatment. After simultaneous injection of heliotrine plus nicotinamide, an early reduction of the ratio again occurred; the fall was intermediate in degree between that with either agent alone.

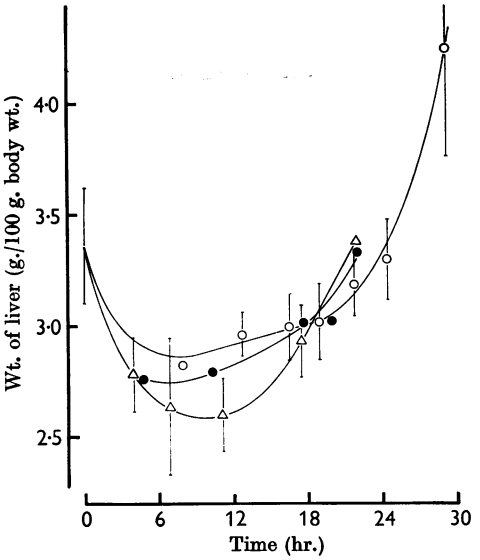


Fig. 3. Changes in liver weight of rats treated with heliotrine and nicotinamide. Liver weight is expressed as g. of liver/100 g. body wt. Rats received heliotrine (320 mg./kg. body wt.) or nicotinamide (500 mg./kg. body wt.). Treatment of rats: ○, heliotrine at 0 hr.; △, nicotinamide at 0 hr.; ●, heliotrine plus nicotinamide at 0 hr. Vertical lines represented ± S.D. population.

The data shown were calculated from measurements of liver weight and body weight at the time of killing the rats. Whereas controls showed negligible changes in body weight over the 24 hr. experimental period, all treated animals (heliotrine only, nicotinamide only, heliotrine plus nicotinamide) lost 10–15 g. body wt. during this interval. If liver weight had been related to initial body weight (measured at the time of injection) the reductions of the ratio would have been greater than those shown.

## DISCUSSION

### *Concentrations of pyridine nucleotide in heliotrine poisoning*

It has been shown (Christie & Le Page, 1962), and confirmed in the present work, that heliotrine (320 mg./kg. body wt.) did not produce definite effects on the concentrations of pyridine nucleotides in rat livers for a period of up to 17 hr., but thereafter the concentrations of $DPN^+$, TPNH and $TPN^+$ fell, whereas that of DPNH temporarily increased. The depression of the total TPN concentration was relatively greater than that of total DPN. Thus a consistent pattern in the concentrations of the individual nucleotides relative to each other was superimposed on the general fall (Table 10).

### *Concentrations of pyridine nucleotide after treatment with heliotrine plus nicotinamide*

*Decrease in $DPN^+$-synthesizing capacity.* It was found possible to alter the magnitude of the initial rise of $DPN^+$ concentration after nicotinamide injection by pretreatment of the rats with a single dose of heliotrine (320 mg./kg. body wt.), but only if the administration of heliotrine was so timed that the expected rise in the concentration of $DPN^+$ due to nicotinamide occurred in the 17–22 hr. period (or later) after the heliotrine injection (Table 3, Fig. 1). This observation could be interpreted as showing that the capacity of the liver to synthesize $DPN^+$ decreases 14–18 hr. after heliotrine administration.

However, lowered concentrations of pyridine nucleotides were observed in rat-liver homogenates 16–18 hr. after the administration of heliotrine only; this has been regarded as a consequence of losses of pyridine nucleotides from cells that had been damaged by the toxin but which had not yet reached an advanced stage of injury (Christie & Le Page, 1962). It is therefore possible that the 'decreased response' in $DPN^+$ concentration to a test dose of nicotinamide, which was observed after 14–18 hr. of pretreatment with heliotrine, could be a consequence of a rapid loss of $DPN^+$, synthetic capacity remaining unimpaired. In support of this, it can be seen from Fig. 1 that the difference between the concentration of liver $DPN^+$ in rats treated with nicotinamide alone (4 hr.) and that in rats treated with heliotrine (14–24 hr.) plus nicotinamide (4 hr.) was similar to the difference in concentration between the untreated controls and the rats treated with heliotrine alone (18–24 hr.). Furthermore, as the data (Tables 1–3, Fig. 1) refer to the concentrations of $DPN^+$ only, the effect of any rise in the concentration of DPNH would not be apparent, yet the DPNH increased for limited periods after treatment with heliotrine only (Christie & Le Page, 1962) and, to a less extent, after heliotrine plus nicotinamide (Table 4). Therefore it is possible that some of the decrease in the concentration of $DPN^+$ at this time may have been due to conversion into DPNH, but the absolute quantities involved are unlikely to have been great enough to affect the conclusions. Thus, assuming that a raised $DPN^+$ concentration after injection of nicotinamide did not protect the liver cells against, or significantly delay the onset of pyridine nucleotide loss in heliotrine poisoning, the observations could be adequately explained as a consequence of increasing nucleotide loss with increasing duration of heliotrine pretreatment.

Preparations of isolated nuclei from livers of rats treated with heliotrine (18 hr.) have a significantly lower capacity to synthesize $DPN^+$ (from

nicotinamide mononucleotide *in vitro*) than preparations from normal rats (G. S. Christie, M. J. Bailie & R. N. Le Page, unpublished work). We have therefore concluded that the progressively lower liver-DPN$^+$ concentrations, observed 4 hr. after a test dose of nicotinamide in rats pretreated with heliotrine for increasing intervals (Fig. 1), resulted from the operation of at least two effects, namely impairment of DPN synthesis and loss of DPN from the cells, and that both of these effects became greater as the duration of pretreatment with heliotrine was extended.

*Failure of nicotinamide to prevent losses of pyridine nucleotides.* During the period 0–17 hr. after heliotrine plus nicotinamide, the concentrations of the individual nucleotides remained typical of those observed in rats treated with nicotinamide only, but after 17 hr. the pattern changed and began to resemble that obtained from rats treated with heliotrine only (Table 10), although it differed in that the changes in the concentration of TPN were accelerated and the changes in concentration of DPN were relatively slower in taking place; thus the value for total DPN remained higher relative to that for total TPN.

Trends in the pyridine nucleotide concentrations of the supernatant fraction after heliotrine-plus-nicotinamide treatment were similar, in general, to those of the whole homogenate. It is possible that alterations of oxidation state during preparation, similar to those demonstrated for mitochondria (Birt & Bartley, 1960; Christie & Le Page, 1962), might also occur in the supernatant, so that the concentrations of individual nucleotides in the supernatant fraction may be a less accurate index of the distribution of the nucleotides *in vivo* than the values obtained from estimations on homogenate preparations.

The decrease of liver-pyridine nucleotide concentrations that is evident from 17 hr. after injection of heliotrine may be due to losses from damaged liver cells *in vivo*, which occur in a similar way to that in which mitochondria (at a comparable stage in the toxic process) lose pyridine nucleotides *in vitro* (Christie & Le Page, 1962). Therefore the administration of nicotinamide did not prevent or delay extensive pyridine nucleotide losses from the livers of rats poisoned with heliotrine.

### *Respiration rate after treatment with heliotrine plus nicotinamide*

Liver mitochondria from rats treated with heliotrine plus nicotinamide failed to carry out DPN-linked oxidations (reversible by DPN$^+$ supplementation of the medium) even when the homogenate-DPN$^+$ concentration was greatly elevated (Table 9); these mitochondria also showed greatly decreased concentrations of pyridine nucleotides (Table 8). The findings may be explained on the assumption that mitochondria, isolated from homogenates of liver (10 %, w/v, in 0·25 M-sucrose) from rats treated with heliotrine plus nicotinamide, had undergone the same change as that in liver mitochondria isolated from rats treated with heliotrine only, and which permitted rapid loss of pyridine nucleotides during isolation or when suspended in the medium used for measuring respiration (Christie & Le Page, 1962). In a liver homogenate the mitochondria would be released into a medium that would then have a concentration of pyridine nucleotides one-tenth of that present in the cytoplasm of the cells originally homogenized. Furthermore,

Table 10. *Summary of changes in concentrations of pyridine nucleotide at 22 hr. after treatment with heliotrine and nicotinamide*

Results are expressed relative to concentrations in similar preparations from normal rats. A minimum number of symbols to suggest degrees of difference considered important has been employed.

| | | Pyridine nucleotides | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Nature of preparation | Treatment of rat | DPN$^+$ | DPNH | TPN$^+$ | TPNH | DPN$^+$ + DPNH | TPN$^+$ + TPNH | Total pyridine nucleotide |
| Homogenate | Nicotinamide only | + + + | + + + | − | + | + + + | ± | + + + |
| | Heliotrine only | − − | + | − | − − | − | − − | − − |
| | Heliotrine plus nicotinamide | ± | ± | − − | − − − − | ± | − − − | − − |
| Supernatant | Nicotinamide only | + + + | + + + | − | + | + + + | ± | + + + |
| | Heliotrine only | − − | ± | − | − − | − | − − | − − |
| | Heliotrine plus nicotinamide | + | + + | ± | − − − | + | − − | ± |
| Mitochondria | Nicotinamide only | True values unknown | | | | ± | ± | ± |
| | Heliotrine only | True values unknown | | | | − | − − | − |
| | Heliotrine plus nicotinamide | True values unknown | | | | − | − − | − |

even when the liver cells initially may have contained twice the normal amount of $DPN^+$, as after heliotrine-plus-nicotinamide treatment (Table 9), a decrease of $DPN^+$ concentration to one-fifth would be expected to take place in the suspending fluid, relative to the normal $DPN^+$ concentration in the cytoplasm of whole liver cells.

This interpretation is compatible with data (Christie & Le Page, 1962) which indicated that mitochondria that had lost about 50% of their content of $DPN^+$ had a greatly reduced respiration rate, and that supplementation of the suspending fluid with a 1–2 mM-$DPN^+$ concentration was required to restore the oxidation rate to near normal.

*Lack of protective action by nicotinamide in heliotrine poisoning*

Gallagher & Simmonds (1959) and Gallagher (1960*a*) found that prior administration of precursors of pyridine nucleotides (nicotinic acid or tryptophan) afforded considerable protection to rats against doses of carbon tetrachloride that were otherwise lethal. Sheep could also be protected against toxic effects of carbon tetrachloride by injections of nicotinic acid (Gallagher, 1960*b*). In the present studies we have not examined the effect of prior treatment with nicotinamide on heliotrine-poisoned rats. However, we did not observe any changes that could be interpreted as indicating a 'protective' effect of nicotinamide against losses of pyridine nucleotides from liver cells, and therefore against this toxic effect of heliotrine, when nicotinamide was administered at the same time as, or later than, heliotrine.

*Changes in liver weight/body weight ratio*

We regard a reduction of the liver wt./body wt. ratio as an indication of a reduction of liver weight. Christie (1958*b*) noted that the fall in liver weight with heliotrine-poisoned rats occurred before liver-cell damage became severe (as judged by histological examination), and suggested that loss of cytoplasmic substance, especially the basophilic material, contributed to the effect at this time (4–10 hr.). Later, however, the loss of weight was arrested by progressive fluid imbibition and small-vacuole fatty change, so that after about 11 hr. the liver weight began to increase. After 15 hr., haemorrhage into damaged parts of the liver (Christie, 1958*a*, 1958*b*) became the predominant factor in the great increase of the liver weight that thereafter occurred.

An unexpected finding in the present studies was the considerable decrease of liver weight after an injection of nicotinamide into rats. One outcome of this, in regard to pyridine nucleotide values (which have been expressed relative to body weight), was that the values were lower compared with control values than they would have been if expressed relative to g. wet wt. of liver. We cannot suggest a reason for the decrease in liver weight after nicotinamide administration other than as a consequence of raised metabolism in response to the very large dose of nicotinamide injected. The injection of heliotrine plus nicotinamide did not produce an additive effect but resulted in a reduction of liver weight comparable with that found in rats injected with either agent alone.

## SUMMARY

1. Spectrophotometric assay indicated a considerable decrease in the concentration of $DPN^+$ in rat liver after treatment with heliotrine alone for 20 hr. or more. After treatment with nicotinamide alone the liver $DPN^+$ content increased 2·5-fold.

2. The increase in liver $DPN^+$ *in vivo* in response to treatment with nicotinamide (4 hr.) was found to decrease with increasing duration of heliotrine pretreatment.

3. Fluorimetric determinations indicated that only the concentration of $DPN^+$ in liver homogenates was raised significantly after nicotinamide treatment.

4. Only after 22 hr. did the heliotrine-plus-nicotinamide-treated rats show a significant decrease in the concentrations of $DPN^+$, TPNH, total DPN, total TPN and total pyridine nucleotide; similar decreases of pyridine nucleotide were found in the soluble supernatant fraction.

5. Mitochondria isolated from the livers of rats treated with heliotrine plus nicotinamide (22 hr.) were found to have lost considerable amounts of pyridine nucleotides.

6. Mitochondria isolated from the homogenates of livers of heliotrine-plus-nicotinamide-treated (22 hr.) rats showed a very low oxidation rate except with added $DPN^+$; this decrease in oxidation rate was correlated with low concentrations of $DPN^+$ and other pyridine nucleotides in the isolated mitochondria.

7. The liver weight/body weight ratio of heliotrine-treated rats was found initially to decrease and then after 10 hr. to increase. After nicotinamide injection a similar decrease in ratio was observed, with a gradual return to normal values in about 24 hr. After heliotrine-plus-nicotinamide injection an initial fall in the ratio was observed.

8. The significance of these observations has been discussed.

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of heliotrine and of rats of the same strain as used in previous studies with heliotrine in this Department. We are also indebted to Dr L. M. Birt of the Department of Biochemistry, University of Melbourne, for valuable advice and discussions. The work was made possible by the financial support of the Anti-Cancer Council of Victoria.

## REFERENCES

Bassham, J. A., Birt, L. M., Hems, R. & Loening, V. L. (1959). *Biochem. J.* **73**, 491.
Birt, L. M. & Bartley, W. (1960). *Biochem. J.* **75**, 303.
Bull, L. B., Dick, A. T. & McKenzie, J. S. (1958). *J. Path. Bact.* **75**, 17.
Christie, G. S. (1958*a*). *Aust. J. exp. Biol. med. Sci.* **36**, 405.
Christie, G. S. (1958*b*). *Aust. J. exp. Biol. med. Sci.* **36**, 413.
Christie, G. S. & Le Page, R. N. (1962). *Biochem. J.* **84**, 25.
Christie, G. S., Le Page, R. N. & Bailie, M. J. (1961). *Nature, Lond.*, **189**, 593.
Ciotti, M. M. & Kaplan, N. O. (1957). In *Methods in Enzymology*, vol. 3, p. 891. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Gallagher, C. H. (1960*a*). *Aust. J. exp. Biol. med. Sci.* **38**, 251.
Gallagher, C. H. (1960*b*). *Aust. J. agric. Res.* **11**, 1009.
Gallagher, C. H. & Simmonds, R. A. (1959). *Nature, Lond.*, **184**, 1407.
Horecker, B. L. & Kornberg, A. (1948). *J. biol. Chem.* **175** 385.
Kaplan, N. O. (1960). In *The Enzymes*, 2nd ed. vol. 3, p. 105. Ed. by Boyer, P. D., Lardy, H. & Myrbäck, K. New York: Academic Press Inc.
Kaplan, N. O., Ciotti, M. M. & Stolzenbach, F. E. (1956*a*). *J. biol. Chem.* **221**, 833.
Kaplan, N. O., Goldin, A., Humphreys, S. R., Ciotti, M. M. & Stolzenbach, F. E. (1956*b*). *J. biol. Chem.* **219**, 287.
Kaplan, N. O., Goldin, A., Humphreys, S. R. & Stolzenbach, F. E. (1957). *J. biol. Chem.* **226**, 365.
Keilin, D. & Hartree, E. F. (1937). *Proc. Roy. Soc.* B, **122**, 298.
Kornberg, A. (1950). *J. biol. Chem.* **182**, 805.
Preiss, J. & Handler, P. (1957). *J. Amer. chem. Soc.* **79**, 4246.
Preiss, J. & Handler, P. (1958*a*). *J. biol. Chem.* **233**, 488.
Preiss, J. & Handler, P. (1958*b*). *J. biol. Chem.* **233**, 493.

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# The Mechanism of Ketone-Body Formation from Butyrate in Rat Liver

BY F. J. R. HIRD AND R. H. SYMONS
*Russell Grimwade School of Biochemistry and School of Agriculture, University of Melbourne, Victoria, Australia*

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Two mechanisms have been proposed for the conversion of acetoacetyl-coenzyme A into acetoacetate in liver tissue. They are the direct hydrolysis of the thioester (Drummond & Stern, 1960) and the pathway via β-hydroxy-β-methylglutaryl-coenzyme A (Lynen, Henning, Bublitz, Sörbo & Kröplin-Rueff, 1958). The results obtained by Hird & Symons (1961) indicated that the β-hydroxy-β-methylglutaryl-coenzyme A pathway was operating in rumen and omasum tissue of the sheep and it was calculated that at least three-quarters of the acetoacetyl-coenzyme A formed from butyrate by these tissues was deacylated in this way.

Most of the literature on this subject has been concerned with liver tissue in which the steps in the oxidation of fatty acids are known in some detail; consequently, there are fewer assumptions in interpreting results obtained with this tissue as compared with those from the rumen and the omasum of the sheep. The experimental procedures used with sheep tissues have therefore been extended to rat-liver slices and mitochondria. The results obtained indicate a similar mechanism of ketone-body formation with each of the preparations. A preliminary account of some of this work on liver preparations has already been published (Hird & Symons, 1960).

## METHODS AND MATERIALS

*Chemicals.* Sodium [1-$^{14}$C]butyrate, sodium [1-$^{14}$C]-acetate, sodium DL-[2-$^{14}$C]lactate and sodium [1-$^{14}$C]-octanoate were obtained from The Radiochemical Centre, Amersham, Bucks. Sodium [3-$^{14}$C]butyrate was obtained from Commissariat à l'Energie Atomique, France. Sodium [2-$^{14}$C]butyrate and sodium [4-$^{14}$C]butyrate were prepared as described by Hird & Symons (1961). Further supplies of sodium [2-$^{14}$C]butyrate were obtained from Research Specialties Co., Richmond, Calif., U.S.A.

Hexokinase (160 000 K.M. units/g.; Kunitz & McDonald, 1946) and ATP were purchased from Sigma Chemical Co.

*Rat-liver slices.* Fresh rat liver was chilled in ice-cold Krebs–Ringer phosphate buffer, pH 7·4, and sliced by hand. The slices were incubated in Krebs–Ringer phos-