3. The relative stability demonstrated *in vivo* is discussed in the light of currently conflicting views of phospholipid turnover in brain.

4. Some aspects of the blood-brain barrier for phosphorus have been reconsidered.

5. It is suggested that the stability of these phospholipids, together with the stability of other brain lipids previously reported, may reflect their structural role in the myelin sheath.

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The Synthesis of Nicotinuric Acid by Rat-Kidney Preparations

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Nicotinuric acid (nicotinoylglycine) was first isolated by Ackermann (1912) from the urine of a dog which had been fed with nicotinic acid, and it has since been found in the urine of other species, including the rat (Huff & Perlzweig, 1942) and man (Melnick, Robinson & Field, 1940). More recent work has shown that it is present only in small quantities in rat urine either normally or after the administration of nicotinamide, but that it is an important urinary metabolite when doses of nicotinic acid are given, especially at short time intervals after administration (Reddi & Kodicek, 1953; Leifer, Roth, Hogness & Corson, 1951; Johnson & Lin, 1953; Lin & Johnson, 1953). No study of nicotinuric acid synthesis *in vitro* has been reported.

In the course of exploratory experiments on nicotinic acid metabolism *in vitro* it was observed that a substance corresponding chromatographically with nicotinuric acid was formed by various tissue preparations. In this paper the identification of the compound with nicotinuric acid and its syn-

thesis by rat-kidney preparations are described. A preliminary report of part of the work has been published previously (Jones & Elliott, 1954).

EXPERIMENTAL

Materials

Nicotinuric acid. This was synthesized by the method of Rohrlach (1951), with methyl nicotinate prepared as described by Levine & Snead (1951).

Nicotinic acid. Commercial nicotinic acid was purified by boiling with decolorizing charcoal and crystallizing it twice from water.

Adenosine triphosphate. The dibarium salt of adenosine triphosphate (ATP) was prepared by the method of Needham (1942) and converted into monobarium ATP (Kerr, 1941). For use, solutions of potassium ATP were prepared by removing the barium with Amberlite IR-100 (H form) and neutralizing to pH 6.8 with KOH. In later experiments crystalline disodium ATP (Schwarz Laboratories Inc., Mount Vernon, N.Y., U.S.A.) was used. It was dissolved in water and neutralized to pH 6.8 with KOH. Solutions of ATP contained added KCl to make them isotonic with rat serum.

Phosphate buffers. These were prepared from solutions of KH_2PO_4 and KOH.

Methods

Chromatography of tertiary nicotinic acid derivatives. Nicotinic acid derivatives having a tertiary ring nitrogen (i.e. nicotinic acid, nicotinamide and nicotinuric acid) were separated by paper chromatography with butan-1-ol saturated with water (butan-1-ol-water) as solvent (Kodicek & Reddi, 1951). The compounds were detected by two methods: procedure (1), by their u.v. absorption, either by direct observation or by contact photography (Markham & Smith, 1949) with unfiltered light from a mercury lamp; procedure (2), by the König reaction (Kodicek & Reddi, 1951), for which the dried paper was placed in an atmosphere of cyanogen bromide for 1 hr. and then sprayed with a 2% solution of *p*-aminobenzoic acid in 0.75 *N*-HCl-ethanol (3:1, v/v).

Measurement of nicotinuric acid synthesis. The standard incubation mixture used in all experiments, except those with tissue slices, had the following composition: 0.3 ml. of 0.1 *M*-phosphate buffer, pH 7.35; 0.03 ml. of 0.1 *M*- MgCl_2 ; 0.6 ml. of 0.1 *M*-potassium fumarate; 0.15 ml. of 0.01 *M*-ATP; 0.1 ml. of 0.154 *M*-potassium nicotinate; 0.2 ml. of 0.308 *M*-glycine; 1 ml. of the tissue preparation; 0.154 *M*-KCl to a total volume of 3.08 ml. Incubations were performed in conical flasks or Warburg manometers shaken at 38° for 2 hr. with a gas phase of air.

After incubation, the mixtures were placed in a boiling-water bath for 5 min., cooled and centrifuged. Samples (0.308 ml.) of the supernatants were taken for the estimation of nicotinuric acid by the method of Jones (1959*b*). Alternatively, after chromatography of the samples, nicotinuric acid was detected qualitatively by procedure (2) above.

Nicotinamide. This was estimated by the method of Jones (1959*b*).

Total nitrogen. This was estimated by the micro-Kjeldahl method.

Preparation of homogenates. Kidneys from female albino rats were washed and cooled in ice-cold 0.154 *M*-KCl or 0.25 *M*-sucrose. After cooling, the medulla was cut away and the kidney cortex blotted, weighed and homogenized in 9 vol. of ice-cold fluid in a Potter & Elvehjem (1936) homogenizer. The homogenate was filtered through either several layers of muslin or a single layer of flannellette before use.

Preparation of tissue fractions. The following procedure, modified from that of Schneider (1948), was used to fractionate homogenates into nuclear, mitochondrial, microsome and soluble fractions. All operations were performed at 0–2°.

Kidney-cortex homogenate was prepared with 8.5 vol. of 0.25 *M*-sucrose plus 0.5 vol. of 0.1 *M*-potassium ethylenediaminetetra-acetate (EDTA), pH 7.4. A sample of the filtered homogenate was set aside at 0° for experiments on the whole homogenate. The remainder of the filtered homogenate was centrifuged at 800 *g* (average value) for 10 min. at 0°. The supernatant was decanted and kept in the cold. The sediment was resuspended in a volume of 0.25 *M*-sucrose equal to half that of the homogenate used and again centrifuged at 800 *g* for 10 min. at 0°. The supernatant was decanted and the sediment suspended in a suitable volume of 0.25 *M*-sucrose (nuclear fraction). The combined supernatants from these centrifugings were centrifuged at 5000 *g* (average value) for 10 min. at 0°. The supernatant was decanted and kept in the cold. The sediment was resuspended in a volume of 0.25 *M*-sucrose equal to half

that of the homogenate originally taken and again centrifuged at 5000 *g* for 10 min. at 0°. The supernatant was decanted and the sediment taken up in a suitable volume of 0.25 *M*-sucrose (mitochondrial fraction). The combined supernatants from the sedimentation and washing of the mitochondria were then centrifuged at 100 000 *g* (average value) for 30 min. at 0°. The sediment was taken up in 0.25 *M*-sucrose (microsome fraction). The supernatant from this centrifuging was used as the soluble fraction. In some experiments the microsome and soluble fractions were not tested separately, and in this event the combined supernatants from the sedimentation of the mitochondrial fraction were used as the microsome plus soluble fraction.

Preparation of mitochondrial suspensions. The mitochondrial fraction was sedimented and washed as described above and the final pellet suspended in a volume of 0.154 *M*-KCl equal to one-fifth of that of the homogenate used. This gave suspensions containing about 1 mg. of total N/ml.

RESULTS

Identification of the nicotinic acid metabolite

The substance formed from nicotinic acid by incubation with slices or homogenates of rat kidney or liver had the same R_f in butan-1-ol-water and in acetone-water (85:15, v/v), and gave the same colours on treatment by procedure (2) as a sample of synthetic nicotinuric acid.

For more rigorous identification the compound was isolated by paper chromatography. Samples of incubation mixtures were applied in a band to Whatman no. 3 paper and the chromatograms developed for 40–48 hr. with butan-1-ol-water. The compound was located by its u.v. absorption, the band was cut out and eluted chromatographically with water. Narrow strips of the chromatograms were subjected to procedure (2) or treated with ninhydrin to show that the compound had been separated from nicotinic acid and from ninhydrin-positive compounds. The eluate was again chromatographed in a band for 24 hr. and the compound eluted as before. This second eluate was used in the subsequent identification.

The spectra of samples of the compound and of synthetic nicotinuric acid were compared over the range 230–280 $m\mu$ after chromatography in butan-1-ol-water for 12 hr. and elution from the paper. Separate samples were eluted with *N*-HCl and with water. The spectrum of the biologically formed compound was identical in both solvents with that of synthetic nicotinuric acid (Fig. 1).

Further samples of the biosynthetic compound were hydrolysed in 6 *N*-HCl at 105° for 18 hr. in sealed tubes and spotted on to paper as described by Consden, Gordon & Martin (1947). Chromatograms developed with butan-1-ol-water and with acetone-water (85:15, v/v) showed the presence in the hydrolysate of nicotinic acid. Another chromatogram was developed in two dimensions with

phenol-0.3% NH_3 followed by methanol-pyridine-water (80:2:20, by vol.), and sprayed with ninhydrin. A single spot having the same R_f as glycine was found. A duplicate chromatogram on which glycine was added to the hydrolysate also showed a single spot, reinforced in intensity. Chromatograms of samples of the unhydrolysed material showed that it contained neither nicotinic acid nor glycine.

These experiments establish the identity of the compound formed from nicotinic acid with nicotinuric acid.

Nicotinuric acid synthesis by rat-kidney slices and homogenates

When slices of rat-kidney cortex were incubated in Krebs-Ringer phosphate solution (Krebs, 1933), pH 7.4, together with nicotinic acid, glycine and fumarate, nicotinuric acid was formed. In a number of experiments the synthesis averaged 8–10 $\mu\text{moles/g. wet wt./2 hr.}$ No synthesis occurred when nicotinic acid was omitted, but in the absence of glycine a small amount of nicotinuric acid was formed. Nicotinuric acid was absent from incubation mixtures stopped at zero time.

Kidney homogenates prepared in 0.154M-KCl or 0.25M-sucrose also synthesized nicotinuric acid in the presence of nicotinic acid, glycine, fumarate and ATP (Table 1). The amounts formed (1.5–2.0 $\mu\text{moles/g. wet wt./2 hr.}$) were rather small compared with those formed by slices, but when potassium EDTA was added to the homogenate (5 mM final concentration in the homogenate) the activity

was increased to a value of 10–15 $\mu\text{moles/g. wet wt./2 hr.}$, which is somewhat higher than that of slices. EDTA was therefore added to the homogenizing fluid in all subsequent experiments.

The requirements for nicotinuric acid synthesis by homogenates were similar whether or not the homogenate contained EDTA (Table 1). Only nicotinic acid was absolutely required for the reaction, but glycine, fumarate and ATP were all necessary for maximum synthesis.

Fractionation of homogenates. The results of a typical experiment, in which the distribution of the ability to form nicotinuric acid under the standard incubation conditions between different subcellular fractions of rat-kidney homogenate was examined, are shown in Table 2. The nuclear and mitochondrial fractions were found to be approximately equally active but no synthesis was observed in the microsome and soluble fractions either together (as in Table 2) or separately. The summed activities of the individual fractions represented 96% recovery of

Table 1. *Nicotinuric acid synthesis by rat-kidney homogenate*

Homogenate A was prepared in 9 vol. of 0.154M-KCl, homogenate B in 8.5 vol. of 0.154M-KCl + 0.5 vol. of 0.1M-potassium EDTA, pH 7.4. Incubation conditions are given in the text. A portion (0.05 ml.) of 0.1M-potassium EDTA, pH 7.4, was added to the incubation mixture where indicated.

Composition of incubation mixture	Nicotinuric acid formed ($\mu\text{moles/g. wet wt. of tissue/2 hr.}$)	
	Homogenate A	Homogenate B
Complete	1.7	10.3
Nicotinic acid and glycine omitted	0	0
Nicotinic acid omitted	0	0
Glycine omitted	0.2	1.1
Fumarate omitted	0.7	2.3
ATP omitted	—	6.0
Complete, EDTA added	11.6	—

Table 2. *Nicotinuric acid synthesis in tissue fractions*

Rat-kidney homogenate was fractionated and incubations were performed as described in the text.

Tissue fraction	Nicotinuric acid formed ($\mu\text{moles/g. wet wt. of tissue/2 hr.}$)	
	($\mu\text{moles/g. wet wt. of tissue/2 hr.}$)	($\mu\text{moles/mg. of total N/2 hr.}$)
Whole homogenate	11.6	0.59
Nuclear fraction	5.95	2.09
Mitochondrial fraction	5.15	1.85
Microsome plus soluble fraction	0	0
Nuclear + mitochondrial fractions	11.2	—
Mitochondrial + microsome plus soluble fractions	5.2	—

Fig. 1. Comparison of the spectra of synthetic nicotinuric acid and the nicotinic acid metabolite in water and $n\text{-HCl}$. Experimentally determined values for the nicotinic acid metabolite have been multiplied by a factor calculated to give the solutions of the metabolite and of synthetic nicotinuric acid the same extinction at 262 $m\mu$ in the same solvent. —, Spectrum of synthetic nicotinuric acid in water; O, nicotinic acid metabolite in water; - - -, synthetic nicotinuric acid in $n\text{-HCl}$; ●, nicotinic acid metabolite in $n\text{-HCl}$.

the activity of the whole homogenate. The amounts of nicotinuric acid formed when the fractions were combined agreed well with the sum of their separate activities, and when samples of the nuclear and mitochondrial fractions were combined the observed synthesis was 97 % of that in the whole homogenate.

In other fractionation experiments 90–110 % of the activity of the homogenate was recovered in the nuclear and mitochondrial fractions, but although the total activity of these fractions was about the same in all experiments, its quantitative distribution between them was rather variable. The ratio of the amount of nicotinuric acid formed per gram of tissue by the nuclear fraction to that formed by the mitochondrial fraction varied between 0.85 and 1.7. A rather similar variation was also found in the amounts of total N per gram of tissue in each fraction, and the activities of each fraction calculated per milligram of total N were much more constant from one experiment to another than those per gram of tissue. In all experiments, including those where only one fraction was tested, the nuclear fraction formed 2.0–2.5 μ moles of nicotinuric acid/mg. of total N/2 hr., whereas the mitochondrial fraction normally formed 1.8–2.5 μ moles/mg. of total N/2 hr., with occasional higher values. In contrast to its stimulating effect when added to homogenates, EDTA was found to inhibit the activity of nuclear and mitochondrial suspensions by about one-third when added to the incubation mixtures at a final concentration of 1.6 mM.

It is possible that the microsome and soluble fractions might fail to synthesize nicotinuric acid because of the rapid hydrolysis of the ATP initially present in the incubation mixtures. Experiments were therefore performed with these fractions in which two methods were used to ensure an adequate constant supply of ATP. First, potassium phosphoglycerate and an enzyme fraction from rabbit muscle (Ratner & Pappas, 1949) were added to the incubation mixture. The muscle fraction contains the enzymes of the glycolytic sequence which convert phosphoglycerate into pyruvate with the concomitant rephosphorylation of adenosine diphosphate to ATP. Neither the microsome fraction nor the soluble fraction formed any nicotinuric acid in the presence of this ATP-regenerating system. Secondly, a sample of mitochondrial suspension was added to the incubation mixture under conditions suitable for oxidative phosphorylation. In such experiments no increase in nicotinuric acid formation over that found with the mitochondrial suspension alone was obtained with either the microsome or the soluble fraction.

Nicotinuric acid synthesis in the nuclear fraction. The nuclear fraction isolated in the manner used here is known to contain whole cells and mitochon-

dria in addition to the nuclei (de Duve & Berthet, 1954), and it seemed possible that the nicotinuric acid synthesis observed in the nuclear fraction might be due to these contaminants rather than to the nuclei.

The formation of nicotinuric acid by the nuclear fraction was shown to be absolutely dependent upon the addition of nicotinic acid, glycine, fumarate and ATP (Table 3). An absolute requirement for all these substances was not found in slices or even in homogenates (Table 1), making it unlikely that nicotinuric acid synthesis in the nuclear fraction was brought about by whole cells.

The succinoxidase activity of the nuclear fraction was used to obtain an estimate of the number of mitochondria in this fraction, since succinoxidase is believed to be present only in mitochondria (Dounce, 1955; Schneider & Hogeboom, 1956). Succinoxidase in the nuclear and mitochondrial fractions was assayed by the method of Schneider & Potter (1943) and nicotinuric acid formation by samples of the same suspensions was measured simultaneously. The results of such an experiment are shown in Table 4. It was found that the nuclear fraction derived from 1 g. of kidney had an activity equivalent to 80 % of the succinoxidase activity of the mitochondrial fraction, whereas nicotinuric

Table 3. *Nicotinuric acid synthesis in the nuclear fraction*

The nuclear fraction of rat kidney was sedimented and washed as described in the text and the final pellet suspended in a volume of 0.154M-KCl equal to one-fifth of that of the homogenate used. Incubation conditions are given in the text.

Composition of incubation mixture	Nicotinuric acid formed (μ moles/mg. of total N/2 hr.)
Stopped at zero time	0
Complete	2.37
Nicotinic acid omitted	0
Glycine omitted	0
Fumarate omitted	0.04
ATP omitted	0.02

Table 4. *Nicotinuric acid synthesis and succinoxidase in the nuclear and mitochondrial fractions*

Activities were measured as described in the text and are given per gram wet weight of tissue. Figures in parentheses are activities per milligram of total N.

Fraction	Nicotinuric acid synthesis (μ moles/2 hr.)	Succinoxidase (O_2 uptake in μ l./hr.)
Whole homogenate	11.0 (0.58)	—
Nuclear fraction	7.15 (2.28)	5800 (1850)
Mitochondrial fraction	4.25 (1.85)	7260 (3160)
Ratio:		
Nuclear fraction	1.68	0.80
Mitochondrial fraction		

acid synthesis in the nuclear fraction was 168 % of that in the mitochondrial fraction. Assuming that mitochondria isolated in the mitochondrial and nuclear fractions have the same activity, the mitochondria present in the nuclear fraction synthesized 3.4 μ moles of nicotinuric acid/g. of kidney/2 hr., whereas the nuclei formed 3.75 μ moles/g. of kidney/2 hr.

Nicotinuric acid synthesis by mitochondrial suspensions

Requirements for synthesis. Nicotinuric acid was synthesized by mitochondrial suspensions in the

Table 5. *Nicotinuric acid synthesis by rat-kidney mitochondria*

Incubation conditions are given in the text.

Composition of incubation mixture	Nicotinuric acid formed (μ moles/mg. of total N/2 hr.)
Stopped at zero time	0
Complete	2.40
Nicotinic acid omitted	0
Glycine omitted	0
Fumarate omitted	0
ATP omitted	0.01

Table 6. *Effect of period of incubation upon nicotinuric acid synthesis and oxygen uptake of rat-kidney mitochondria*

Incubation conditions were as given in the text, except that 0.3 ml. of 0.1 M-potassium fumarate was used. Incubations were performed in Warburg manometers. Potassium nicotinate and glycine were placed in the side arm and tipped after equilibration.

Time of incubation (min.)	Uptake of O ₂ (μ l./mg. of total N)	Nicotinuric acid formed (μ moles/mg. of total N)	Uptake of O ₂ (μ l./ μ mole of nicotinuric acid formed)
0	0	0	0
30	222	1.10	202
60	426	2.13	200
90	575	2.71	212
120	630	3.07	205
150	610	2.90	210
180	662	2.70	245

Table 7. *Effect of fumarate concentration upon nicotinuric acid synthesis and uptake of oxygen of rat-kidney mitochondria*

Incubations were performed in Warburg manometers under the conditions given in the text. Potassium nicotinate and glycine were placed in the side arm and tipped after equilibration.

Concn. of fumarate (M)	Uptake of O ₂ (μ l./mg. of total N/2 hr.)	Nicotinuric acid formed (μ moles/mg. of total N/2 hr.)	Uptake of O ₂ (μ l./ μ mole of nicotinuric acid formed)
0	0	0	—
0.005	440	1.63	270
0.01	625	2.20	284
0.02	780	2.40	324

presence of nicotinic acid, glycine, fumarate and ATP. If any of these components were omitted from the incubation mixture, no nicotinuric acid was formed and none was present in the mixture stopped at zero time (Table 5).

Relation of synthesis to energy supply. Table 6 shows the nicotinuric acid synthesis and the oxygen uptake by mitochondrial suspensions after different periods of incubation. Both proceeded linearly for about 90 min., and after 2 hr. the oxygen uptake continued to rise only very slowly and no further nicotinuric acid synthesis was observed. The oxygen uptake per μ mole of nicotinuric acid formed was constant irrespective of the length of incubation (Table 6). When the concentration of fumarate was varied the oxygen uptake per μ mole of nicotinuric acid formed was again constant and was independent of the fumarate concentration (Table 7).

Nicotinuric acid synthesis was inhibited by 2,4-dinitrophenol at concentrations of 20 μ M to 0.5 mM, concentrations which uncouple oxidative phosphorylation. No synthesis occurred when the incubation was performed anaerobically.

Effect of concentration of substrates. The effect upon nicotinuric acid synthesis of varying the nicotinic acid concentration is shown in Fig. 2. Maximum synthesis was obtained at a concentration of 3 mM, but at higher nicotinic acid concentrations the synthesis was inhibited and at 0.04 M-nicotinic acid it was half of that at 3 mM. At low concentrations a large proportion of the nicotinic acid was converted into nicotinuric acid; in the experiment illustrated in Fig. 2, 71 % conversion was observed at 1 mM and in other experiments up to 95 % conversion was found at lower concentrations.

Fig. 2. Effect of nicotinic acid concentration upon nicotinuric acid synthesis by rat-kidney mitochondria. Incubation conditions are given in the text.

Fig. 3 shows the nicotinuric acid synthesis at different glycine concentrations. Maximum synthesis was obtained at about 0.04 M-glycine and no inhibition was observed at concentrations up to 0.08 M. The K_m of the system towards glycine was about 7.5 mM.

The effect of different ATP concentrations is illustrated in Fig. 4. In this experiment, maximum synthesis was obtained with a concentration of 1 mM, and at higher concentrations inhibition occurred, the synthesis at 2 mM-ATP being 65 % of the maximum. The cause of this inhibition is not known, but it seems possible that it was due to an impurity in the ATP preparations rather than to

Fig. 3. Effect of glycine concentration upon nicotinuric acid synthesis by rat-kidney mitochondria. Incubation conditions are given in the text.

Fig. 4. Effect of ATP concentration upon nicotinuric acid synthesis by rat-kidney mitochondria. Incubation conditions are given in the text.

ATP itself since optimum synthesis was obtained at slightly different ATP concentrations with different ATP preparations.

Inhibitors of nicotinuric acid synthesis. Nicotinuric acid formation by mitochondrial suspensions was inhibited by potassium fluoride, 82 % inhibition being observed at a concentration of 10 mM (Table 8). The effect of magnesium concentration is also shown in Table 8. In the absence of fluoride, a 39 % decrease in synthesis was found when the magnesium concentration was raised from 1 to 5 mM, but when fluoride was present the synthesis was unaffected by changing the magnesium concentration.

The formation of nicotinuric acid was strongly inhibited by benzoic acid, inhibition being 14 % at 0.1 mM-, 33 % at 0.3 mM- and 98 % at 1 mM-benzoic acid. The degree of inhibition caused by 0.1 mM-benzoic acid was unaffected by variation of the nicotinic acid concentration over a fourfold range, suggesting that the inhibition was non-competitive. Benzoic acid and hippuric acid were shown not to interfere with the estimation of nicotinuric acid.

Effect of ammonia. The addition of ammonium chloride to the incubation mixtures at concentrations of 3 mM to 0.03 M had no effect upon the nicotinuric acid synthesis. No nicotinamide was formed from nicotinic acid and ammonia by these preparations, either in the presence or in the absence of glycine.

DISCUSSION

The experiments described show that rat kidney and liver contain an enzyme system which forms nicotinuric acid from nicotinic acid and glycine. With slices and homogenates some nicotinuric acid was formed from nicotinic acid in the absence of added glycine, showing that the tissues contain sufficient free glycine for nicotinuric acid synthesis to occur *in vivo* so long as nicotinic acid is available. These observations are a further proof of the ability of the rat to form nicotinuric acid, and remove any remaining doubt on this point (Ellinger & Abdel Kader, 1949).

Table 8. *Effect of magnesium and fluoride ions upon nicotinuric acid synthesis by rat-kidney mitochondria*

Incubation conditions are given in the text.

Concn. of KF (mM)	Nicotinuric acid formed (μmole/mg. of total N/2 hr.)	
	With 1 mM-MgCl ₂	With 5 mM-MgCl ₂
0	1.97	1.19
1	1.54	—
5	0.61	0.60
10	0.36	0.33

Distribution of nicotinuric acid synthesis between tissue fractions. The results show that the nicotinuric acid-synthesizing ability of the homogenate is localized in the nuclear and mitochondrial fractions, being approximately equally divided between the two. None was found in the microsome or soluble fractions even when an external source of energy was added. This distribution differs from those found for other similar reactions forming peptide-like bonds. For example, Kielley & Schneider (1950) showed that in mouse liver *p*-aminohippuric acid synthesis is located almost entirely in the mitochondrial fraction, whereas taurocholic acid synthesis in guinea-pig liver occurs in the microsomes but not in the mitochondria (Elliott, 1956*a*, *b*). These differences may be due to different enzymes being involved in the several reactions or may merely reflect variations in the distribution of the same enzymes in different tissues.

The synthetic activity of the nuclear fraction is of particular interest since the only energy-requiring reaction of known mechanism previously shown to occur in this fraction is the synthesis of diphosphopyridine nucleotide from ATP and nicotinamide mononucleotide, which is almost exclusively located there in rat liver (Hogeboom & Schneider, 1952).

The evidence that some of the activity of the nuclear fraction was due to the nuclei themselves and some to contaminating mitochondria is based upon the differences in the distribution of succinoxidase and nicotinuric acid-synthesizing activities between the nuclear and mitochondrial fractions. The interpretation of this experiment is based upon certain assumptions. First, that the succinoxidase was, in fact, all in the mitochondria, since its localization in these structures has been demonstrated only in liver. Secondly, it was assumed that a given number of mitochondria had the same succinoxidase and nicotinuric acid-synthesizing activities whether they were isolated in the nuclear or the mitochondrial fraction; that is to say that these activities were uninfluenced by the other components of the nuclear fraction.

It appeared possible that the second assumption particularly might not be true and that the nuclei might exert some protective action on the mitochondria in the nuclear fraction, for example by binding an inhibitor, which would result in these mitochondria being more active, unit for unit, than those in the mitochondrial fraction. This suggestion was, however, not borne out by the evidence. First, the possibility that the nuclei act by binding an inhibitory metal ion appeared unlikely in view of the observation that with both the nuclear and mitochondrial fractions the addition of EDTA to the incubation mixture caused an inhibition of nicotinuric acid synthesis. Secondly, it would be expected that any protective action of the nuclei

would be fully effective in a homogenate, but that when the nuclei were separated from the other components of the homogenate by fractionation, the sum of the activities of the individual fractions would be less than that of the whole homogenate. In fact, 90–110% of the activity of the whole homogenate was always recovered in the fractions. Furthermore, it would also be expected that the addition of a sample of the nuclear fraction to the mitochondrial fraction would lead to a greater synthesis than that calculated from their separate activities. No such effect was ever observed. These two last-named facts particularly make the possibility of the nuclei having a protective action on the mitochondria appear most unlikely.

The simplest explanation of all these observations seems to be that both the nuclei and the mitochondria possess the ability to synthesize nicotinuric acid, but a definite answer to this question must await the isolation of uncontaminated rat-kidney nuclei.

The variable distribution of the synthetic activity and of total N between the nuclear and mitochondrial fractions was probably due to the sedimentation of variable numbers of mitochondria with the nuclear fraction. Hers, Berthet, Berthet & de Duve (1951) have noted that kidney homogenates are particularly liable to clumping of the particles, which causes the mitochondria to sediment with the nuclear fraction, on account of their tendency to form acid rapidly, or, if buffer is added to counteract the lowering of pH, because of the increased ionic strength of the homogenizing medium. In these experiments, the homogenizing medium contained a small amount of buffer (5 mM-potassium EDTA) and so clumping would probably occur for both reasons. The degree of clumping would presumably depend upon the precise treatment of the homogenate in question, particularly upon the time of standing between homogenization and centrifuging, and might vary appreciably from one experiment to another, leading to differences in the numbers of mitochondria sedimented in the nuclear fraction.

Nicotinuric acid synthesis in mitochondrial suspensions. The close relationship between nicotinuric acid synthesis and oxidation is shown by the constancy of the oxygen uptake per μ mole of nicotinuric acid formed, in spite of variations in the period of incubation and the fumarate concentration, and by the absence of any synthesis under anaerobic conditions. The inhibition by 2:4-dinitrophenol shows that, with mitochondrial suspensions, oxidative phosphorylation is required for the synthesis. In these suspensions, oxidative phosphorylation cannot be adequately replaced by added ATP, presumably owing to the rapid hydrolysis of the latter.

A possible explanation of the non-competitive

inhibition of nicotinuric acid formation by benzoic acid has been suggested by the observation of Schachter & Taggart (1954) that the condensation of various acyl-coenzyme A compounds with glycine to form the corresponding acyl-glycine compounds is inhibited non-competitively by hippuric acid. If an enzyme with similar properties participates in nicotinuric acid synthesis, it is possible that benzoic acid was first converted into hippuric acid, a conversion which these mitochondrial suspensions were able to perform, and that hippuric acid was the true inhibitor of nicotinuric acid synthesis.

The present investigation of nicotinuric acid synthesis was undertaken partly in the hope that an activated form of nicotinic acid might be found which reacted not only with glycine to form nicotinuric acid but also with ammonia to form nicotinamide. The absence of any effect of ammonia upon nicotinuric acid synthesis and the failure to find any nicotinamide synthesis when glycine was replaced by ammonia show that this is not the case in rat-kidney mitochondria.

The mechanism of nicotinuric acid synthesis is discussed in the next paper (Jones, 1959a).

SUMMARY

1. Slices and homogenates of rat kidney and liver have been shown to synthesize nicotinuric acid from nicotinic acid and glycine. Nicotinuric acid was identified chromatographically, by its spectrum and by hydrolysis to nicotinic acid and glycine. The synthesis in homogenates was stimulated by fumarate and adenosine triphosphate.

2. The synthetic activity of rat-kidney homogenate was located entirely in the nuclear and mitochondrial fractions. None was found in the microsome and soluble fractions even when an energy-supplying system was added. About half the activity of the nuclear fraction appeared to be due to the nuclei, the remainder being caused by contaminating mitochondria.

3. Nicotinuric acid synthesis by rat-kidney mitochondria was completely dependent upon added nicotinic acid, glycine, fumarate and adenosine triphosphate. The synthesis was inhibited by 2:4-dinitrophenol, benzoic acid and potassium fluoride. The properties of the synthetic system in mitochondria are described.

4. Nicotinamide was not synthesized by the enzyme system which synthesized nicotinuric acid.

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