5. Experiments with coupled oxidation showed that hydrogen peroxide is formed when histaminase acts on histamine.

6. The antihistamine drugs Benadryl, Antergan, Neo-antergan and the preparation R.P. 3015 do not inhibit histaminase preparations. Partial inhibition was obtained with Antistin and R.P. 3277.

I wish to thank Prof. J. H. Gaddum, F.R.S., Prof. D. Keilin, F.R.S., and Dr H. Laser for their valuable help and kind interest in this work. My thanks are also due to Dr Malcolm Dixon, F.R.S., for a preparation of split D-amino-acid oxidase and to Dr H. Laser for a sample of D-amino-acid oxidase as well as for a purified preparation of catalase. I am grateful to Miss Mairi A. Mackay for technical assistance, and very much indebted to the Manager of the Fleshing Department, St Cuthbert's Co-operative Society, Edinburgh, for the generous supply of hog's kidneys. The tenure of a whole-time grant from the Medical Research Council is gratefully acknowledged.

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Nicotinamide Metabolism in Mammals

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(*Received* 2 *April* 1948)

A number of compounds have been described as metabolites of nicotinic acid or nicotinamide in mammals. These are nicotinic acid (Komori & Sendju, 1926), nicotinamide, nicotinuric acid (Ackermann, 1913), trigonelline (Ackermann, 1913), nicotinamide methochloride (Najjar & Wood, 1940; Huff & Perlzweig, 1943*a*; Ellinger & Coulson, 1943; Coulson & Ellinger, 1943) and, recently, 1-methyl-6-pyridone-3-carbonamide (Knox & Grossmann, 1946, 1947). Dinicotinyl ornithine is a metabolite in the chick (Dann & Huff, 1947) and nicotinamide methochloride is formed by insects and bacteria (Ellinger, Fraenkel & Abdel Kader, 1947). In the earlier papers the metabolites were isolated from urine and identified by chemical analysis. When the physiological importance of nicotinamide was established, large series of analyses were carried out by methods allowing routine assay. Most of them were based on the König (1904) principle. The majority of the investigators were satisfied to estimate 'total nicotinic acid' which generally includes nicotinic acid, nicotinamide, nicotinuric acid and trigonelline. When nicotinamide methochloride was found to be a metabolite, this substance was assayed by special methods (Huff & Perlzweig, 1943*b*; Coulson, Ellinger & Holden, 1944; Najjar, 1944). It soon became evident that most of the metabolite previously described as trigonelline was in fact nicotinamide methochloride (Sarett, 1943; Huff & Perlzweig, 1943*a*). Although single observations have been carried out on man, dog, ferret, rat, mouse, calf, horse, rabbit and guinea pig, apart from the investigations of Johnson, Hamilton & Mitchell (1945) on man, and of Johnson, Wiese, Mitchell & Nevens (1947) on the calf, no distinction was made between

* Some of the results presented in this paper were communicated to the Biochemical Society on 24 January 1948 (Ellinger & Abdel Kader, 1948).

urinary nicotinamide and nicotinic acid. In this paper the elimination of all possible nicotinamide metabolites, with the exception of 1-methyl-6-pyridone-3-carbonamide, was determined under normal conditions, and after the ingestion of nicotinamide, nicotinic acid, nicotindiethylamide (nikethamide) and a few other compounds in man, dog, cat, rat, rabbit and guinea pig.

EXPERIMENTAL

Plan of experiments

Man. From a normal healthy male (subject no. 1, Table 2*a*) 24 hr. samples of urine were tested for nicotinic acid, nicotinamide, nicotinuric acid, trigonelline and nicotinamide methochloride. After a preliminary period of 3 weeks, single doses of nicotinamide, nicotinic acid or nikethamide were given orally or subcutaneously at intervals of 3–4 days. In addition 24 hr.-urine samples were collected from seven other healthy persons, and 1 diabetic balanced by insulin and diet; there was no extradietary ingestion of nicotinamide derivatives in the latter experiments.

Animals. These were kept in metabolism cages in which 24 hr.-urine samples were collected separately from the faeces. Particulars of the weights of the animals, their diets and particularly their intake of nicotinic acid and nicotinamide are given in Table 1. Since all metabolites were found to be stable in urine at low temperature, no preservative was added and the samples were kept at 5°. Two rats of the same strain were kept in the same cage on account of their small urine volume. All extradietary administrations were given intraperitoneally. The animals were given single test doses of nicotinamide, nicotinic acid, nikethamide or occasionally nicotinuric acid or nicotinamide methochloride at intervals of 3–4 days. Rats were on some occasions given methionine with the nicotinamide derivatives. In addition the dogs were given 9 g. of nicotinic acid, dissolved as sodium salt in the milk; 1 g. was given on the first and 2 g. on each of the 4 following days. The urine was collected during these 5 and the following 2 days and examined for metabolites.

Estimation of metabolites

In view of the large number of quantitative estimations of the various metabolites to be performed, routine methods had to be found and their specificity examined. The designation of the metabolites should correctly be given as 'compound reacting like nicotinic acid' rather than as 'nicotinic acid', etc. As techniques based on the König (1904) principle have been criticized by various authors, the methods of Bandier & Hald (1939), Bandier (1939), Melnick, Robinson & Field (1940), Perlzweig, Levy & Sarett (1940), Sarett, Huff & Perlzweig (1942), Sarett (1942), Huff & Perlzweig (1942), Wang & Kodicek (1943) and Carter & O'Brien (1945) were tested. The various methods used for removing interfering pigments were also studied, the colour developed with various aromatic amines was tested for stability and intensity, and the effect of the different types of hydrolysis and the colour intensities obtained with the different metabolites before and after hydrolysis were examined. As the result of our findings the following procedure was adopted.

Without extradietary administrations of nicotinamide derivatives the urine contained the various metabolites in low concentration; a portion of the 24 hr. urine was, therefore, concentrated *in vacuo* at 80° to 50–60% of its volume. The concentrate was saturated with NaCl and acetone (9 vol.) was added. The precipitated pigments were removed by centrifugation and acetone by evaporation *in vacuo* at 40°. The aqueous solution was made up with water to a definite volume and portions of it were used for the

Table 1. *Weights and diets of the animals used*

Species	No. of animals	Weight (kg.)	Diet (food given/day)	Dietary intake of: Nicotinic acid (mg./day)	Dietary intake of: Nicotinamide (mg./day)
Dog	2	1·8–3·5	Meat, bread and vegetables, 300 g.; milk, fresh, 200 ml.	6·1	10·0
Cat	3	0·76–0·88	Powdered milk, 15 g.; fish, 70 g.; bread, 30 g.	4·5	1·3
Rat (adult):					
Albino (stock of P.E.)	21	0·20–0·26	Grain mixture, 4·0 g.; extracted liver residue 3·5 g.; wheat germ, 1·0 g.; dried yeast 0·3 g.; salt mixture (McCallum 185), 0·5 g.; maize oil, 0·6 g.; cod-liver oil, 0·1 g.; national bread, 13·0 g.; milk powder, 1·5 g.	0·37	0·21
Hooded (Lister stock)	21	0·20–0·34			
Rat (young):					
Albino	5	Average 0·069			
Hooded	5	Average 0·039			
Rabbit	8	2·90–3·90	Either (*a*) cabbage, 200 g.; swedes, 100 g.; or (*b*) oats, 100 g.	1·60 0·34	1·60 0·21
Guinea pig	6	0·40–0·47	Pellets (containing grass meal 30%, bran 15%, ground-nut cake 15%, linseed cake 10%, barley meal 20%, meat and bone meal 8%, $CaCO_3$ 1%, NaCl 1%), 50 g.; cabbage, 50 g.	0·32	0·88

All animals obtained water *ad lib.*

different estimations, without hydrolysis and after hydrolysis with N-HCl, N-NaOH and 10N-NaOH with addition of urea.

To develop the colour in each solution the pH was adjusted to 5·5 with KH_2PO_4 and NaOH using bromocresol purple as external indicator: 4 and 8 ml., respectively, of these solutions were used, the former being made up to 8 ml. with water; the solutions were heated to 75–80° and 0·8 ml. of freshly prepared CNBr was then added in a test tube graduated at 10 ml.; the solution was heated to 75–80° for another 5 min. After cooling to room temperature the volume was made up to 10 ml. with water and 0·4 g. *p*-methylaminophenol sulphate (metol) was added and allowed to dissolve completely. Blanks were developed simultaneously by adding to similar volumes of urine 0·15 ml. N-H_2SO_4 instead of the metol. Standards were made from solutions containing 2 and 4 μg./ml., respectively of nicotinic acid. After 1 hr. in the dark the concentration was measured by comparing the extinction with that of the standard in a Bausch and Lomb 'Colorimeter' used as a photometer with a Wratten filter no. 39.

On an equimolecular basis, nicotinamide (*Nam*) gave 50% of the extinction given by nicotinic acid (*Nac*) before and 100% after hydrolysis with N-HCl or N-NaOH. Nicotinuric acid (*Nur*) gave values of 30% before hydrolysis and no increase after hydrolysis with N-HCl, but 100% after hydrolysis with N-NaOH. Trigonelline (*Trig*) and nicotinamide methochloride (*NM*) gave no coloured compounds before or after hydrolysis with N-acid, N-NaOH or 10N-NaOH. After hydrolysis with 10N-NaOH, in the presence of urea, a colour equivalent to 63% of that of nicotinic acid was obtained.

Calculation of the concentrations of the different metabolites was carried out as follows: If the value obtained before hydrolysis is called *A*, that after hydrolysis with acid *B*, that after hydrolysis with N-NaOH *C*, and that after hydrolysis with 10N-NaOH in the presence of urea *D*, then these equations follow from the figures given above:

$$A = 1\ Nac + 0{\cdot}5\ Nam + 0{\cdot}3\ Nur,$$

$$B = 1\ Nac + 1\ Nam + 0{\cdot}3\ Nur,$$

$$C = 1\ Nac + 1\ Nam + 1\ Nur,$$

$$D = 1\ Nac + 1\ Nam + 1\ Nur + 0{\cdot}63\ (Trig + NM),$$

$$Nam = 2\ (B - A),$$

$$Nur = \frac{C - B}{0{\cdot}7},$$

$$Nac = C - 2\ (B - A) - \frac{C - B}{0{\cdot}7},$$

$$Trig + NM = \frac{D - C}{0{\cdot}63},$$

NM is estimated separately by a method described later, and

$$Trig = \frac{D - C}{0{\cdot}63} - NM \text{ (in terms of } Nac)$$

Since all values are obtained in terms of nicotinic acid, the values have to be multiplied by the mol.wt. of the metabolite and divided by that of nicotinic acid. The values obtained in that way in terms of μg./ml. are multiplied by the urine volume to obtain the value of the 24-hr. elimination. The maximum error of the method, as estimated from recovery tests of each of the metabolites added to urine, is in the region of ±10%, when all metabolites are present; when only nicotinic acid and nicotinamide have to be estimated, the maximum error found was less than ±5%. The smallest concentration which can be estimated by this method is 0·8 μg./ml.

Nicotinamide methochloride was estimated by a modification of the acetone method of Huff & Perlzweig (1947), since non-fluorescent KCl was unobtainable and the method of Coulson *et al.* (1944) could thus not be used. The procedure differed from that described by Huff & Perlzweig in one respect: the fluorescence was compared visually with that of standards prepared from synthetic nicotinamide methochloride in a similar manner. Recoveries of 95–105% were obtained with added nicotinamide methochloride. Filter paper was chosen which neither adsorbed nicotinamide methochloride nor released fluorescent pigments, the charcoal used adsorbed interfering pigments; but not nicotinamide methochloride. Acetone was freed from fluorescent pigments by distillation over $KMnO_4$. The maximum error was about ±5%. The range of the method was found to be 0·01–1·0 μg./ml. Urines containing higher concentrations were diluted before the estimation. All analyses were carried out in duplicate. Negative or dubious results obtained with the König reaction were checked microbiologically with the method of Barton-Wright (1944) using *Lactobacillus arabinosus*. The presence of the coenzymes which give a positive reaction with the acetone method was checked microbiologically by the method of Pittman & Fraser (1940) using *Haemophilus parainfluenzae* (National Collection of Type Cultures, no. 4101).

RESULTS

Man. In man (Table 2*a*) nicotinamide methochloride is the main end product of nicotinamide metabolism forming on the average 71·2%, whilst nicotinamide represents 24·2% and nicotinic acid 4·6% of the output. These values may be compared with those of Johnson *et al.* (1945), who found, however, absolute values for nicotinamide methochloride (average 18·7 mg.) far higher than either those found in the present work or any values published by previous investigators (Ellinger & Coulson, 1944; Ellinger, Benesch & Hardwick, 1945; Hochberg, Melnick & Oser, 1945; Ellinger & Hardwick, 1947). Ingested nicotinic acid (Table 2*b*) is amidated and nikethamide is de-ethylated. In all cases nicotinamide methochloride forms the main metabolite. After subcutaneous administration the elimination is greater than after oral administration, in agreement with the findings of Ellinger & Hardwick (1947). The total recovery of the ingested compounds is much lower than in any other mammal examined. This might be due to the fact that part of the methylated compound is immediately oxidized to the corresponding pyridone (Rosen, Perlzweig & Handler, 1948) and not determined by the methods of assay used. It might also be due to a relatively low saturation state of the subject examined, indicated by the considerable rise in output of the three metabolites in the course of administration of nicotinamide derivatives. Similar observations have

Table 2*a*. *Daily elimination of nicotinamide derivatives by man*

Subject				Metabolites eliminated (mg./24 hr.)			
No.	Sex	Age (yr.)	Wt. (kg.)	Nicotinamide	Nicotinic acid	Nicotinamide methochloride*	Remarks
1†	♂	27	80	2·000	0·810	8·630	No smoking, no coffee
R1‡	♂	27	80	1·342	0·360	6·681	
R1§	♂	27	80	3·622	1·233	11·420	
2	♀	61	50	3·320	0·680	6·160	No smoking, coffee
3	♀	19	70	1·400	0·480	9·360	No smoking, no coffee
4	♀	19	65	2·180	0·370	9·440	
5	♂	47	67	1·980	0·215	12·040	
6	♂	22	70	1·590	0·264	12·480	
7	♀	25	58	1·175	0·190	10·840	
8	♂	53	64	2·540	0·145	6·050	
9	♂	60	68	3·000	0·440	5·040	Balanced diabetic; no smoking, no coffee
Average				**2·132**	**0·399**	**8·894**	

Neither trigonelline nor nicotinuric acid were eliminated.
* Absolute, not in terms of nicotinic acid.
† Average of daily output estimated on 22 control days spread over the whole period.
‡ Average of 3 days before the first injection.
§ Average of 3 days, 14 days after last injection.

Table 2*b*. *Response to extradietary nicotinamide derivatives by man* (*subject no.* 1)

Compound administered	Dose (mg.)	Route	Metabolites eliminated (% of ingested)* Nicotinamide	Nicotinic acid	Nicotinamide methochloride	Total
Nicotinamide	100	Oral	0	0	18·9 [3]	18·9
		Subcutaneous	1·8	0	24·9 [3]	26·7
Nicotinic acid	100	Oral	2·3	0·8	16·5 [2]	19·6
		Subcutaneous	2·1	0·9	21·1 [2]	24·1
Nicotindiethylamide	100	Oral	6·2	0	5·8 [2]	12·0
		Subcutaneous	6·3	0	15·5 [2]	21·8

[ ] indicates time in days over which elimination occurred.
* *Note.* Dose of administered compound is in terms of nicotinic acid. The response (percentage of ingested), also in terms of nicotinic acid, is calculated as

$$\frac{\text{(output until predosing level is reached)} - \text{(average predosing output} \times \text{days of increased output)}}{\text{dose administered}} \times 100.$$

Neither trigonelline nor nicotinuric acid was eliminated.

been made by Ellinger & Hardwick (1947). The smaller elimination of metabolites after oral than after parenteral administration indicates destruction by the intestinal flora which has been observed by Koser & Baird (1944), Benesch (1945), Ellinger & Emmanuelowa (1946), Ellinger (1947) and Ellinger, Abdel Kader & Emmanuelowa (1947).

Dog. In this species (Table 3) nicotinamide and nicotinamide methochloride are the only urinary metabolites; the relative proportion of the two compounds eliminated differed considerably in the two animals examined, but was fairly constant from day to day in the same animal. The total elimination was high compared with that of other species. After administration of nicotinamide and nikethamide (Table 4), the only metabolites found were nicotinamide and nicotinamide methochloride, while after nicotinic acid injection some unchanged nicotinic acid appeared in the urine. This, and particularly the absence of nicotinuric acid, contradicts the findings of Ackermann (1913), Komori & Sendju (1926) and Sarett (1942). The last-named found a recovery of 75 % in the form of 'trigonelline' and 25 % in that of nicotinuric acid. When Ackermann's (1913) experiment was repeated, neither dog eliminated either trigonelline or nicotinuric acid. Dogs gave a much higher response to injected compounds than man. The relatively greater elimination of the methylated product with smaller than with larger doses suggests that the methylating mechanism is the limiting factor. The relative proportion of the methylated to the unmethylated metabolites is considerably greater after administration of nicotinamide and nikethamide than after that of nicotinic acid. The relative total response is greater after small than after big doses.

Table 3. *Average daily urinary elimination of nicotinamide derivatives by various mammalian species*

Species	Metabolites eliminated (Averages, limiting values in parentheses) (mg./day) Nicotinamide	Nicotinic acid	Nicotinamide methochloride
Dog	4·590 (3·480–5·720)	0	5·370 (1·000–10·250)
Cat	0	0	0·290 (0·070–0·400)
Rat (albino):			
Adult	0·080 (0·054–0·130)	0	0·100 (0·030–0·260)
Young	0·035	0	0·292
Rat (hooded):			
Adult	0·140 (0·100–0·170)	0	0·960 (0·450–1·700)
Young	0·0214	0	0·150
Rabbit (On cabbage)	0	0·880 (0·500–1·350)	0*
(On oats)	0	1·190 (1·010–1·540)	0*
Guinea pig	0	0	0*

Neither trigonelline nor nicotinuric acid were eliminated.
* Indicates a small positive reaction with the acetone, but none with the König (1904) method.

Table 4. *Average response to extradietary nicotinamide derivatives by dogs*

Compound administered	Dose* (mg.)	Metabolites eliminated (% of ingested)* Nicotinamide	Nicotinic acid	Nicotinamide methochloride	Total
Nicotinamide	200	31·7 [2]	0	50·1 [2]	81·8
	500	43·0 [2]	0	31·6 [3]	74·6
Nicotinic acid	200	48·8	13·1	31·6 [2]	93·5
	500	49·0 [2]	16·7	21·5 [2]	87·2
	9000†	10·0	49·4	5·6	65·0
Nicotindiethylamide	100	43·5	0	52·1 [2]	95·5
Nicotinuric acid	67·7	31·4	41·8	18·1 [2]	91·2

[ ] indicates time in days over which elimination occurred, if more than one.
Neither trigonelline nor nicotinuric acid were eliminated.
* See note to Table 2*b*.
† Orally within 5 days.

Table 5. *Average response to extradietary nicotinamide derivatives by cats*

Compound administered	Dose* (mg.)	Metabolites eliminated (% of ingested)* Nicotinamide	Nicotinic acid	Nicotinamide methochloride	Total
Nicotinamide	40	34·5	0	28·3 [3]	63·3
	80	37·6 [2]	0	20·4 [2]	58·0
	200	47·0 [2]	0	34·1 [4]	81·1
Nicotinic acid	40	12·8	10·5	24·1 [2]	47·4
	100	24·1 [2]	29·0	18·7 [3]	71·8
Nicotindiethylamide	40	19·0	0	16·1 [2]	35·0

[ ] indicates time in days over which elimination occurred, if more than one.
Neither trigonelline nore nicotinuric acid were eliminated.
* See note to Table 2*b*.

Cats normally eliminate small amounts of nicotinamide methochloride only (Table 3). Nicotinamide and nicotinamide methochloride were eliminated after administration of nicotinamide and nikethamide and in addition some nicotinic acid when this compound was given (Table 5). As in dogs, the relative proportion of methylated to unmethylated metabolites decreases with rising doses. The total response was lower than in dogs.

similar to those observed by Huff & Perlzweig (1942), though the experiments were not directly comparable. Nicotinuric acid was found to be split as observed by Huff & Perlzweig (1942), but not so completely as by dogs. Nicotinamide methochloride was eliminated unchanged but only to the extent of 63 %, confirming the findings of Ellinger (1947), Ellinger & Coulson (1944) and Perlzweig & Huff (1945) in man. The effect of methionine administered,

Table 6. *Response to extradietary nicotinamide derivatives by two strains of rats*

Compound administered	Dose* (mg.)	Metabolites eliminated (% of ingested)*				
		Nicotinamide	Nicotinic acid	Nicotinamide methochloride	Nicotinuric acid	Total
		Albino				
Nicotinamide	20	23·7	0	9·3	0	33·0
	20 (+30 DL-methionine)	19·2	0	12·5	0	31·7
	50	31·6	0	15·0 [2]	0	46·6
Nicotinic acid	20	23·8	26·9	1·8	0	52·5
	20 (+30 DL-methionine)	20·4	49·5	1·0 [2]	0	70·9
	50	31·9 [2]	35·4	1·0 [2]	0	68·3
Nicotindiethylamide	20	36·7	0	9·8 [2]	0	46·5
	20 (+30 DL-methionine)	48·0	0	4·9 [2]	0	52·9
	50	38·8 [2]	0	3·4 [2]	0	42·2
Nicotinamide methochloride	17·5	0	0	71·0	0	71·0
Nicotinuric acid	33·9	13·0	2·1	3·2 [2]	72·8	91·0
		Hooded				
Nicotinamide	20	15·0	0	29·7	0	44·7
	20 (+30 DL-methionine)	16·5	0	59·7	0	76·2
	50	19·2	0	61·6 [2]	0	80·8
Nicotinic acid	20	24·1	27·7	16·0	0	67·8
	20 (+30 DL-methionine)	26·0	37·8	8·9 [2]	0	72·7
	50	25·4	19·4	5·7	0	50·5
Nicotindiethylamide	20	36·0	0	33·4 [2]	0	69·4
	50	34·4 [2]	0	13·7 [2]	0	48·1
Nicotinamide methochloride	17·5	0	0	63·2	0	63·2
Nicotinuric acid	33·9	11·4	2·0	3·1 [2]	62·2	79·6

[ ] indicates time in days over which elimination occurred, if more than one.
No trigonelline was eliminated.
* See note to Table 2*b*.

Rat. The two strains of rat examined differed mainly in the efficiency of the methylating mechanism. Rats, like dogs, eliminated only nicotinamide and nicotinamide methochloride (Table 3). The relative proportion of the latter to the former was 1·25 in the albino and 6·85 in the hooded strain. Relative to weight the output is high compared with that of cats; for the albino it was similar to that found by Huff & Perlzweig (1942) and for the hooded strain it was much higher. Both strains eliminated injected nicotinamide and nikethamide as nicotinamide and nicotinamide methochloride; when nicotinic acid was given it was eliminated partly unchanged (Table 6). The total response was greater in the hooded than in the albino strain. The values found for response to nicotinamide and nicotinic acid were together with the nicotinamide derivatives on the formation of nicotinamide methochloride, was irregular. The elimination of nicotinic acid after administration of nicotinic acid was always increased by simultaneous administration of methionine. When large doses (100 mg.) of nicotinamide were administered the nicotinamide methochloride output was increased by rising doses of methionine; this indicates the exhaustion of methyl donors by methylation as shown for man by Ellinger & Hardwick (1947). In all cases the elimination of nicotinamide methochloride was far greater in the hooded than in the albino strains. On no occasion was nicotinamide deaminated or nicotinic acid formed from nikethamide. DL-Methionine proved to be toxic to rats. LD_{50} found was about 700 mg./kg. The hooded rats

died earlier and were more strongly affected by sublethal doses than the albino. Macroscopic post-mortem examination showed the kidneys congested with blood, confirming observations by Simmonds, Cohn & du Vigneaud (1947).

Young rats (Table 3), like adults, eliminated nicotinamide and nicotinamide methochloride but no nicotinic acid. This conversion of nicotinic acid into nicotinamide makes the observation of Handler & Dann (1942) that the growth of young rats is inhibited by nicotinamide but not by nicotinic acid even less explicable.

tinamide methochloride. It is concluded, therefore, that a substance is present in the urine giving the acetone but not the König reaction. A test for co-enzymes which fulfils this condition gave negative results. The possibility has, therefore, to be considered that there is present an unknown factor which might be a metabolite of a nicotinamide derivative. It will be extremely difficult to isolate or identify it since it is present in minute quantities and no way has been found to increase its elimination markedly. It is possible that the 'F_2' which, according to Handler (1944), is eliminated by young

Table 7. *Average response to extradietary nicotinamide derivatives by rabbits*

Compound administered	Dose* (mg.)	Metabolites eliminated (% of ingested)* Nicotinamide	Nicotinic acid	Trigonelline	Total
		Diet of cabbage			
Nicotinamide	100	2·9	3·3 [2]	5·1 [2]	11·3
	200	6·9	14·5 [3]	9·3 [3]	30·6
	400	7·5	18·6 [3]	9·4 [3]	35·5
Nicotinic acid	100	0	35·5 [2]	48·0 [2]	83·5
	200	0	63·4 [2]	33·9 [2]	97·3
Nicotindiethylamide	50	1·6	5·7	3·3	10·6†
	100	9·2	15·1	6·4	30·7†
	200	4·9	10·0 [2]	7·0 [2]	21·8†
	2 hourly doses of 100				
	600	7·5	21·7 [2]	2·7 [2]	30·9†
	6 hourly doses of 100				
		Diet of oats			
Nicotinamide	100	2·2	3·5 [2]	3·3	9·5‡
	200	7·5 [2]	13·0 [3]	6·5 [3]	27·0‡
Nicotinic acid	100	0	32·9 [2]	54·8 [2]	87·7
	200	0	52·4 [2]	29·8 [2]	82·2

[ ] indicates time in days over which elimination occurred, if more than one.
Neither nicotinamide methochloride nor nicotinuric acid were eliminated.
* See note to Table 2*b*.
† Indicates that after injection of nicotindiethylamide an unknown substance of high fluorescent efficiency was eliminated interfering with the application of the acetone method.
‡ Indicates that after injection of nicotinamide into rabbits fed on oats and possibly after that of nicotindiethylamide a substance was eliminated to a slightly increased extent giving a positive reaction with the acetone but not with the König (1904) method.

Rabbits (Table 3) independently of the diet, cabbage or oats, normally only eliminated nicotinic acid. In addition a substance was regularly eliminated, and to a slightly increased extent after injection of nicotinamide by rabbits fed on oats, which gave a positive reaction by the acetone method for nicotinamide methochloride. The amount was very small, just within the limits of the method, but the substance appeared to be always present. The König (1904) assay failed to show the presence of trigonelline or nicotinamide methochloride in the same urines. This might be due to insufficient sensitivity of the König reaction; however, this is improbable. The concentrations found with the acetone method were large enough to give positive results with the König method if the substance were nico-

rabbits and guinea pigs, but not increased after nicotinamide administration, and the nicotinamide methochloride found by Johnson *et al.* (1947) in the urine of young calves, which also is not increased by administration of nicotinamide, is in fact this unknown substance.

Extradietary nicotinamide and nikethamide were found to be deaminated to nicotinic acid which is methylated to trigonelline (Table 7). This observation contradicts the results of Komori & Sendju (1926) who, after feeding nicotinic acid to rabbits, isolated nicotinic acid and nicotinuric acid from the urine, but no trigonelline. The observation is also at variance with the assumption of Huff & Perlzweig (1943*a*) that rabbits are unable to carry out this methylation. The elimination of known metabolites

is low after ingestion of nicotinamide and nikethamide, but almost complete after the administration of nicotinic acid. The deamination might lead to a thorough disintegration of the pyridine ring. The type of diet seems to have very little influence on the qualitative and quantitative elimination of metabolites except for the increase in 'pseudo-nicotinamide methochloride' after administration of nicotinamide, which occurs only in rabbits fed on oats. The increased elimination of trigonelline following nicotinic acid injection is relatively higher after smaller than after bigger doses and indicates that the methylating mechanism is exhaustible.

The urine passed by rabbits after administration of nikethamide exhibits without any treatment a strong purplish blue fluorescence in the long ultraviolet which increases in intensity with rising nikethamide administration. The urine ceases to be fluorescent about 16 hr. after the injection. Some preliminary data of the isolation and properties of the substance responsible for this fluorescence have been obtained. The fluorescent substance is not extractable from neutral, acid or alkaline urine by ethyl acetate, benzene, *iso*butanol, light petroleum, chloroform or ether and is stable to boiling in neutral, acid or alkaline solution. It is not adsorbed on decalso or charcoal, but is adsorbed on Al_2O_3 (Brockmann) from dry ethanol solution. For its isolation the urine was acidified with HCl, filtered through a decalso column, and shaken with charcoal to remove as many of the other urine constituents as possible. The colourless filtrate was evaporated to dryness, extracted with hot dry ethanol and filtered through an Al_2O_3 column, the fluorescent material being completely adsorbed. The column was thoroughly washed with dry ethanol. Two fluorescent zones were seen on the upper part of the column, a narrow one of purplish blue fluorescence on the extreme top and a wider one of bright blue fluorescence clearly separated from it farther down. The top layer was cut off and both layers were eluted with 50% (v/v) ethanol for the top layer and 85% (v/v) ethanol for the other one. Both eluates were evaporated to a small volume *in vacuo* and treated with non-fluorescent acetone. In both cases part of the fluorescent material was precipitated and another part remained dissolved in acetone. The precipitates from the top layer (*A*) and from the lower layer (*B*) were filtered off, washed with dry ethanol and twice recrystallized from hot ethanol. The main fluorescent material (*C*) dissolved in acetone was brought to dryness by evaporation *in vacuo*, dried *in vacuo* over P_2O_5 and recrystallized three times from hot dry ethanol. The three substances had the following properties.

Substance *A*: m.p. 166·5–168·5°; ultraviolet absorption in water; no band between 260–380 mμ., general absorption from 280 to 220 mμ. with shoulder at 260 mμ., $E^{1\%}_{1\,cm.} = 2·5$.

Substance *B*: m.p. 325° with decomposition and sublimation; ultraviolet absorption in water: similar to *A* with much smaller general absorption.

Substance *C*: very hygroscopic, colourless, turns brown when exposed to air. M.p. 96·5°; if heated to 108·5°, it does not resolidify on cooling; ultraviolet absorption in water: distinct band at 336 mμ., $E^{1\%}_{1\,cm.} = 4·6$, with general absorption from 280 to 220 mμ., shoulder at 265 mμ., $E^{1\%}_{1\,cm.} = 3·4$ and indications of a peak at 220 mμ., $E^{1\%}_{1\,cm.} = 17·0$. In a cataphoretic experiment it formed a uniform band and moved slowly to the anode. It has not been possible so far to collect sufficient quantities of the fluorescent pigments for analysis and estimation of the mol.wt.

Niketamide is toxic to rabbits and larger doses have to be divided; the total amount which can be administered is, therefore, small. Moreover, the mechanism involved in the production of the fluorescent pigments seemed to be quickly exhausted; the animals used have to rest for a considerable time before they can be used with advantage for a second collection. None of the substances seems to be identical with 1-methyl-2 (or 6)-pyridone-3-carbondiethylamides; these differ from the pigments by their fluorescence and ultraviolet absorption. Perhaps the pigments are derivatives of a dihydropyridine which, according to Warburg & Christian (1936), has a band at 340 mμ.

Guinea pigs differed from all other species examined by not eliminating any metabolite except 'pseudo-nicotinamide methochloride' (Table 3) and by not methylating any extradietary nicotinamide derivative (Table 8). Slight increase of the 'pseudo-nicotinamide methochloride' occurred after nicotinamide and nikethamide, but not after nicotinic acid administration. Nicotinamide was completely de-

Table 8. *Average response to extradietary nicotinamide derivatives by guinea pigs*

Compound administered	Dose* (mg.)	Metabolites eliminated (% of ingested)* Nicotinamide	Nicotinic acid	Nicotinamide methochloride	Total
Nicotinamide	100	0	32·4	0*	32·4
	200	0	24·2 [2]	0* [2]	24·2
	400	0	38·9 [2]	0* [2]	38·9
Nicotinic acid	100	0	60·5 [2]	0	60·5
	200	0	82·2 [2]	0	82·2
	400	0	66·7 [2]	0	66·7
Nicotindiethylamide	25	12·9	12·7	0†	25·6
	50	10·3	20·4 [2]	0†	30·7

[ ] indicates time in days over which elimination occurred, if more than one.
Neither trigonelline nor nicotinuric acid were eliminated.
* See note to Table 2*b*.
† Indicates that after administration of nicotinamide or nicotindiethylamide a substance was eliminated to a slightly increased extent giving a positive reaction with the acetone, but not with the König (1904) method.

aminated to nicotinic acid. Of the ingested nicotinamide or nikethamide 25–38 % was eliminated and of the nicotinic acid about 60–80 %.

DISCUSSION

The most surprising result is that the two compounds described by other authors (Ackermann, 1913; Komori & Sendju, 1926; Linneweh & Reinwein, 1932*a*, *b*; Melnick *et al.* 1940 and Perlzweig *et al.* 1940) as the metabolites of nicotinic acid or nicotinamide in dogs, men or rats, viz. trigonelline and nicotinuric acid, are not eliminated by any of these species normally or after administration of the tested compounds. It is easy to understand that authors using the König (1904) method for assay have described the occurrence of trigonelline in the urine before nicotinamide methochloride had been established as a metabolite. The two compounds cannot be distinguished by the König method. However, it has been claimed that these two substances can be isolated from urine and analyses for the compounds or derivatives have been given. Ackermann (1913) e.g. analyzed the gold salt of a substance obtained from urine of dogs after feeding nicotinic acid for gold, carbon and hydrogen, but not for nitrogen, and claimed to have identified it as a salt of trigonelline. The theoretical values of gold, carbon and hydrogen are almost identical for the gold salts of trigonelline and nicotinamide methochloride, while the nitrogen value of the latter is about twice that of the former. The same omission explains the results of Komori & Sendju (1926) who only made an analysis for gold. The results of Linneweh & Reinwein (1932*a*, *b*), who have produced analytical values for carbon, hydrogen and nitrogen in agreement with trigonelline itself for a substance isolated from human urine, are more difficult to explain. This probably was pure nicotinamide methochloride. With regard to nicotinuric acid, Ackermann's (1913) results on dogs are inexplicable since injected nicotinuric acid is completely split by the dog and eliminated as nicotinamide, nicotinic acid and nicotinamide methochloride (Table 4). It cannot, therefore, be an end product of nicotinic acid metabolism in the dog. This breakdown to nicotinamide, however, explains the anti-blacktongue (Woolley, Strong, Madden & Elvehjem, 1938) and antipellagra activities (Elvehjem & Teply, 1943) of nicotinuric acid. The findings of Melnick *et al.* (1940) that man eliminates nicotinuric acid after ingestion of doses of 500 mg. and more of nicotinic acid have not been tested. It is probable that their method of assay is not suitable for separate determination of nicotinamide and nicotinuric acid.

The contradictory results of the many investigations are mainly due to the faults of the assay methods based on the König principle. The conditions of hydrolysis are of great importance for a clean separate determination of the metabolites as well as for securing low blanks. Melnick *et al.* (1940), Perlzweig *et al.* (1940), Sarett *et al.* (1942), Sarett (1942) and Huff & Perlzweig (1942) use for the hydrolysis of nicotinamide and nicotinuric acid N-hydrochloric acid and 5N-hydrochloric acid respectively. The latter produces strongly coloured hydrolysates and, consequently, such high blank values that correct absorption readings are almost impossible. This can be avoided by using N-sodium hydroxide for the hydrolysis of nicotinuric acid. Bandier & Hald's (1939) method for the removal of interfering pigments is satisfactory, while Wang & Kodicek's (1943) method is tedious and results in a strong yellow tint interfering with the assay. The modification of the method of Bandier & Hald (1939) by Carter & O'Brien (1945) with regard to pH and amount of metol is advantageous. By systematic study of the effect of pH and amount of metol used on the reproducibility and intensity of colour, the method has been further improved. Contrary to the findings of Bandier & Hald (1939), exposure to daylight, even direct sunlight, does not interfere with the colour intensity.

Considering the metabolism of nicotinamide derivatives, the mammalian species examined so far can be classified into two groups; man, dog, cat and rat which aminate nicotinic acid to nicotinamide, whilst rabbit and guinea pig deaminate nicotinamide to nicotinic acid. Before drawing any further conclusions more species have to be examined; but it is attractive to base on this and similar fundamental functions a classification of species on the basis of their biochemical properties. It has already been mentioned that some mammalian species such as rabbit and horse do not methylate nicotinic acid or nicotinamide whilst other species do so. Whether the type of diet (carnivore, omnivore or herbivore) is responsible for these differences is doubtful. It cannot play any role in the process of methylation since herbivores like rabbit and guinea pig differ in this respect. The mechanisms of methylation and amination of these compounds have been elucidated and their sites located in the liver (Perlzweig, Bernheim & Bernheim, 1943) and kidney and brain (Ellinger, 1946, 1948), respectively. It would be interesting to see whether the methylating enzyme is absent from guinea-pig liver and where and how the deamination occurs in rabbit and guinea pig. It is possible that aminating and deaminating enzymes are present in both types of species, the one being inhibited in normal conditions in the living animal as has been shown for the sulphonamide acylating and deacylating enzymes by Krebs, Sykes & Bartley (1947). The instability of nicotinamide in rabbit and guinea pig suggests that the nicotinamide moiety of the coenzymes might be replaced by nicotinic acid in these species.

The metabolism of nikethamide particularly in man, rabbit and guinea pig shows, as already suggested by Ellinger *et al.* (1947), that this compound is broken down by de-ethylation to nicotinamide followed, in rabbit and guinea pig, by deamination of the latter compound.

SUMMARY

1. The metabolism of nicotinamide derivatives in men, dogs, cats, rats, rabbits and guinea pigs has been studied under normal conditions and after administration of nicotinamide, nicotinic acid, nikethamide and, in some instances, of nicotinamide methochloride and nicotinuric acid. The metabolites tested for have been nicotinamide, nicotinic acid, nicotinuric acid, trigonelline and nicotinamide methochloride.

2. Under normal conditions men eliminate nicotinamide, nicotinic acid and mainly nicotinamide methochloride, dogs and rats nicotinamide and nicotinamide methochloride, cats only the latter compound, rabbits nicotinic acid and guinea pigs none of these compounds. No nicotinuric acid or trigonelline was found in the urine. Rabbits and guinea pigs, however, eliminate small amounts of an unknown substance, giving a positive reaction by the acetone method.

3. Extradietary nicotinamide was eliminated by men almost exclusively as nicotinamide methochloride, by dogs, cats and rats partly as nicotinamide, partly as nicotinamide methochloride, by rabbits as nicotinamide, nicotinic acid and trigonelline and by guinea pigs as nicotinic acid; in the last-named species the unknown compound giving a positive reaction with the acetone method was slightly augmented.

4. Nicotinic acid was eliminated by men mainly as nicotinamide methochloride and to a very small extent unchanged and as nicotinamide; by dogs, cats and rats in the form of all three metabolites, by rabbits partly unchanged, partly as trigonelline and by guinea pigs unchanged.

5. Nikethamide is broken down to nicotinamide and accordingly further metabolized by all species. In rabbits after the injection of nikethamide, purplish blue fluorescent pigments occur in the urine, three of which have been isolated.

6. Men, dogs, cats and rats aminate nicotinic acid; rabbits and guinea pigs deaminate nicotinamide; all species except guinea pigs methylate the metabolites to nicotinamide methochloride or trigonelline, respectively; nicotinuric acid is broken down by dogs and rats to nicotinic acid and nicotinamide and cannot, therefore, be an end product of metabolism, while nicotinamide methochloride is eliminated unchanged by rats.

7. DL-Methionine has no constant effect on the metabolism of the various derivatives in rats and is toxic to these animals in larger doses.

We wish to thank Dr E. M. F. Roe and Mr R. N. Beale for carrying out ultraviolet-absorption examinations of the fluorescent pigments, Dr R. L. M. Synge for helping us with the cataphoretic examination of one of the pigments, Dr C. C. Vass and his staff for providing facilities for the dog experiments and Prof. F. G. Young for supplying metabolism cages for the dogs. We wish to acknowledge gifts of nicotinuric acid from Bayer Products Ltd. and Ward, Blenkinsop and Co. Ltd.

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Sulphur Compounds of the Genus *Allium*

DETECTION OF *n*-PROPYLTHIOL IN THE ONION. THE FISSION AND METHYLATION OF DIALLYL DISULPHIDE IN CULTURES OF *SCOPULARIOPSIS BREVICAULIS*

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(*Received* 29 *April* 1948)

The chief constituent of the essential oils of onion and garlic is usually stated to be allyl sulphide (Whitmore, 1937; Bernthsen & Sudborough, 1931; Vass, 1939). This is an erroneous view arising from a statement by Wertheim (1844) and was first contradicted by Semmler (1892). He found no allyl sulphide, but by distilling the oil of onions, *Allium cepa*, under reduced pressure and collecting various fractions he concluded from the boiling point and other physical properties that the main constituent is a disulphide, $C_6H_{12}S_2$, probably propyl allyl disulphide. The oil amounted to only 0·005% of the whole weight of the onions. Semmler (1892) states that oil of garlic, *A. sativum*, contains 6% propyl allyl disulphide, 60% diallyl disulphide, 20% diallyl trisulphide, and some diallyl tetrasulphide.

Kooper (1910) found thiocyanic acid and also allyl thiocarbimide in freshly expressed, weakly acid onion juice. No formaldehyde, acetaldehyde or acrolein was found. Platenius (1935), Platenius & Knott (1941), Sherratt (1943), Currier (1945) and Dyer, Taylor & Hamence (1941) estimated the total volatile sulphur in onions by conversion to sulphate. Walker, Lindegren & Bachmann (1925) found the fungicidal principles in onion juice to be of two types, one of which is non-volatile and stable to heat. The other is volatile, and passes off from the extracted juice at room temperature within a few hours. No attempt was made by these later authors to identify the compounds in question.

The work of recent investigators. Kohmann (1947) reports the presence of propionaldehyde in onions from evidence based on the melting points and analyses of the 4-nitro- and 2:4-dinitro-phenylhydrazones. He also states that the lachrymatory principle is probably a thioaldehyde, and that sulphur analyses indicate that this may be thiopropionaldehyde. It seems possible that, during the distillation under reduced pressure in presence of water at 50° which Kohmann employed, propionaldehyde might have arisen from propylthiol by the following series of reactions:

$$\begin{aligned} &CH_3.CH_2.CH_2.SH \xrightarrow{\frac{1}{2}[O_2]} (CH_3.CH_2.CH_2S-)_2 \xrightarrow{H_2O} \\ &CH_3.CH_2.CH_2SH \\ &\qquad + CH_3.CH_2.CH_2SOH \xrightarrow{-H_2S} CH_3CH_2CHO. \\ &\qquad \text{propylsulphenic acid} \end{aligned}$$

This type of reaction was observed by Schöberl (1933, 1936) with many disulphides under mild alkaline conditions and by Challenger & Rawlings (1937) with diethyl disulphide in a closed copper vessel at 210°.