The Relation between Riboflavin and Tryptophan Metabolism, Studied in the Rat

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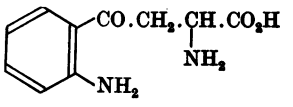
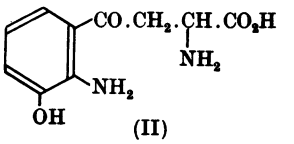
Dietary tryptophan is the precursor of nicotinic acid in a wide variety of living species, including mammals. The general outline of the route by which tryptophan is converted to nicotinic acid is becoming clear (for review see Dalgliesh, 1951) but much of the detailed mechanism remains obscure. One of the stages requiring investigation is the nature of the hydroxylation reaction. It is generally assumed that kynurenine (I) is hydroxylated to 3-hydroxykynurenine (II), and that (II) by the action



(I)



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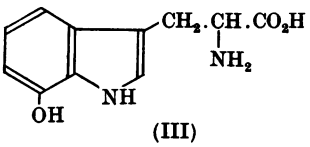

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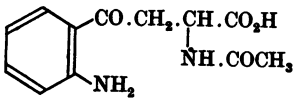

(IV)

of kynureninase gives 3-hydroxyanthranilic acid and hence nicotinic acid. But it is also possible that in part hydroxylation occurs at the tryptophan stage to give 7-hydroxytryptophan (III), which is then degraded to (II), or that the hydroxylation reaction occurs not with (I) itself, but with a derivative such as N^{α}-acetylkynurenine (IV), which is hydroxylated to the corresponding derivative of (II) (Dalgliesh, 1951, 1952). Porter, Clark & Silber (1948) and Henderson, Weinstock & Ramasarma (1951) have examined the effect of various B vitamins on tryptophan metabolism. Porter *et al.* showed that riboflavin deficiency markedly affected the excretion of kynurenine, kynurenic acid and xanthurenic acid. Henderson *et al.* showed that riboflavin deficiency, like pyridoxin deficiency, greatly reduced the conversion of tryptophan and kynurenine, but not of 3-hydroxyanthranilic acid, to pyridine compounds. Riboflavin and pyridoxin therefore function at some stage in the conversion of kynurenine to 3-hydroxyanthranilic acid. Pyridoxin deficiency is known to produce its effect by the inhibition of kynureninase, for which pyridoxal phosphate is the coenzyme (e.g. Dalgliesh, Knox & Neuberger, 1951; Dalgliesh, 1952), and Henderson *et al.* suggested that riboflavin might be concerned with the conversion of kynurenine to hydroxykynurenine. The association of riboflavin with an oxidative process is reasonable, and further information was therefore sought using techniques recently developed (Dalgliesh, 1952). The results, some of which have been briefly reported (Charconnet-Harding, Dalgliesh & Neuberger, 1952*a*, *b*), show that riboflavin deficiency does not inhibit formation of hydroxylated metabolites, but does have a marked effect on tryptophan metabolism, for which a possible explanation is suggested. The work has also involved investigation of the metabolic fate of anthranilic acid.

METHODS

Animals and diets. The rats were Institute albino stock strain. Both young and adult rats were used, the young rats being placed on the diet at the age of 6 weeks. The diet of the control animals had the following composition: casein (extracted), 12%; fat mixture, 8%; sucrose, 36%; maize starch, 40%; salt mixture, 4%. The fat mixture and salt mixture have been described (Dalgliesh, 1952). To each kg. of diet was added a vitamin mixture of the following composition: thiamine, 10 mg.; riboflavin, 10 mg.; pyridoxin, 10 mg.; nicotinic acid, 10 mg.; *p*-aminobenzoic acid, 10 mg.; calcium pantothenate, 50 mg.; inositol, 100 mg.; pteroylglutamic acid, 2 mg.; biotin, 0·1 mg.; α-tocopherol, 40 mg.; vitamin K, 1 mg.; and choline, 500 mg. The vitamin-deficient animals were fed the same diet with omission of the appropriate vitamins (riboflavin, or riboflavin and pyridoxin). In addition, all animals received vitamin B_{12} equivalent to 0·05 μg./rat/day. When vitamin deficiency was established, as shown by loss of weight and onset of alopecia, tryptophan supplements (usually 0·1 g. L- or 0·2 g. DL-tryptophan) were added to the diet as appropriate. In

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later experiments it was found that more consistent onset of vitamin deficiency was obtained if the maize starch in the diet was replaced by sucrose.

The rabbits were Institute Himalayan stock strain. Before feeding anthranilic acid they were starved for 24 hr., and were then given their normal diet, which had previously been soaked in an ethanolic solution of anthranilic acid (corresponding to 1 g./rabbit/day), and the ethanol allowed to evaporate. When fed to rats the anthranilic acid was mixed in the diet at the rate of 0·2 g./rat/day.

Examination of the metabolites. The methods for isolation of the urinary metabolites, and their chromatographic and chemical examination, have already been described (Dalgliesh, 1952). In general, the mercuric acetate precipitation step was omitted, the metabolites being adsorbed directly from the acidified urines on to deactivated charcoal. For routine chromatograms the butanol/acetic acid mixture was used.

Xanthurenic acid estimation. This was carried out directly on the urines (which were collected as quantitatively as possible) using essentially the method of Rosen, Lowy & Sprince (1951).

RESULTS

Anthranilic acid metabolism

The first experiments showed that in riboflavin-deficient rats fed supplementary tryptophan large amounts of three substances were excreted having a powerful purple fluorescence in ultraviolet light. One of these substances was readily identified as anthranilic acid and the others were thought likely to be derivatives of anthranilic acid, as indeed proved to be the case. It was of some importance to establish whether these metabolites were intermediates between tryptophan and anthranilic acid, or whether anthranilic acid and these metabolites were formed from tryptophan by different routes, or whether they were merely secondary ('detoxication') products of anthranilic acid. The metabolism of anthranilic acid itself was therefore examined. On adding anthranilic acid to the diet of normal rats and subsequent examination of the urine the same three fluorescent substances were observed, and in the same relative proportions (judged by visual inspection of the chromatograms), as appeared in the urines of tryptophan-fed riboflavin-deficient rats. These same substances were also excreted after feeding anthranilic acid to the riboflavin-deficient rats. They are therefore to be considered as metabolites of anthranilic acid rather than of tryptophan, their formation is not dependent on riboflavin deficiency, and their appearance after feeding tryptophan is only further evidence of a large output of anthranilic acid. It was, however, considered desirable to identify the anthranilic acid derivatives. When anthranilic acid was added to the diet of normal rabbits it was found that the urines contained the same three fluorescent substances as were found with rats, and because of the larger quantities thereby available rabbits were mainly used in these experiments.

The chromatographic behaviour of the three fluorescent metabolites, called α, β and γ, is summarized in Table 1. The metabolite α was present in largest amount, and the amounts of β and γ were similar in magnitude. The substance γ was readily shown to be anthranilic acid. The colour of fluorescence and behaviour to Ehrlich's and Ekman's reagents (Dalgliesh, 1952) showed that α and β were conjugated derivatives of anthranilic acid in which the aromatic amino group was intact, whilst the colour of fluorescence and the absence of reaction with Pauly's diazo reagent or ammoniacal silver nitrate showed that neither α nor β was hydroxylated in the aromatic ring. The principal metabolite of anthranilic acid in the dog and rabbit was shown by Mitsuba & Ichihara (1927) to be anthranilic acid glucuronide. Substance α was readily hydrolysed by acid or alkali to anthranilic acid and the hydrolysate gave a strong positive Tollens reaction (naphthoresorcinol-HCl). Substance α is therefore the glucuronide and the low R_F compared with anthranilic acid, and other properties (Table 1), are those to be expected. The glucuronide was partially purified to give a syrup solidifying to a glass. This had not crystallized after some months. A similar failure to crystallize the substance was recorded by Bray, Lake, Neale, Thorpe & Wood (1948). Anthranilic acid glucuronide has recently been reported under similar circumstances by Mason (1952),

Table 1. *Properties of anthranilic acid metabolites excreted by the rabbit and rat, and comparison with known substances*

(All show a powerful purple fluorescence under ultraviolet light, give a magenta colour with Ekman's reagent, and an immediate strong yellow colour with Ehrlich's reagent, but no reaction with Pauly's reagent, and no rapid reduction of ammoniacal silver nitrate.)

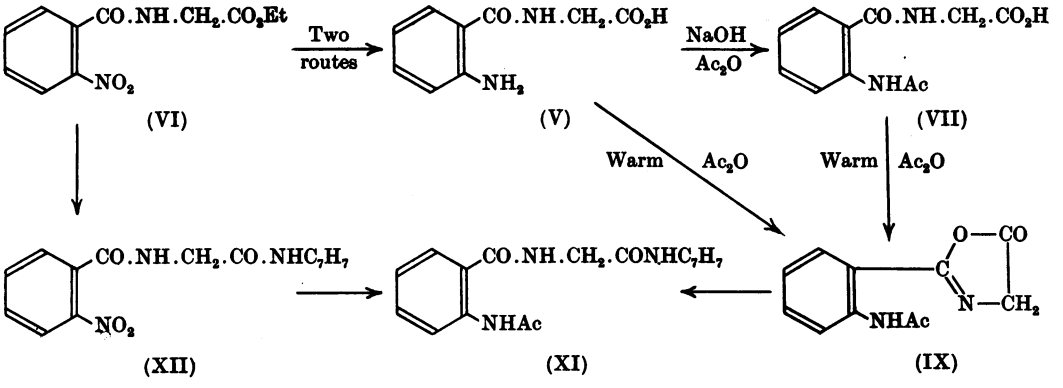
Substance	R_F values: Butanol/acetic acid/water (1:1:5)	R_F values: Amyl alcohol/pyridine/water (35:35:30)	R_F values: Phenol/water (5:2)	Tollens reaction on hydrolysate	Patton-Foreman reaction on hydrolysate
α	0·52	0·45	.	+	−
β	0·75	0·50	0·73	−	+
o-Aminohippuric acid	0·75	0·50	0·73	−	+
γ	0·91	0·85	0·88	−	−
Anthranilic acid	0·91	0·85	0·89	−	−

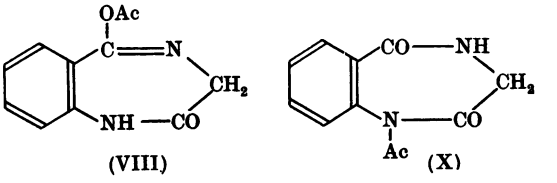
and, as its identity was not in doubt, no attempt was made to prepare crystalline derivatives.

Substance β was resistant to hydrolysis under the mild conditions sufficing for hydrolysis of the glucuronide, but hydrolysis was complete using 5N-hydrochloric acid in a sealed tube for 2·5 hr. Chromatographic examination of the hydrolysate revealed only two substances, anthranilic acid and a ninhydrin-positive substance indistinguishable from glycine using numerous solvent mixtures, and showing the characteristic green colour given by glycine with *o*-phthalaldehyde (Patton & Foreman, 1949). This suggested that β was *o*-aminohippuric acid, and comparison of β with synthetic (see below) material showed their identical behaviour (Table 1). Glycine conjugation with anthranilic acid was stated not to occur by Muenzen, Cerecedo & Sherwin (1926), and to be inhibited for all *ortho*-substituted benzoic acids for steric reasons (Quick, 1932). However, salicyluric acid (*o*-hydroxyhippuric acid) was isolated as long ago as 1856 (Bertagnini, 1856; cf. Williams, 1949) as the principal metabolite of salicylic acid in man, and the occurrence of glycine conjugation with many other *ortho*-substituted benzoic acids has since been recorded (e.g. Bray, Clowes, Thorpe, White & Wood, 1952). Glycine conjugation with anthranilic acid was suspected to occur by Bray *et al.* (1948) but could not be demonstrated, probably due to the use of too vigorous conditions in working up their preparations. In view of these conflicting reports it was considered desirable to identify β unambiguously.

o-Aminohippuric acid (V), not previously recorded in the literature, was obtained either by hydrolysis of its ester prepared by hydrogenation of *o*-nitrohippuric ester (VI) or by hydrolysis of (VI) to *o*-nitrohippuric acid and subsequent hydrogenation. The product obtained was chromatographically and analytically pure, but considerable difficulty was experienced in its recrystallization. Treatment with acetic anhydride under Schotten-Baumann conditions gave the readily crystallizable *N*-acetyl derivative (VII).

Isolation of substance β from the urine gave a gum. In view of the difficulty already experienced in crystallizing authentic (V) the gum was treated with acetic anhydride and gently warmed, giving orange-coloured crystals quite different from (VII). However, when authentic *o*-aminohippuric acid (V) or its acetyl derivative (VII) were gently warmed with acetic anhydride, the same orange-coloured substance was obtained. Analysis showed that loss of a molecule of water from (VII) had occurred. The ready formation from (VII) made it likely that the acetyl group was on the aromatic amino group, rearrangement to a substance such as (VIII) being improbable, and the product was therefore either the oxazolone (IX) or the acetylated cyclic lactam (X). As hippuric acid on treatment with warm acetic anhydride readily gives 2-phenyloxazolone (Cornforth, 1949), structure (IX) was most probable. This was supported by the colour of the product and by its ready reaction with benzylamine to give *o*-acetamidohippurylbenzylamide (XI) identical with material made by treatment of the ester (VI) with benzylamine to give the benzylamide (XII), followed by hydrogenation and acetylation. The oxazolone formulation was confirmed by the ready condensation with benzaldehyde to give a benzal derivative, and the structure of the latter was confirmed by reduction and hydrolysis to yield anthranilic acid and phenylalanine. The formation of an oxazolone from *o*-aminohippuric acid is





probably responsible for the failure of previous attempts to identify this substance. Thus it was observed when working up urine preparations that in the final stages of evaporation of the metabolite solution considerable darkening occurred if the temperature was allowed to rise. This darkening was probably to a large extent due to cyclization of the *o*-aminohippuric acid to the oxazolone, a type of structure known readily to undergo degradative reactions (cf. Cornforth, 1949).

Besides the three substances α, β and γ, small quantities of several other fluorescent substances were observed. Some of these showed fluorescence resembling that of anthranilic acid, whilst the fluorescence of others was compatible with their being *N*-acylanthranilic acids. These substances were not investigated, but the absence of any appreciable quantity of hydroxyanthranilic acid derivatives was confirmed (cf. Bray *et al.* 1948).

Experimental

Melting points are uncorrected, and were taken in a capillary unless otherwise stated.

o-*Aminohippuric acid* (V). Ethyl *o*-nitrohippurate (VI) (prepared by treating *o*-nitrobenzoic acid with $SOCl_2$, removing excess $SOCl_2$, and treating the resultant acid chloride with glycine ester) was dissolved in ethanol and hydrogenated at atmospheric pressure over palladium-charcoal. The theoretical amount of H_2 was taken up smoothly. Catalyst and solvent were removed and the residual oil evaporated in a desiccator, whereupon it solidified. Recrystallization from ethanol gave stout colourless prisms of *ethyl* o-*aminohippurate*, m.p. 78°. (Found: C, 59·8; H, 6·4. $C_{11}H_{14}O_3N_2$ requires C, 59·6; H, 6·3%.) The *picrate* separated as yellow leaflets from ethanol, and melted at 152° to give a red melt. (Found: N, 15·6. $C_{11}H_{14}O_3N_2.C_6H_3O_7N_3$ requires N, 15·5%.)

This ester was shaken with the calculated quantity of N-NaOH until a clear solution was obtained. One equiv. of N-HCl was added, the mixture extracted with ethyl acetate, the extract dried (Na_2SO_4) and solvent removed to give o-*aminohippuric acid* (V). (Found: C, 55·2; H, 5·1; N, 14·4. $C_9H_{10}O_3N_2$ requires C, 55·7; H, 5·1; N, 14·4%.)

A more satisfactory procedure was to hydrolyse the ester (VI) to *o*-nitrohippuric acid which on hydrogenation as above gave (V). (Found: C, 55·7; H, 4·7; N, 14·2%.) *o*-Nitrohippuric acid was characterized as its *benzylamine salt*, small needles from ethanol, m.p. 172°. (Found: C, 58·0; H, 5·1; N, 12·7. $C_9H_8O_5N_2.C_7H_9N$ requires C, 58·0; H, 5·1; N, 12·7%.)

When heated in a capillary in the usual way *o*-aminohippuric acid shrank and finally decomposed at about 280°. If the melting point was taken on a preheated block it was found to melt at 148°, resolidify, and decompose at about 280°. The higher value probably represents the melting point of an anhydro derivative of the type of (IX) or (X). The amino-acid appeared to be stable in solution at room temperature. On heating a concentrated solution, decomposition may occur. One preparation, e.g. in which the ethanol was removed from the hydrogenation filtrate in a stream of warm air, gave an orange glass. On rubbing with ethanol part separated as an orange powder, decomposing at about 320°, and part went into solution. The latter contained (V), identified as its *picrate*, yellow needles from ethanol, melting at 156° (block), partially solidifying, and decomposing at about 220°. (Found: C, 42·5; H, 3·8; N, 17·0. $C_9H_{10}O_3N_2.C_6H_3O_7N_3$ requires C, 42·6; H, 3·1; N, 16·5%.) The orange powder on warming with aqueous acetic acid gave a scarlet insoluble product. (Found: C, 70·2; H, 4·3; N, 14·8%.) The solution deposited orange needles, decomposing at about 290°. (Found: C, 60·2; H, 4·6; N, 15·3%.) The structure of these substances was not examined.

o-*Acetamidohippuric acid* (VII). The amino-acid (V) was dissolved in 2·4 equiv. of NaOH, shaken with acetic anhydride (1·2 equiv.), the solution acidified (HCl), and the product extracted with ethyl acetate. After drying (Na_2SO_4), removal of solvent, and recrystallization (ethanol-charcoal) o-*acetamidohippuric acid* was obtained as colourless crystals, m.p. 180°. (Found: C, 55·9; H, 5·3; N, 12·0. $C_{11}H_{12}O_4N_2$ requires C, 55·9; H, 5·1; N, 11·9%.)

2-(2′-*Acetamidophenyl*)*oxazolone* (IX). The amino-acid (V) was warmed gently on the water bath with acetic anhydride, whereupon it dissolved to give an orange solution. Sufficient ethanol was added to decompose excess acetic anhydride, and the mixture set aside at 0°. (A better, but more time-consuming, method is to remove the excess anhydride in a vacuum desiccator over NaOH.) Needles separated, orange or bronze-coloured depending on the crystal size, of 2-(2′-*acetamidophenyl*)*oxazolone*, m.p. 185° (block); 181° (capillary). (Found: C, 60·6; H, 4·8; N, 12·9. $C_{11}H_{10}O_3N_2$ requires C, 60·5; H, 4·6; N, 12·9%.) The same compound was obtained on similar treatment of the acetamido acid (VII) with acetic anhydride.

o-*Acetamidohippurylbenzylamide* (XI). Benzylamine (1 ml.) was added to the oxazolone (1·1 g.). The mixture became hot and the initially orange colour faded. Dry benzene was added and the mixture set aside at 0° overnight. Recrystallization of the product from ethanol gave small colourless needles of o-*acetamidohippurylbenzylamide*, m.p. 187°. (Found: C, 66·2; H, 5·6; N, 12·9. $C_{18}H_{19}O_3N_3$ requires C, 66·5; H, 5·8; N, 12·9%.)

o-Aminohippurylbenzylamide (1·35 g. see below) was dissolved in warm acetic acid (5 ml.), cooled to room temperature, and acetic anhydride (0·46 ml.; 1 equiv.) added. After 45 min. the mixture was poured into excess water and set aside at 0°. Recrystallization of the precipitate from ethanol gave small colourless needles of m.p. 187° identical with the material prepared from the oxazolone. (Mixed m.p. 187°. Found: C, 66·8; H, 5·9; N, 12·9%.)

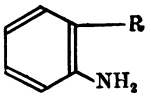
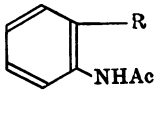
o-*Nitrohippurylbenzylamide* (XII). The ester (VII) was warmed in a test tube over a flame with benzylamine to give a homogeneous mixture. The tube was sealed and the whole heated for 3 hr. at 100°. On treating the resultant gum with ethanol it crystallized and was recrystallized from ethanol to give colourless fine needles of o-*nitrohippurylbenzylamide* of m.p. 160°. (Found: C, 61·0; H, 4·7; N, 13·1. $C_{16}H_{15}O_4N_3$ requires C, 61·3; H, 4·8; N, 13·4%.)

o-*Aminohippurylbenzylamide*. The nitro compound (XII) was dissolved in 2-methoxyethanol (methyl cellosolve) and hydrogenated at atmospheric pressure using palladium-charcoal. After removal of the catalyst the mixture was poured into a large excess of water and set aside at 0°. The precipitated oil soon solidified and was recrystallized from ethanol to give stout colourless prisms of o-*aminohippurylbenzylamide* of m.p. 111°. (Found: C, 67·6; H, 6·5; N, 14·3. $C_{16}H_{17}O_2N_3$ requires C, 67·8; H, 6·1; N, 14·8%.)

2-(2′-*Acetamidophenyl*)-4-*benzaloxazolone*. To the oxazolone (IX) (1 g.) and benzaldehyde (0·6 ml.) in ethanol (4 ml.) was added a drop of pyridine, and the mixture heated on the water bath and then set aside. The precipitated solid was recrystallized from ethanol-butanol to give small glistening flattened yellow needles of 2-(2′-*acetamidophenyl*)-4-*benzaloxazolone* of m.p. 240°. (Found: C, 70·8; H, 4·6; N, 9·4. $C_{18}H_{14}O_3N_2$ requires C, 70·6; H, 4·6; N, 9·2%.) The structure of the benzaloxazolone was confirmed by reduction of a small amount with phosphonium iodide, followed by hydrolysis (cf. Gillespie & Snyder, 1943). Paper-chromatographic comparison with authentic substances in acidic, neutral and basic solvents showed the formation of anthranilic acid and phenylalanine.

Ultraviolet absorption spectra. These were determined on solutions in spirits of wine (95% ethanol, free of substances absorbing light at wavelengths above 250 mμ.), using a Unicam spectrophotometer. The results are recorded in Table 2.

Table 2. *Ultraviolet absorption maxima of various anthranilic acid derivatives, determined in solution in spirits of wine*

		First maximum		Second maximum	
	R =	mμ.	*E*	mμ.	*E*
	—CO_2H	249	6030	336	4200
	—$CO.NH.CH_2.CO_2H$ (V)	249	7860	329	3870
	—$CO.NH.CH_2.CO_2Et$	249	9720	329	4320
	—$CO.NH.CH_2.CO.NH.C_7H_7$	253	8940	330	4500
	—CO_2H	252	14370	305	5000
	—$CO.NH.CH_2.CO_2H$ (VII)	252	14370	299	3400
	—$CO.NH.CH_2.CO.NH.C_7H_7$ (XI)	253	13590	297	3290
	 (IX)	261	11000	311	4500

o-Acetamidohippuric acid (VII) showed maxima at 252 and 299 mμ., whilst the oxazolone (IX) showed maxima at 261 and 311 mμ. The two curves were similar in shape, that for the oxazolone being displaced by about 10 mμ. to longer wavelengths. The curves for hippuric acid and 2-phenyloxazolone were examined for comparison. Both showed only end absorption, but here again the oxazolone curve was displaced by about 10 mμ. to longer wavelengths. The *o*-aminohippuryl derivatives all showed spectra resembling that of anthranilic acid under the same conditions, whilst the *o*-acetamidohippuryl derivatives showed spectra resembling that of acetylanthranilic acid.

Separation of the urinary metabolites of anthranilic acid. Various procedures were tried, of which the following was most convenient.

The urine from rabbits fed anthranilic acid was acidified (effervescence), filtered, and the filtrate treated with deactivated charcoal. The charcoal was separated, washed with water and the metabolites eluted with 5% (w/v) aqueous phenol in the normal way (Dalgliesh, 1952). The eluate was concentrated *in vacuo* on the water bath, further water being added during the process to ensure removal of the phenol. As the solution became more concentrated it was important to keep the temperature low, otherwise considerable darkening occurred. The final concentration to a thin syrup was carried out in a vacuum desiccator. The syrup was applied to the starting line of thick sheets of chromatographic paper (Whatman 3 MM) and the sheets developed with butanol-acetic acid in the usual way. The regions of the dried sheets containing α and (β + γ) were then marked under ultraviolet light, the appropriate parts cut out, and eluted in a Soxhlet apparatus with aqueous ethanol or ethanol respectively. The eluate containing α was concentrated to dryness, rubbed successively with ether, ethyl acetate and ethanol, and the residue kept in a vacuum desiccator, to form a glass which did not crystallize. It was essentially pure anthranilic acid glucuronide, as judged by the absence of other fluorescent substances, but contained some urea (revealed by Ehrlich's reagent). The tendency of urea to associate with glucuronides has been noted previously (e.g. Jaffé, 1874, 1878–9). Urea and the glucuronide move at similar speeds in the butanol-acetic acid mixture used in this work, but can be separated using other solvents. Treatment of an appropriately buffered urine concentrate with commercial urease removed the urea but also caused appreciable hydrolysis of the glucuronide. Celite columns (Perrone, 1951) were made with 0·2M-phosphate buffer, pH 6, as the stationary phase. Solvent was removed from the (β + γ) eluate described above, and the residue taken up in buffer-saturated ethyl acetate and applied to the column. The development of the column was followed in ultraviolet light, the column being shielded from the light when not under observation. Elution with ether caused the γ band to move rapidly through the column, whereas β moved much more slowly. The appropriate fraction containing γ was collected, placed in an evaporating basin, and allowed to evaporate in the open. A crystalline residue remained, which was purified by high-vacuum sublimation to give colourless crystals agreeing in melting point, mixed melting point and chromatographic behaviour with authentic anthranilic acid.

When the γ fraction had been eluted from the column, elution was continued with ethyl acetate. The appropriate fraction containing β was collected and shown by chromatography in many solvents to be free of other fluorescent substances. The solvent was removed at the pump, the flask being immersed in water at room temperature, and the residual oil set aside in a vacuum desiccator. Only partial solidification occurred and the product was therefore warmed with acetic anhydride as described for the synthetic *o*-aminohippuric acid above. The orange-coloured product, recrystallized from ethyl acetate, had m.p. 183° (block) undepressed by authentic 2-(2′-acetamidophenyl)oxazolone (Found: C, 60·8; H, 5·3%.)

Isolation occurred smoothly by the above procedure, but if the initial rough separation on paper was omitted the separation on celite gave discoloured and unsatisfactory products. The paper stage removes much of the dark-coloured products formed in working up the urine, these products probably being mainly due to decomposition of the *o*-aminohippuric acid present. The latter is not solely responsible, however, as experiments with rat urines were in general cleaner, but were considered less convenient because of the smaller quantities available.

Tryptophan metabolism

On feeding tryptophan to the riboflavin-deficient animals the urines contained (*a*) N^{α}-acetylkynurenine (IV) with, occasionally, kynurenine, (*b*) kynurenic and xanthurenic acids and (*c*) the three anthranilic acid metabolites, α, β and γ, described above. The amount of xanthurenic acid excreted was sufficient for appreciable amounts to separate on acidification of the urine.

As this hydroxylated substance was not expected under these conditions the product was isolated and its identity confirmed by comparison of its melting point, and the ultraviolet absorption spectrum of the iron complex, with those of authentic xanthurenic acid.

The urines of rats deficient in both riboflavin and pyridoxin contained, as expected, no appreciable amounts of the anthranilic acid metabolites. Kynureninase inhibition, due to pyridoxin deficiency, provides the most favourable conditions for the excretion of kynurenine analogues and derivatives. In these rats, besides N^{α}-acetylkynurenine, the corresponding N^{α}-acetylhydroxykynurenine was also excreted, with, occasionally, hydroxykynurenine, and even larger amounts of xanthurenic acid than in riboflavin deficiency alone. Typical results for the excretion of xanthurenic acid by adult rats are given in Table 3. With young riboflavin-deficient rats even higher values were observed than those shown in the table. On administering riboflavin to the tryptophan-fed riboflavin-deficient rats the xanthurenic acid excretion immediately returned to normal levels.

The unexpected appearance of xanthurenic acid in the urines of riboflavin-deficient rats might have been due to oxidation of kynurenic acid by some different mechanism from that operating in kynurenine hydroxylation. Both quinoline (Scheunemann, 1923) and atophan (2-phenylcinchoninic acid; Dohrn, 1912) have been shown to be metabolized to their 8-hydroxy derivatives, but in the present experiments no xanthurenic acid was formed when kynurenic acid was added to the diet

Table 3. *Xanthurenic acid excretion of adult rats*

(Three animals in each group, mean values in mg./rat/day.)

Substance administered	L-Tryptophan (100 mg.) added to diet	DL-Kynurenine (100 mg.) by intraperitoneal injection	Kynurenic acid (100 mg.) added to diet
Period of deficiency (weeks)	5–6	9	8
Control	0·2	4·3	Very small
Riboflavin-deficient	3	5·2	Very small
Riboflavin- and pyridoxin-deficient	8	19·2	Very small

Table 4. *The excretion of tryptophan metabolites after feeding tryptophan to rats on various vitamin-deficient diets*

(+, present in the urine; −, absent; ~, sometimes present or present in only small amount.)

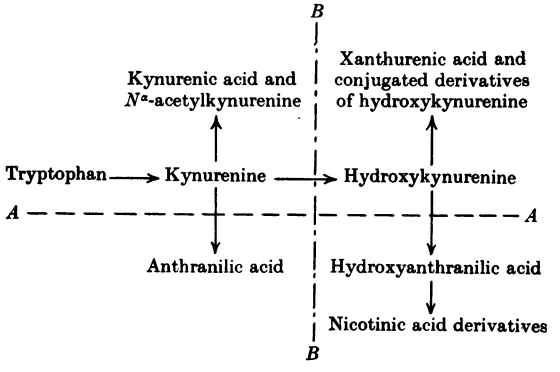
Metabolite	Pyridoxin deficiency (Dalgliesh, 1952)	Riboflavin deficiency	Riboflavin and pyridoxin deficiency
Kynurenine			
(*a*) Free	+	~	~
(*b*) N^{α}-Acetyl derivative	+	+	+
Kynurenic acid	+	+	+
Anthranilic acid			
(*a*) Free	−	+	−
(*b*) Glycine conjugate	−	+	−
(*c*) Glucuronide	−	+	−
Hydroxykynurenine			
(*a*) Free	+	−	~
(*b*) N^{α}-Acetyl derivative	+	~	+
(*c*) *O*-Sulphate	+	−	−
(*d*) *O*-Glucuronide	+	−	−
Xanthurenic acid	+	+	+

(Table 3). The experiment is not conclusive, as the insolubility of kynurenic acid added to the diet might make it behave differently from kynurenic acid formed in the cell, but the present finding agrees with the results of earlier workers (see Neuberger, 1944, for collected references).

The excretion of tryptophan metabolites in various vitamin-deficient states is summarized in Table 4. It is interesting to note that the N^{α}-acetyl derivatives of kynurenine and hydroxykynurenine often appear when free kynurenine and hydroxykynurenine are not observed, lending support to the suggestion (e.g. Dalgliesh, 1951) that these acetyl derivatives have greater significance in tryptophan metabolism than would mere 'detoxication' products.

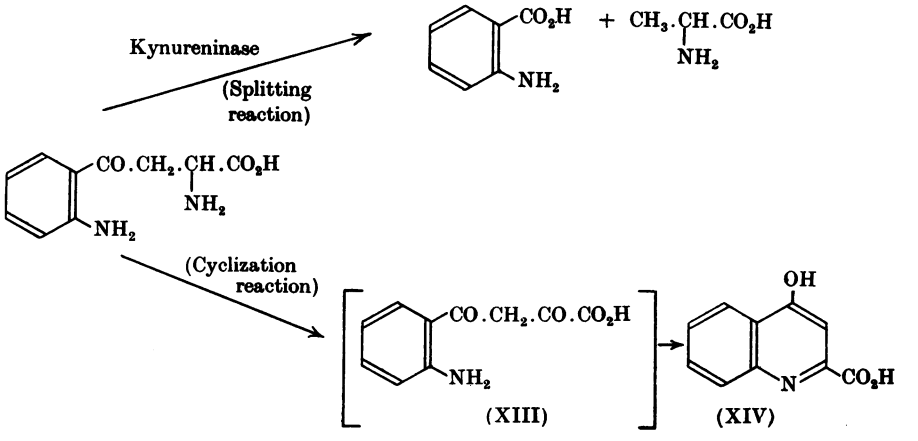
DISCUSSION

The known facts about the metabolism of tryptophan in the rat may be summarized thus:



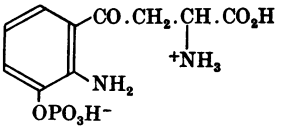
In pyridoxin deficiency only those metabolites are excreted which lie above the line *AA*. The line *AA* represents the stage at which the enzyme kynureninase functions, this enzyme being dependent on pyridoxal phosphate. By analogy, if the hydroxylation were carried out by an enzyme involving riboflavin in its prosthetic group, it would be expected that in riboflavin deficiency those tryptophan metabolites would be excreted which lie to the left of line *BB*, and that no excretion, or a much reduced excretion, would occur of the hydroxylated metabolites lying to the right of *BB*. Similarly, in a combined riboflavin and pyridoxin deficiency it would be expected that only those metabolites would be excreted which lie above *AA* and to the left of *BB*. The high excretion of anthranilic acid derivatives observed in riboflavin-deficient rats is in agreement with such a direct function for riboflavin. On the other hand, excretion of hydroxylated metabolites lying to the right of *BB* not only occurred in these rats, but the amount excreted of one of these metabolites, xanthurenic acid, considerably exceeded normal values. The simple theory that hydroxylation of kynurenine to hydroxykynurenine is carried out by a riboflavin-dependent enzyme therefore becomes unlikely.

Wiss (1952) has shown that the two pathways for kynurenine degradation involve two distinct enzymes. One of these enzymes, kynureninase, leads to anthranilic acid and alanine, whilst the other (or others), either by oxidative deamination or transamination (or both), leads (or lead) to formation of the corresponding α-keto acid (XIII) which spontaneously cyclizes to kynurenic acid (XIV) (Musajo, Spada & Bulgarelli, 1950). These two reactions, which will be referred to as the splitting and cyclization reactions, will therefore compete for any kynurenine present. With hydroxykynurenine the splitting and cyclization reactions give hydroxyanthranilic acid and xanthurenic acid respectively, and of these products hydroxyanthranilic acid, the precursor of nicotinic acid derivatives, is useful to the animal, whereas xanthurenic acid is, metabolically speaking, waste. Obviously the more the



splitting reaction is favoured as compared with the cyclization reaction the more efficiently will the animal be utilizing tryptophan. Kynureninase is a relatively unspecific enzyme, and besides kynurenine it has been shown to attack 3-hydroxykynurenine (Wiss & Fuchs, 1950; Dalgliesh *et al.* 1951), 3:4-dihydroxykynurenine (Butenandt & Schlossberger, 1952), γ-phenyl-γ-keto-α-aminobutyric acid (Wiss & Fuchs, 1949; Wiss, Viollier & Waldi, 1951) and even the acid $CH_3.CO.CH_2.CH(NH_2).CO_2H$, which contains no aromatic ring (Wiss & Fuchs, 1950). However, the rates of attack of the various substrates vary.

Our results may be explained if (1) kynurenine is normally converted not to hydroxykynurenine itself but to some derivative, X, of hydroxykynurenine, and if (2) the ratio of the rates of the splitting and cyclization reactions is greater when the substrate is X rather than hydroxykynurenine itself. The possible identity of X is suggested by the work of Hellmann & Wiss (1952) who have shown that although rat-liver preparations convert hydroxykynurenine to hydroxyanthranilic acid, both tryptophan and kynurenine are converted by these preparations to a hydroxyanthranilic acid phosphate. This suggests that a hydroxykynurenine phosphate, and not hydroxykynurenine itself, is the normal physiological substrate for kynureninase. Thus our results would be explained if the normal metabolic oxidation of kynurenine were not a simple oxidation but an oxidative phosphorylation to give, e.g. (XV), and if the ratio of the rates of the splitting



(XV)

and cyclization reactions were greater when (XV) was substrate rather than hydroxykynurenine. If such were the case the 'normal' metabolism of tryptophan, via (XV), would follow the most useful pathway with the production of but little xanthurenic acid, whereas in riboflavin deficiency oxidation might continue to give unphosphorylated hydroxykynurenine for which the relative rates of splitting and cyclization reactions are so altered that appreciably more xanthurenic acid is formed than in normal animals. In other words, it is considered possible that riboflavin is concerned with a phosphorylative rather than an oxidative step.

Our results do not exclude the possibility that riboflavin has no specific action, but produces changes in cell organization such that excretion occurs of intermediates not normally accumulated, or the much less likely possibility that riboflavin exerts its effect by modifying reabsorption of the metabolites in the kidney. On the available evidence, however, we consider that the observed results of riboflavin deficiency can be best explained in the manner already outlined, but further work is clearly required.

SUMMARY

1. A study is reported of the urinary metabolites excreted after feeding tryptophan to (*a*) normal, (*b*) riboflavin-deficient and (*c*) riboflavin- and pyridoxin-deficient rats, and of the metabolites excreted after feeding anthranilic acid to (*a*) normal and (*b*) riboflavin-deficient rats and (*c*) normal rabbits.

2. Both rats and rabbits excrete anthranilic acid partly unchanged, partly as its glucuronide and partly as *o*-aminohippuric acid. The latter has been isolated and characterized as 2-(2′-acetamidophenyl)oxazolone, and has also been synthesized and its chemistry examined.

3. Tryptophan is excreted in the urines of riboflavin-deficient rats as N^{α}-acetylkynurenine, kynurenic acid, xanthurenic acid, and the anthranilic acid metabolites described above. Rats deficient in both riboflavin and pyridoxin excrete N^{α}-acetylkynurenine, N^{α}-acetyl-3-hydroxykynurenine, kynurenic acid and xanthurenic acid. Kynurenine and hydroxykynurenine sometimes occur in addition to their N^{α}-acetyl derivatives.

4. Riboflavin-deficient rats fed tryptophan excrete much larger amounts of xanthurenic acid than normal rats. On administration of riboflavin the excretion immediately returns to normal values.

5. The function of riboflavin in tryptophan metabolism is discussed in relation to these results. In the explanation considered most likely riboflavin is concerned with a phosphorylative rather than an oxidative step.

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A Method of Measuring the Yield of Oxidative Phosphorylation

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Oxidative phosphorylation may be defined as the phosphorylation of adenosinediphosphate (ADP) to adenosinetriphosphate (ATP) which accompanies the oxidation of a number of intermediary metabolites. It is now clear (Slater, 1950; Barkulis & Lehninger, 1951; Kielley & Kielley, 1951; Lindberg & Ernster, 1952; Slater & Holton, 1953) that ADP is phosphorylated much more rapidly than adenosinemonophosphate (AMP).

The overall oxidative phosphorylation reaction may be written

$$S\mathrm{H_2} + \mathrm{O} + x\mathrm{H_3PO_4} + x\mathrm{ADP} \rightarrow S + (x+1)\,\mathrm{H_2O} + x\mathrm{ATP}, \qquad (1)$$

where $S\mathrm{H_2}$ is the oxidizable substrate and S the product of the oxidation. The yield of oxidative phosphorylation is expressed by the value of x, the number of atoms of inorganic phosphorus esterified per atom of oxygen consumed, usually called the P:O ratio.

Because ATP is relatively unstable in the preparations used for these studies, many investigators add hexokinase and glucose, so that the labile terminal phosphate group is transferred to glucose to form hexosemonophosphate (HMP) by reaction (2)

$$\mathrm{ATP} + \mathrm{glucose} \rightarrow \mathrm{ADP} + \mathrm{HMP}. \qquad (2)$$

The overall reaction is now reaction (3), the sum of reactions (1) and (2)

$$S\mathrm{H_2} + \mathrm{O} + x\ \mathrm{glucose} + x\mathrm{H_3PO_4} \rightarrow S + (x+1)\,\mathrm{H_2O} + x\mathrm{HMP}. \qquad (3)$$

This method has the further advantage that ADP acts as a catalyst and its concentration remains relatively constant during the measurement.

In all previous studies, the yield of oxidative phosphorylation has been measured by determining the disappearance of inorganic phosphate. While this method has the advantage of simplicity and is sufficiently precise and sensitive for most investigations, it is unsuitable for certain purposes. For example, the kinetics of the early stages of the reaction cannot be studied because the percentage disappearance of inorganic phosphate is too low