QUARTERLY REVIEWS

BIOLOGICAL DEGRADATION OF TRYPTOPHAN

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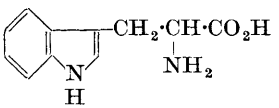
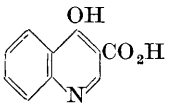
Tryptophan (I), first isolated [1] in 1901, assumed considerable biochemical importance when it was shown that it could not be synthesised by mammals but had to be supplied in the diet. Recently it has become apparent that tryptophan acts in many widely different living species as the precursor of one of the B vitamins, nicotinic acid (XVI). The metabolic conversion of the indole ring present in tryptophan into the pyridine ring present in nicotinic acid is of considerable interest to the organic chemist as well as to the biochemist. Moreover this transformation has been shown to be intimately connected with another of the B vitamins, pyridoxine. It is the purpose of this Review to consider the metabolism of tryptophan with especial reference to its function as a precursor of nicotinic acid derivatives: the particular derivative produced in any given case, *e.g.*, nicotinic acid, nicotinamide, *N'*-methylnicotinamide or 1-methyl-2-pyridone-5-carboxyamide, and the inter-relations of these derivatives or their further metabolism, are not considered to come within its scope.

Early Work (up to 1944).—Not long after the discovery of tryptophan, Ellinger [2] reported the isolation of a tryptophan metabolite, kynurenic acid, from the urine of rats to which tryptophan had been fed. This substance, which as long ago as 1853 had been observed by Liebig [3] in the urine of dogs, was assigned by Ellinger the structure (II), the correct structure 4-hydroxyquinoline-2-carboxylic acid (III) being later established by



(I.)



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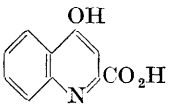
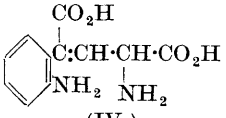


(III.)

Homer.[4] In 1931 kynurenine, a substance discovered earlier,[5] was shown to be a tryptophan metabolite by Y. Kotake and Iwao [6] who obtained it, after subcutaneous injection of (I), from the urine of rabbits on a polished-rice diet. Here again the structure originally [6] assigned, (IV), was sub-

[1] Hopkins and Cole, *J. Physiol.*, 1901, **27**, 418. See also Ellinger and Flammand, *Ber.*, 1907, **40**, 3029.
[2] *Z. physiol. Chem.*, 1904, **43**, 325.
[3] *Annalen*, 1853, **86**, 125.
[4] *J. Biol. Chem.*, 1914, **17**, 509.
[5] Matsuoka and Yoshimatsu, *Z. physiol. Chem.*, 1925, **143**, 206.
[6] *Ibid.*, 1931, **195**, 139.

sequently shown to be incorrect, and the correct structure, α-amino-β-*o*-aminobenzoylpropionic acid or *o*-aminophenacylglycine (V), was determined by Butenandt and his co-workers [7] in 1942 and confirmed by synthesis. This synthesis involved the condensation of *o*-nitrophenacyl bromide with sodiophthalimidomalonic ester to give (VI), followed by hydrolysis and catalytic reduction. Subsequently the synthesis was improved by the use of benzamidomalonic ester in the place of phthalimido-



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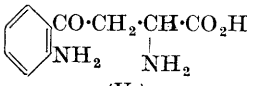

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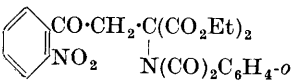

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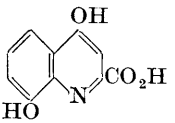


(VII.)

malonic ester.[8] Kynurenine contains one asymmetric carbon atom, and the L-isomer was obtained in a very neat manner by formation of its complex with sucrose.[9] That such a complex was formed had been observed previously.[10] An alternative synthesis of (V) involving addition of ammonia to β-*o*-nitrobenzoylacrylic acid has been reported by Sakan.[11,11a] A procedure for the isolation of L-kynurenine and kynurenic acid from the urine of rabbits after administration of tryptophan has been given by Kallio and Berg.[12]

A third tryptophan metabolite, xanthurenic acid, was isolated in 1938 by Musajo [13] from the urine of rats fed on fibrin, and was shown to be 4 : 8-dihydroxyquinoline-2-carboxylic acid (VII). In common with other 8-hydroxyquinoline derivatives, this forms metal complexes, the intense green colour given with ferrous salts providing a method for its determination and enabling its presence to be demonstrated in the urines of many other species.[14] Musajo suspected that his rats were suffering from a vitamin deficiency. That this vitamin was pyridoxine became clear when Lepkovsky and Nielsen [15] reported the occurrence, in the urine of pyridoxine-

[7] Butenandt, Weidel, and Derjugin, *Naturwiss.*, 1942, **30,** 51 ; *idem* with Weichert, *Z. physiol. Chem.*, 1943, **279,** 27.

[8] Butenandt, Weidel, and Neckel, *ibid.*, 1944, **281,** 120.

[9] Butenandt and Weichert, *ibid.*, p. 122.

[10] Tatum and Haagen-Smit, *J. Biol. Chem.*, 1941, **140,** 575.

[11] *J. Chem. Soc. Japan,* 1942, **63,** 1545.

[11a] A preliminary report has recently been published (Butenandt and Hellmann, *Z. Naturf.*, 1950, **5,** *b*, 445) of another synthesis of kynurenine which proceeds in good yield. *o*-Nitroacetophenone is condensed with ethyl oxalate, and the resultant diketo-ester converted into its monoxime, which is hydrogenated to kynurenine ethyl ester, and this is hydrolysed. 3-Hydroxykynurenine may also be prepared by this method.

[12] *J. Biol. Chem.*, 1949, **181,** 333.

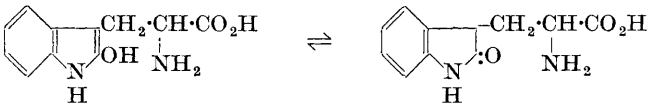
[13] *Atti R. Accad. Lincei,* 1935, **21,** 368 ; *Gazzetta,* 1937, **67,** 165, 171, 182.

[14] For collected references, see ref. 22.

[15] *J. Biol. Chem.*, 1942, **144,** 135.

deficient rats receiving tryptophan, of a green pigment which was subsequently shown [16] to be the iron complex of xanthurenic acid. Xanthurenic acid may be conveniently synthesised from oxaloacetic ester and *o*-anisidine.[17]

Other lines of work having a direct bearing on tryptophan metabolism were also being carried on. Hydrolysis of phalloidine, a toxid peptide obtained [18] from the deathcap, *Amanita phalloides*, was shown [19] to give, amongst other products, 2-hydroxytryptophan (VIII*a*) [more correctly described [85] as β-3-oxindolylalanine (VIII*b*)]. Kotake had previously



(VIII*a*.) (VIII*b*.)

suggested that this substance was an intermediate in the conversion of tryptophan into kynurenine and kynurenic acid, and this was rendered more plausible by the isolation of the substance from a natural product.

Further support for the view that oxindolylalanine (VIII) was a metabolite was forthcoming from genetical experiments on the eye-pigments of mutants of *Drosophila melanogaster* and *Ephestia kuhniella*. In mutants lacking one or more of the appropriate enzyme systems the brown eye-pigment, ommochrome, was derived from tryptophan by way of the so-called V^+-substance and the cn^+-substance :

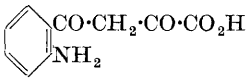
Tryptophan $\longrightarrow$ V^+-Substance $\longrightarrow$ cn^+-Substance $\longrightarrow$ Ommochrome

The V^+-substance was identified as kynurenine,[10, 20] and soon afterwards it was reported [21] that oxindolylalanine acted as a "prokynurenine," causing pigment formation.

Meanwhile tryptophan was receiving renewed attention in its metabolic aspects. The work in this field has been reviewed by Neuberger [22] and the main results obtained up to 1944 can be summarised here (for collected references see refs. 22 and 23). It had been shown that kynurenic acid is formed from L-, but not, in most species, from D-tryptophan. Xanthurenic acid is also formed from L-, but not from D-tryptophan, but it is not formed from kynurenic acid. The appearance of kynurenine and xanthurenic acid in the urine seemed to depend on pyridoxine-deficiency, but it appeared likely that kynurenine was a normal metabolite the further breakdown of which was prevented in pyridoxine-deficiency. Both kynurenic and xanthurenic acids appeared to be formed in side-reactions. At this stage

[16] Lepkovsky, Roboz, and Haagen-Smit, *J. Biol. Chem.*, 1943, **149**, 195.
[17] Musajo and Minchilli, *Ber.*, 1941, **74**, 1839 ; Furst and Olsen, *J. Org. Chem.*, 1951, **16**, 412.
[18] Lynen and U. Wieland, *Annalen*, 1937, **533**, 93.
[19] H. Wieland and Witkop, *ibid.*, 1940, **543**, 171.
[20] Butenandt, Weidel, and Becker, *Naturwiss.*, 1940, **28**, 63.
[21] *Idem*, *ibid.*, p. 447. [22] *Ann. Reports*, 1944, **41**, 237.
[23] Steward and Pollard, *ibid.*, 1934, **31**, 341.

(1944) it was therefore reasonable to postulate the following series of changes : tryptophan (I) → oxindolylalalanine (VIII) → kynurenine (V) → kynurenic acid (III). It was suggested [7,22] that the diketo-acid (IX) may be an intermediate between kynurenine and kynurenic acid, whereas xanthurenic acid (VIII) might be derived from 3-hydroxykynurenine (X) or the dihydroxytryptophan (XI).



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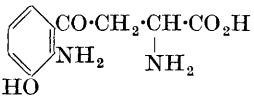

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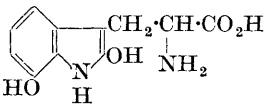

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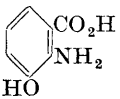


(XII.)

Establishment of the Relation between Tryptophan and Nicotinic Acid.— In 1945 it was shown by Elvehjem and his co-workers [24] that rats deficient in nicotinic acid would grow if given tryptophan, and soon afterwards Rosen, Huff and Perlzweig [25] demonstrated an increased excretion of nicotinic acid derivatives in the urine of rats to which tryptophan had been administered. These reports aroused widespread interest, and numerous confirmatory reports appeared in rapid succession showing that tryptophan was the dietary precursor of nicotinic acid derivatives in the rat, cotton-rat, horse, dog, pig, and man.[26] These experiments did not in themselves show that formation of nicotinic acid had occurred in the animal tissues, it being possible either that the change was brought about by the bacterial flora of the intestine, or that the tryptophan acted as a growth factor for the bacteria without being itself a precursor. Participation of the bacterial flora has been advocated especially by Elvehjem and by Ellinger.[27]

The transformation of tryptophan into nicotinic acid within animal tissues has now been conclusively proved. In 1948 Schweigert *et al.*[28] demonstrated the conversion in the developing chick embryo ; and it was subsequently demonstrated in rats from which the entire intestinal tract had been removed.[29] Moreover it took place to the same extent in infants whether the tryptophan was administered orally or intravenously,[30] and could be shown to occur in liver slices.[31] Nicotinic acid and tryptophan are

[24] Krehl, Teply, Sarma, and Elvehjem, *Science*, 1945, **101**, 489.
[25] *J. Biol. Chem.*, 1946, **163**, 343.
[26] For collected references see Spector, *ibid.*, 1948, **173**, 659.
[27] Ellinger and Emmanuelowa, *Lancet*, 1946, **251**, 716 ; Ellinger, Kader, and Emmanuelowa, *Brit. J. Exp. Path.*, 1947, **28**, 261 ; Ellinger, *Experientia*, 1950, **6**, 144.
[28] Schweigert, German, and Garber, *J. Biol. Chem.*, 1948, **174**, 383.
[29] Henderson and Hankes, *Proc. Soc. Exp. Biol. Med.*, 1949, **70**, 26 ; Hundley, *ibid.*, p. 592.
[30] Snyderman, Ketron, Carretero, and Holt, *ibid.*, p. 569.
[31] Hurt, Scheer, and Deuel, *Arch. Biochem.*, 1949, **21**, 87.

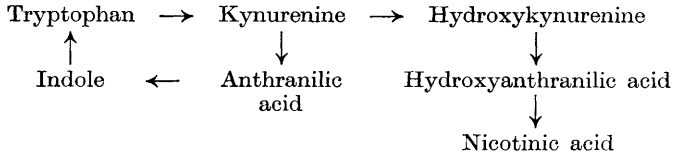
interchangeable in counteracting the growth-retarding effects of 3-acetylpyridine.[32] This does not necessarily imply that the bacterial flora cannot play a subsidiary part, and bacterial action has been suggested, especially by Elvehjem, as responsible for variations obtained with different diets.

During the nutritional work various other results of interest emerged. No increased excretion of *N'*-methylnicotinamide was observed by Rosen *et al.*[33] after administration of kynurenine, kynurenic acid, or xanthurenic acid. However, Kallio and Berg [12] found that kynurenine did function as a precursor of nicotinic acid, and Reid *et al.*[34] found it to act as a precursor of xanthurenic acid. More recently Krehl *et al.*[35] report that kynurenine is not a precursor of nicotinic acid, contrary to the results of Swiss workers.[60] Axelrod *et al.*[36] showed that tryptophan was converted by normal dogs into kynurenine and kynurenic acid with no xanthurenic acid, and by pyridoxine-deficient dogs into kynurenine and xanthurenic acid with no kynurenic acid. More recently Musajo *et al.*[37] have demonstrated the conversion into xanthurenic acid, in rats, of hydroxykynurenine, and to a somewhat lesser extent of kynurenine.

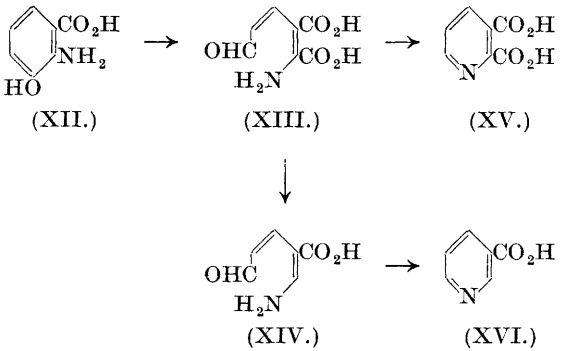
Experiments with Moulds, Bacteria, and Insects.—The probable course of the tryptophan–nicotinic interconversion first became clear from experiments with lower organisms, mainly carried out by Mitchell, Nyc, Bonner, and their co-workers. In 1947 a mutant of *Neurospora crassa* was obtained [38] which required either tryptophan or nicotinic acid for growth, and it was found that the growth requirement could equally be satisfied by kynurenine, the activity of the DL- being half that of the L-isomer. Moreover, administration of excess of kynurenine gave rise to nicotinic acid. It was considered possible that the pyridine ring in nicotinic acid might be derived by oxidation of the benzene ring of kynurenic or xanthurenic acid. Many pyridine carboxylic acids were therefore tested for growth-promoting properties and as a result it was possible to conclude [39] that the pyridine ring was not formed by oxidation of the quinoline derivatives, and also that the oxidation which has occurred in position 8 in the xanthurenic acid molecule preceded closing of the quinoline ring. It was therefore suspected that 3-hydroxykynurenine (X) might be an intermediate, which on oxidation could give 3-hydroxyanthranilic acid (XII). When synthetic [40] (XII) was tested, high growth-promoting activity was found. At the same time Bonner [41] confirmed by direct comparison the identity of 3-hydroxyanthranilic acid with a precursor of nicotinic acid previously shown [42] to

[32] Woolley, *J. Biol. Chem.*, 1946, **162,** 179.
[33] Rosen, Huff, and Perlzweig, *J. Nutrit.*, 1947, **33,** 561.
[34] Reid, Lepkovsky, Bonner, and Tatum, *J. Biol. Chem.*, 1944, **155,** 299.
[35] Krehl, Bonner, and Yanofsky, *J. Nutrit.*, 1950, **41,** 159.
[36] Axelrod, Morgan, and Lepkovsky, *J. Biol. Chem.*, 1945, **160,** 155.
[37] Musajo, Chiancone, and Coppini, *Science*, 1951, **113,** 125.
[38] Beadle, Mitchell, and Nyc, *Proc. Nat. Acad. Sci.*, 1947, **33,** 155.
[39] Mitchell and Nyc, *ibid.*, 1948, **34,** 1.
[40] Nyc and Mitchell, *J. Amer. Chem. Soc.*, 1948, **70,** 1847.
[41] *Proc. Nat. Acad. Sci.*, 1948, **34,** 5.
[42] Bonner and Beadle, *Arch. Biochem.*, 1946, **11,** 319.

be accumulated by a mutant of *Neurospora*. Haskins and Mitchell [43] soon afterwards produced evidence for hydroxykynurenine as a tryptophan metabolite, and also showed that anthranilic acid is formed from tryptophan by *Neurospora* and may be used for resynthesis of indole, and hence of tryptophan. Apart from nicotinic acid formation there exists in *Neurospora* a " cycle " thus :



Evidence for the method of formation of the pyridine ring was produced by Bonner and Yanofsky.[44] It had previously been shown [45] that on administration of tryptophan to rats a substance was excreted in the urine, which, after being heated in an autoclave in acid solution, had nicotinic acid activity, and this substance had been identified as quinolinic acid (XV).[46] Its function in *Neurospora* was therefore examined. A mutant was found which accumulated quinolinic acid as the result of a genetic block, but this acid showed either little or no activity according to the strain examined, so it was suggested that the following changes occurred :



These results were confirmed by Henderson.[68] That the conversion of hydroxyanthranilic acid may be more complicated than is represented above is, however, suggested by experiments in which unlabelled hydroxyanthranilic acid and ^{15}N-labelled ammonium chloride were fed to *Neurospora*.[47] About half the nitrogen of the derived nicotinic acid was shown to originate from the labelled ammonia, and it was suggested that a symmetrical diamino-compound may be an intermediate. The nature of such an intermediate is rather difficult to visualise, and more recent work [47a] suggests

[43] *Proc. Nat. Acad. Sci.*, 1949, **35**, 500. [44] *Ibid.*, p. 576.
[45] Singal, Briggs, Sydenstricker, and Littlejohn, *J. Biol. Chem.*, 1946, **166**, 573.
[46] Henderson, *ibid.*, 1949, **178**, 1005.
[47] Leifer, Langham, Nyc, and Mitchell, *ibid.*, 1950, **184**, 589.
[47a] Yanofsky and Bonner, *ibid.*, 1951, **190**, 211.

that the incorporation of ^{15}N was due to the active growth of the organism (which was not exacting in its growth requirements) during the experiment, with consequent biosynthesis by the organism of ^{15}N-labelled tryptophan from the $^{15}NH_3$. Under conditions when no growth is occurring the nitrogen of nicotinic acid appears to be entirely derived from the nitrogen of hydroxyanthranilic acid.[47a]

Stanier and Tsuchida,[48] using a species of *Pseudomonas*, obtained further evidence for the intermediary function of kynurenine. They adapted their organism to use L-tryptophan as sole source of carbon, and showed that it was also adapted to L-kynurenine and to kynurenic acid. An organism adapted to L-kynurenine was similarly adapted to tryptophan, but an organism adapted to kynurenic acid was not adapted to tryptophan or to kynurenine. The pathway, tryptophan → kynurenine → kynurenic acid, was thereby established, and it was also shown that there is a pathway for kynurenine metabolism other than that through kynurenic acid. In further experiments the pathways, tryptophan (*a*) → indole + serine (reverse of synthesis), (*b*) → indole + ammonia + pyruvic acid (as in *Escherichia coli*), and (*c*) → indolylpyruvic acid, were all eliminated.

At this stage of development interest shifted to the establishment of an analogous metabolic pathway for tryptophan in animals. Before considering this, however, it is convenient to record some further results obtained with lower organisms.

Yanofsky and Bonner [49] obtained a *Neurospora* mutant which could use hydroxykynurenine or hydroxyanthranilic acid, but not tryptophan or kynurenine, as its source of nicotinic acid. If kynurenine is an intermediate this mutant should accumulate kynurenine or a simple derivative or metabolite thereof. It was found that the strain did in fact produce a substance which was identified as N^{α}-acetylkynurenine (XXVI) (p. 241).

Japanese workers [50] showed that in *Pseudomonas* tryptophan is converted into anthranilic acid by way of kynurenine. In *Xanthomonas pruni* the tryptophan–nicotinic conversion resembles that in *Neurospora*,[51] whereas neither kynurenine nor hydroxyanthranilic acid can replace nicotinic acid in *Leuconostoc mesenteroides*, *Streptococcus fæcalis*, *Proteus vulgaris*, or *Torula cremaris*.[52] Tryptophan is converted into nicotinic acid by green leaves.[53]

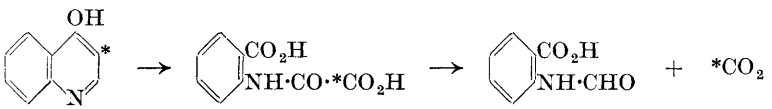
Tryptophan metabolism in insects received attention largely in connection with investigations on eye pigments. Hydroxykynurenine was isolated by Butenandt *et al.*[54] from the pupae of *Calliphora erythroencephala* and identified as the cn$^+$-substance (cf. p. 229). The same workers reported,[54] without experimental details, the synthesis of 3-hydroxy-DL-kynurenine (X) by a method analogous to the synthesis [7] of kynurenine by that

[48] *J. Bacteriol.*, 1949, **58**, 45. [49] *Proc. Nat. Acad. Sci.*, 1950, **36**, 167.
[50] Suda, Hayaishi, and Oda, *Med. J. Osaka Univ.*, 1950, **2**, 21.
[51] Davis, Henderson, and Powell, *J. Biol. Chem.*, 1951, **189**, 543.
[52] Volcani and Snell, *Proc. Soc. Exp. Biol. Med.*, 1948, **67**, 511.
[53] Gustafson, *Science*, 1949, **110**, 279.
[54] Butenandt, Weidel, and Schossberger, *Z. Naturforsch.*, 1949, **4***b*, 242 ; Butenandt, *Angew. Chem.*, 1949, **61**, 262.

school.[54a] A complete report has appeared of a similar synthesis,[55] and an alternative but longer one has been published by the Japanese workers [56] based on the Japanese synthesis [11] of kynurenine. Hydroxykynurenine has also been isolated from larvæ of the silkworm, *Bombyx mori*.[57]

Isotopic and Other Experiments in Animals.—Soon after it had been shown by Mitchell and Nyc that 3-hydroxyanthranilic acid (XII) was a precursor of tryptophan in *Neurospora*,[39] the same workers were able to announce as a result of dietary experiments [58] that the acid also acted as a precursor of nicotinic acid in the rat, and this was confirmed by Albert, Scheer, and Deuel [59] who showed that its administration to rats resulted in increased excretion of *N'*-methylnicotinamide. Further confirmation was provided by Wiss, Viollier, and Müller [60] who showed that hydroxyanthranilic acid, given either by mouth or subcutaneously, could replace nicotinic acid as a growth-factor for rats. Much smaller doses were required when administered subcutaneously, demonstrating once again that the intestinal flora played no part. Anthranilic acid is definitely not a precursor of urinary nicotinic acid in the rat.[61]

Powerful confirmatory evidence for the general pathway of tryptophan metabolism which was now emerging came from isotopic experiments by Heidelberger and his co-workers. Tryptophan was synthesised which was labelled with ^{14}C in the β-position of the side chain.[62] Part was administered to rabbits, and kynurenine isolated from the urine; part was administered to dogs, and urinary kynurenic acid isolated; and part was administered to rats, and urinary *N'*-methylnicotinamide isolated. Isotopically labelled kynurenine (from the rabbit experiments) was also fed to the rats, and *N'*-methylnicotinamide was subsequently isolated. The kynurenine was degraded by means of the iodoform reaction, and the kynurenic acid degraded by decarboxylation to 4-hydroxyquinoline, which was oxidised in alkaline solution to *o*-carboxyoxanilide, and this was decarboxylated:



The results showed unequivocally (1) that the β-carbon atom of the tryptophan side chain becomes the β-carbon atom of the kynurenine side chain, and (2) that the side chain of tryptophan is lost and does not become part of the nicotinic acid molecule.[63] The latter result was confirmed by

[54a] For another, recent synthesis see ref. 11*a*.
[55] Musajo, Spada, and Casini, *Gazzetta*, 1950, **80**, 171.
[56] M. Kotake, Sakan, and Senoh, *J. Amer. Chem. Soc.*, 1951, **73**, 1832.
[57] Hirata, Nakanishi, and Kikkawa, *Science*, 1950, **112**, 307.
[58] Mitchell, Nyc, and Owen, *J. Biol. Chem.*, 1948, **175**, 433. [59] *Ibid.*, p. 479.
[60] *Helv. Physiol. Pharmacol. Acta*, 1949, **7**, *C* 64; *Helv. Chim. Acta*, 1950, **33**, 771.
[61] Hankes, Lyman, and Elvehjem, *J. Biol. Chem.*, 1950, **187**, 547.
[62] Heidelberger, *ibid.*, 1949, **179**, 139.
[63] Heidelberger, Gullberg, Morgan, and Lepkovsky, *ibid.*, p. 143.

Hundley and Bond,[70] using tryptophan labelled with ^{13}C in the carboxyl group. In further experiments [64] tryptophan was synthesised with ^{14}C in the 3-position of the indole nucleus,[62] and this was fed to rats. The *N'*-methylnicotinamide isolated from the urine was converted into nicotinic acid, which was decarboxylated. The *N'*-methylnicotinamide was radioactive, confirming unambiguously the reality of tryptophan → nicotinic acid conversion. Carbon atom 3 of the indole ring, which is the expected precursor of the carboxyl group of hydroxyanthranilic acid, was shown to become the carboxyl-carbon atom of nicotinic acid. It was therefore clear that tryptophan metabolism in the rat and in *Neurospora* follows similar pathways.

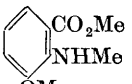
Later, Schayer [65] synthesised [^{15}N]indole, and hence [1-^{15}N]tryptophan, which he fed to rabbits and rats. The results showed that L-tryptophan was converted into kynurenine, kynurenic acid and, in pyridoxine-deficiency, xanthurenic acid with only slight dilution. D-Tryptophan was not used by the rabbit, but was used by the rat. This result is to be expected, as inversion of D-tryptophan is known to be possible in the rat,[66] and was confirmed in this case by administration of labelled D-tryptophan and subsequent isolation of labelled L-tryptophan from the body-protein. Labelled kynurenine was shown to be converted into xanthurenic acid in the rat without dilution, and into kynurenic acid in the rabbit with significant dilution. It was also shown that after administration of the labelled tryptophan the concentration of ^{15}N in the urinary ammonia was four times as high as in the urinary urea, suggesting the existence of an enzyme in rat kidney and liver which splits ammonia from the indole ring of tryptophan, the ammonia then being excreted before it is appreciably converted into urea.

Evidence for the role of quinolinic acid in the metabolic pathway in the rat was also soon obtained. Henderson and Hirsch [67] demonstrated the occurrence of quinolinic acid in the urine of rats fed on a low-casein diet supplemented with tryptophan, and Henderson [68] confirmed the slight but significant effect of quinolinic acid both in *Neurospora* and in the rat. The conversion of hydroxyanthranilic acid into quinolinic acid in rat-liver slices or homogenates was shown by Henderson and Ramasarma,[69] but the conversion of tryptophan, kynurenine, or hydroxyanthranilic acid into nicotinic acid in liver slices could not be demonstrated, contrary to a previous report.[31] Henderson pointed out [68] that the conversion of tryptophan, kynurenine, or hydroxyanthranilic acid into nicotinic acid is low. As quinolinic acid passes through the body of the rat mainly unchanged it was suggested that the decarboxylation of quinolinic acid may be the limiting factor, or alternatively, as was suggested in the case of *Neurospora*, that quinolinic acid is the product of a reversible side-reaction such as ring-closure of an intermediate, *e.g.*, (XIII), between hydroxyanthranilic and nicotinic acids.

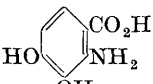
[64] Heidelberger, Abraham, and Lepkovsky, *J. Biol. Chem.*, 1949, **179**, 151.
[65] *Ibid.*, 1950, **187**, 777.
[66] du Vigneaud, Sealock, and van Etten, *ibid.*, 1932, **98**, 565.
[67] *Ibid.*, 1949, **181**, 667. [68] *Ibid.*, p. 677. [69] *Ibid.*, p. 687.
[70] *Arch. Biochem.*, 1949, **21**, 313.

Bokman and Schweigert [71] showed that hydroxyanthranilic acid is converted into quinolinic acid by rat-liver homogenates or acetone-dried powder to the extent of 73—100%. These authors also developed a convenient method for estimation of 3-hydroxyanthranilic acid based on its fluorescence. 3-Hydroxyanthranilic acid has also been shown [72] to be oxidised enzymically to a red product by rat-liver homogenate.

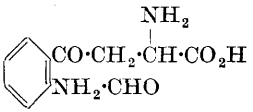
It is of some interest that the trimethyl derivative of hydroxyanthranilic acid is a natural product, occurring as the alkaloid damascenine (XVII).[73]



(XVII.)



(XVIII.)



(XIX.)

The conversion of hydroxyanthranilic into nicotinic acid can reasonably be expected to involve oxidative cleavage of the benzene ring. Makino, Itoh, and Nishi [74] considered that if this were so 3 : 4-dihydroxyanthranilic acid (XVIII) might be an intermediate, and they synthesised it from *o*-aminoveratric acid. Using rat-liver slices they were able to show that both hydroxy- and dihydroxy-anthranilic acids give rise to nicotinic acid whereas, as was to be expected, anthranilic acid did not. But it cannot yet be said that the dihydroxy-acid is a true metabolic intermediate as Mitchell [75] found previously that it does not function as such in *Neurospora*.

Pathway of the Tryptophan → Kynurenine Conversion.—Information on the detailed mechanism of the tryptophan–kynurenine conversion has been largely obtained by Knox and his co-workers. In 1949 it was reported [76] that kynurenine was formed from tryptophan in liver homogenates under aerobic conditions, and soon afterwards detailed studies of the steps involved were published.[77, 78] L-Tryptophan is converted by the supernatant liquid of a fresh liver homogenate of all animal species tested into a diazotisable aromatic amine, identified as kynurenine, with uptake of one molecule of oxygen and formation of one molecule of formic acid, but no carbon dioxide. D-Tryptophan is not metabolised and negative results with other indole and tryptophan derivatives showed the reaction to be highly specific. Under normal conditions the reaction is slow, but if tryptophan is fed to the animal some hours before isolation of the liver enzymes an increase of up to 10-fold in overall activity occurs, a result which is very probably due to a genuine enzymic adaptation.[79] Peroxide generated *in situ* was required

[71] *J. Biol. Chem.*, 1950, **186**, 153.
[72] Viollier and Sullmann, *Helv. Chim. Acta*, 1950, **33**, 776.
[73] Henry, "Plant Alkaloids", 3rd edn., Churchill, London, 1939, pp. 559–560.
[74] *Nature*, 1951, **167**, 115.
[75] Unpublished results cited in *Vitamins and Hormones*, 1950, **8**, 144.
[76] Knox, Mero, Grossmann, and Auerbach, *Fed. Proc.*, 1949, **8**, 214.
[77] Knox and Mehler, *J. Biol. Chem.*, 1950, **187**, 419.
[78] Mehler and Knox, *ibid.*, p. 431.
[79] Knox and Mehler, *Science*, 1951, **113**, 237.

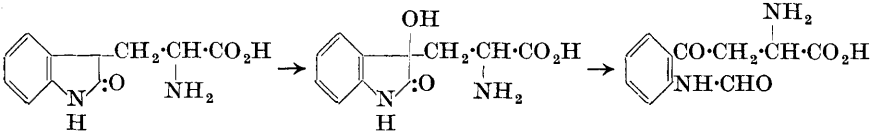
by the system, free added hydrogen peroxide being ineffective. The complete system was separable into two fractions. One fraction converted tryptophan into a substance identified [78] as formylkynurenine (XIX), which is split by a second enzyme, formylase, into kynurenine and formic acid. Formylase, like the first enzyme, shows high specificity. It is of interest to note that Stanier and Hayaishi [80] have since demonstrated the existence of formylase in many bacterial species. The first enzyme system was shown to involve two steps ; in the first tryptophan is converted by peroxide under the action of a peroxidase into an unknown intermediate, A, and this step is followed by a second, coupled reaction in which A is oxidised to formylkynurenine with simultaneous formation of peroxide which, in the absence of other sources of hydrogen peroxide, may be used in the first step. The three stages therefore are :

(*a*) Tryptophan + H_2O_2 $\xrightarrow{\text{peroxidase}}$ A

(*b*) A $\xrightarrow[\text{oxidase}]{O_2}$ Formylkynurenine + H_2O_2

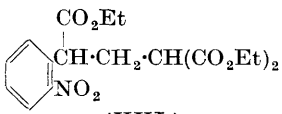
(*c*) Formylkynurenine $\xrightarrow{\text{Formylase}}$ Kynurenine + $H{\cdot}CO_2H$

Evidence already described had suggested that oxindolylalanine (VIII) was a tryptophan metabolite, and it was reasonable to suspect that it might be the above intermediate, A. The 2-position of the indole ring is fairly reactive, and the conversion of (I) into (VIII*a*) would involve hydroxylation, the occurrence of which in the body was not unreasonable. (VIII*a*) is tautomeric with (VIII*b*), which is an oxindole, and oxindoles are well known to undergo oxidation to dioxindoles ; [81] in this case (VIII*b*) would give the substance (XX), which is a cyclic α-ketol tautomeric with formyl-

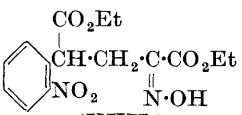


(VIII*b*.) (XX.) (XIX.)

kynurenine (XIX). Oxindolylalanine (VIII), however, long resisted attempts at synthesis. In 1947 Witkop [82] succeeded in obtaining it in low yield by per-acid oxidation of tryptophan, but more recently two convenient



(XXI.)

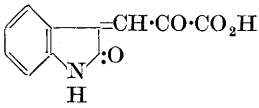


(XXII.)

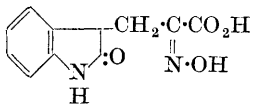
syntheses have appeared. Kotake, Sakan, and Miwa [83] condensed *o*-nitrophenylacetic ester with ethyl methylenemalonate to give the tri-ester (XXI). This on treatment with ethyl nitrite gave the oximino-ester (XXII), reduc-

[80] Private communication to Dr. W. E. Knox.
[81] Sumpter, *Chem. Reviews*, 1945, **37**, 443.
[82] *Annalen*, 1947, **558**, 98.
[83] *J. Amer. Chem. Soc.*, 1950, **72**, 5085.

tion and hydrolysis of which were followed by cyclisation to (VIII). In a rather shorter synthesis English workers [84] condensed isatin with pyruvic ester to give 3-oxindolidenepyruvic acid (XXIII). This on reduction with dithionite and treatment with hydroxylamine gave the oximino-acid (XXIV), reduction of which gave (VIII). The product was shown to exist in the oxindole form (VIII*b*), rather than the hydroxyindole form (VIII*a*).[85]



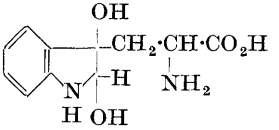
(XXIII.)



(XXIV.)

With the availability of synthetic oxindolylalanine it rapidly became clear that it is not a normal tryptophan metabolite. Sakan and Hayaishi [86] showed that it was not metabolised by *Pseudomonas* which had been adapted to tryptophan or kynurenine. Amano, Torii, and Iritani [87] confirmed this, and showed that formylkynurenine was metabolised by tryptophan-adapted cells but not by kynurenine-adapted cells, as was to be expected from its postulated position in the metabolic chain. (Formylkynurenine has so far only been synthesised in very low yield by ozonisation of tryptophan.[90,78,87]) They also quoted unpublished results of Kikkawa that pure oxindolylalanine is not a precursor of insect eye-pigment. Mason and Berg [88] in experiments with rat-liver slices showed that the metabolism of oxindolylalanine was quite different from that of tryptophan or kynurenine, and the same result was obtained with intact animals by Dalgliesh, Knox, and Neuberger.[89] Moreover the latter workers showed that oxindolylalanine gives rise to some kynurenine by slow spontaneous decomposition in solution, which might account for its apparent low activity [21] as a " prokynurenine " in insect eye-pigment formation. Even its apparent occurrence as a natural product in phalloidine was shown spectroscopically to be doubtful, as it was probably formed by hydrolysis of a 2-substituted tryptophan derivative.[85, 89]

The identity of the unknown intermediate, A, which is the first product of tryptophan metabolism therefore remains open to question. It has been



(XXV.)

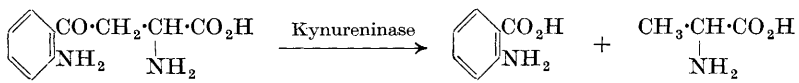
suggested by Knox *et al.*[77, 89] that peroxide adds to tryptophan to give 2 : 3-dihydro-2 : 3-dihydroxytryptophan (XXV), which by dehydrogenation

[84] J. W. Cornforth, R. H. Cornforth, Dalgliesh, and Neuberger, *Biochem. J.*, 1951, **48**, 591.
[85] J. W. Cornforth, Dalgliesh, and Neuberger, *ibid.*, p. 598.
[86] *J. Biol. Chem.*, 1950, **186**, 177.
[87] *Med. J. Osaka Univ.*, 1950, **2**, 45.
[88] *J. Biol. Chem.*, 1951, **188**, 783.
[89] *Nature*, 1951, **168**, 20.
[90] Witkop and Graser, *Annalen*, 1944, **556**, 103.

with simultaneous ring opening could then give formylkynurenine. The suggestion is attractive, but verification must await the synthesis or isolation of the diol.

Kynurenine, Pyridoxine, and Removal of the Side Chain.—That pyridoxine-deficiency causes marked alterations in tryptophan metabolism was evident from early work. Attempts were made to throw light on the problem by means of nutritional experiments,[91] but many of the results appeared contradictory or inconclusive. To some extent this was due to incomplete knowledge of nicotinic acid metabolism, and the consequent basing of conclusions on estimations of an inappropriate derivative. The particular nicotinic acid derivative produced varies in different species, and in man, for example, where the principal metabolite is 1-methyl-2-pyridone-5-carboxyamide [92] significant results may not be obtained by estimating *N'*-methylnicotinamide. However, it was known that pyridoxine-deficiency in general caused excretion of xanthurenic acid where a normal animal would have excreted kynurenic acid. Recent improved methods of estimation [93] show that kynurenic and xanthurenic acids are not mutually exclusive metabolites and are frequently excreted together.

In 1947 two groups of workers showed that pyridoxine-deficient rats [94, 33] and mice [94] had a greatly decreased ability to convert tryptophan into nicotinic acid derivatives. Later reports [95] claimed that pyridoxine had no effect on the conversion, but this result may be due to the fact that on very-low-tryptophan diets the effect of pyridoxine becomes less marked.[96] Pyridoxine-deficiency also decreases the conversion of kynurenine into nicotinic acid derivatives,[33] and the decreased conversion of both tryptophan and kynurenine has since been confirmed.[97]



(XXIX.)

The first indications of the mode of action of pyridoxine emerged from enzymic experiments. Kotake and Nakayama [98] in 1941 found an enzyme in cat liver and kidney which converts kynurenine into anthranilic acid (XXIX), and this enzyme they called kynureninase. It was further studied by Braunshtein in Russia and Wiss in Switzerland. Braunshtein *et al.*[99]

[91] The nutritional experiments were usually primarily concerned with the larger problem of anti-pellagra activity, and have been excellently reviewed by Chick, *Nutrit. Abs. and Reviews*, 1951, **20**, 523.

[92] Knox and Grossmann, *J. Biol. Chem.*, 1946, **166**, 391 ; Holman and de Lange, *Biochem. J.*, 1949, **45**, 559 ; *Nature*, 1950, **165**, 112, 604.

[93] Musajo and Coppini, *Experientia*, 1951, **7**, 20 ; and references therein.

[94] Schweigert and Pearson, *J. Biol. Chem.*, 1947, **168**, 555.

[95] Spector, *ibid.*, 1948, **173**, 659 ; Heimberg, Rosen, Leder, and Perlzweig, *Arch. Biochem.*, 1950, **28**, 225.

[96] Scheer and Deuel, *J. Nutrit.*, 1948, **35**, 239.

[97] Henderson, Weinstock, and Ramasarma, *J. Biol. Chem.*, 1951, **189**, 19.

[98] *Z. physiol. Chem.*, 1941, **270**, 76.

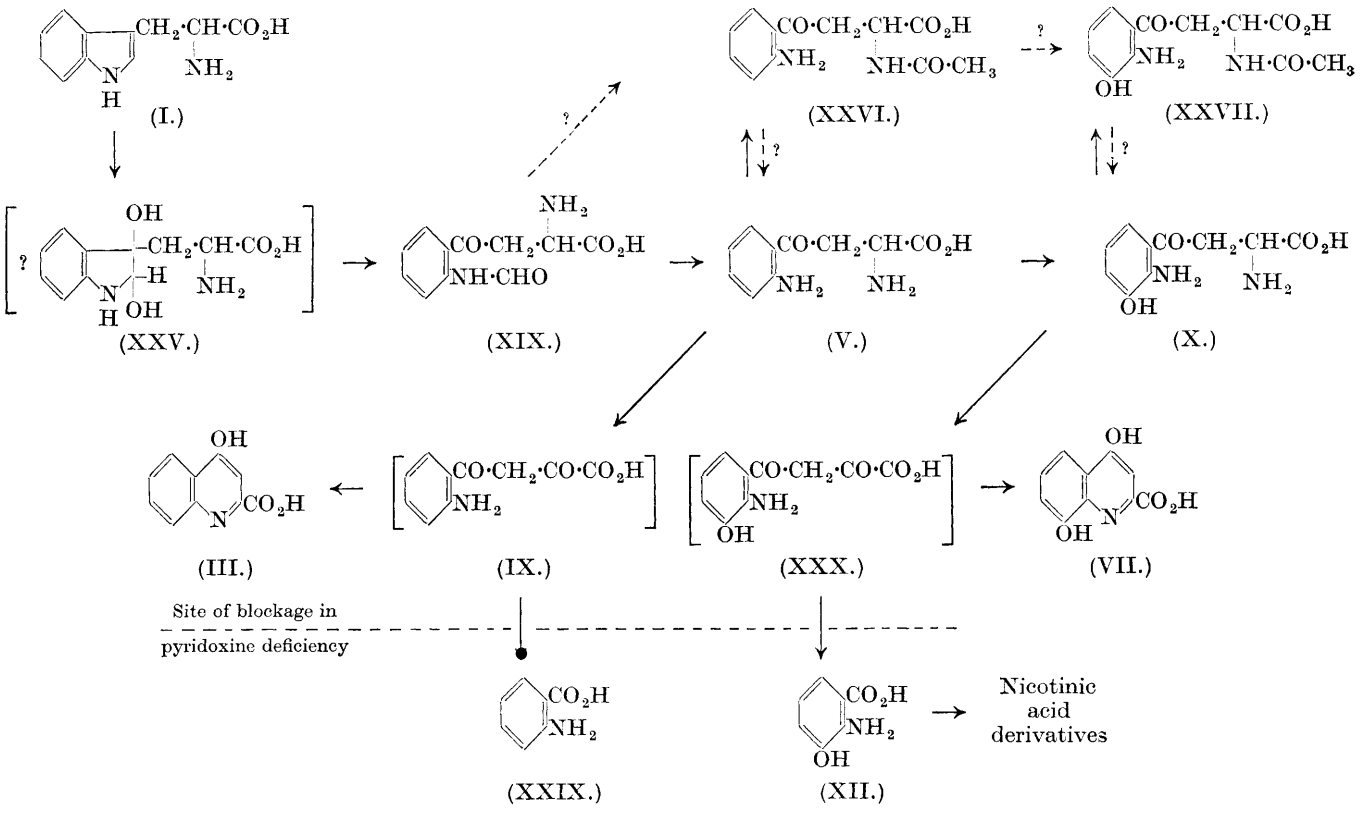
[99] Braunshtein, Goryachenkova, and Pashkina, *Biokhimiya*, 1949, **14**, 163.

found that kynureninase converted kynurenine into alanine as well as anthranilic acid, and that the enzyme was present in the liver and kidney of man and of all other animals studied. Wiss and his co-workers [100] found the same overall reaction and later demonstrated analogous reactions with other compounds containing the —CO·CH_2·CH(NH_2)·CO_2H grouping,[101, 102] including hydroxykynurenine.[102] Braunshtein *et al.*[99] found that pyridoxine did not affect the conversion of tryptophan into kynurenine, and showed that the kynureninase activity of the liver of pyridoxine-deficient animals fell to a small fraction of the activity in normal animals, but that it could be restored *in vitro* by the addition of pyridoxal phosphate. These results have now been confirmed by Knox *et al.*,[89] who resolved the enzyme from normal animals and verified the requirement for pyridoxal phosphate. These authors found that after prolonged pyridoxine-deficiency the kynureninase activity was not only much reduced, but could not be restored *in vitro* by the addition of pyridoxal phosphate. The latter result probably accounts for the fact that after a prolonged period of pyridoxine-deficiency the nutritional effect may not be reversed by addition of pyridoxine to the diet [*e.g.*, 33, 96]. As pyridoxine-deficiency is known [97] to have no effect on the conversion of hydroxyanthranilic acid into nicotinic acid, the site of action of pyridoxine therefore becomes clear.

The study of tryptophan metabolism has recently been greatly facilitated by the techniques of paper-chromatography. The separation and identification of kynurenine and tryptophan were recorded in 1950 [77, 103] and investigations of the possible role of oxindolylalanine led to further study of the separation and identification of tryptophan metabolites.[85, 104] The approach has since been widely developed.[88, 89, 105] The majority of tryptophan metabolites show characteristically fluorescent spots under ultra-violet illumination. By application of ninhydrin, Ehrlich's, Pauly's, and Hellman's reactions (diazotisation followed by coupling with ethyl-α-naphthylamine [104]) considerable information on the chemical nature of the metabolites can be obtained.

Using paper-chromatographic and enzymic techniques Dalgliesh, Knox, and Neuberger [89] showed that pyridoxine-deficient rats converted tryptophan into a large number of substances all of which still carried the carbon skeleton of the original tryptophan side chain. Amongst other products they demonstrated the occurrence of hydroxykynurenine (X), N^{α}-acetylkynurenine (XXVI) and probably of N^{α}-acetyl-3-hydroxykynurenine (XXVII) (see scheme, p. 241). (It is of interest that kynurenine isolated from urine has frequently [*e.g.*, 6, 65] given analytical figures suggesting the presence of more oxygen than the formula (V) requires. It is possible that in these cases the kynurenine was contaminated with hydroxykynurenine.) Kynureninase was purified many-fold, and the results of Braunshtein *et al.*

[100] Wiss and Hatz, *Helv. Chim. Acta*, 1949, **32**, 532; Wiss, *ibid.*, p. 1694.
[101] Wiss and Fuchs, *ibid.*, p. 2553. [102] *Idem, Experientia*, 1950, **6**, 472.
[103] Tabone, Robert, Thomassey, and Mamounas, *Bull. Soc. Chim. biol.*, 1950, **32**, 529.
[104] Hellman, *Z. physiol. Chem.*, 1951, **287**, 205.
[105] Mason and Berg, *Fed. Proc.*, 1951, **10**, 221.


(I.)
(XXV.)
(XIX.)
(V.)
(X.)
(XXVI.)
(XXVII.)
(III.)
(IX.)
(XXX.)
(VII.)
Site of blockage in
pyridoxine deficiency
(XXIX.)
(XII.)
Nicotinic
acid
derivatives

were extended to hydroxykynurenine. Furthermore, kynurenic acid was shown always to be present amongst the products formed by kynureninase. To simplify discussion the various compounds and their inter-relation are summarised on p. 241. Substances merely postulated, or as yet undemonstrated, are enclosed in square brackets.

The transformation of kynurenine into anthranilic acid and alanine is a surprising reaction if it takes place in one step. Pyridoxal phosphate is well known to be concerned with, amongst other reactions, transaminations [106] and it is possible to satisfy the overall stoicheiometric result by the following series of transamination reactions [89] (where R = *o*-aminophenyl, Pyd·CHO = pyridoxal, and Pyd·CH_2·NH_2 = pyridoxamine):

(1) R·CO·CH_2·CH(NH_2)·CO_2H + Pyd·CHO → R·CO·CH_2·CO·CO_2H + Pyd·CH_2·NH_2
(V.) (IX.)

(2) R·CO·CH_2·CO·CO_2H + H_2O → R·CO_2H + CH_3·CO·CO_2H
(IX.) (XXIX.)

(3) CH_3·CO·CO_2H + Pyd·CH_2·NH_2 → CH_3·CH(NH_2)·CO_2H + Pyd·CHO

Such postulated reactions would explain both the formation of alanine and anthranilic acid (XXIX), and the need for pyridoxine. Enzymes splitting $\alpha\gamma$-diketo-acids of the type of (IX) have previously been demonstrated.[107] By an analogous series of reactions hydroxykynurenine could give the diketo-acid (XXX), which might be split to give hydroxyanthranilic acid (XII) and alanine. Moreover the diketo-acids (IX) and (XXX) might be expected to cyclise very readily to give kynurenic acid (III) and xanthurenic acid (VII), and in fact the only recorded attempt [108] to synthesise (IX) resulted in spontaneous cyclisation to (III), none of the diketo-acid being isolated. These changes would therefore explain why formation of anthranilic acid is always accompanied by formation of kynurenic acid in the enzymic experiments.[89] It has not yet proved possible to get conclusive evidence either for or against this mechanism, but definite evidence might be obtained if a synthesis of the diketo-acid (IX) were accomplished. As pyridoxine is also concerned with decarboxylation reactions involving breaking of a carbon–carbon bond, direct fission of kynurenine by kynureninase remains a possible reaction mechanism.[108a]

The identification of the acetyl compounds (XXVI) and (XXVII) as mammalian excretory products is of some interest, especially as the former has been previously isolated from *Neurospora*.[49] These compounds may indeed only be detoxication products, but alternatively acetylation may be a mechanism by which the body directs the chain of metabolic reactions. Thus, if formylkynurenine (XIX) were normally converted into N^{α}-acetyl-

[106] Cohen, "The Enzymes", Academic Press, New York, 1951, Vol. I, Pt. II, p. 1040.

[107] Meister, *J. Biol. Chem.*, 1949, **178**, 577.

[108] Musajo, Spada, and Bulgarelli, *Gazzetta*, 1950, **80**, 161.

[108a] Cf. Meister, Sober, and Tice, *J. Biol. Chem.*, 1951, **189**, 577, for a possibly analogous reaction.

kynurenine (XXVI), either by coupled acetylation and deformylation or by way of the corresponding N^{α}-acetyl-N^{1}-formyl derivative, then the acetyl compound (XXVI) may be the substrate which undergoes hydroxylation to give N^{α}-acetyl-3-hydroxykynurenine (XXVII), this subsequently undergoing deacetylation to give hydroxykynurenine (X). By this means the body could prevent formation of kynurenic and anthranilic acids, which are substances not giving rise to nicotinic acid. Moreover, this might explain the contradictory results which, as already shown, have been obtained in investigations of the availability of kynurenine as a nicotinic acid precursor. If the metabolic pathway includes the acetyl compounds (XXVI) and (XXVII), kynurenine would no longer be a normal intermediate, and its availability as a precursor would depend on the possibility of acetylation. However, the suggestion can at present only be regarded as speculative, as must be also the suggestion [79] that variations in the apparent availability of tryptophan as a precursor are caused by the adaptive nature of the enzymes.

Little has yet been published on the conversion of kynurenine into hydroxykynurenine. It has been suggested that riboflavin is involved in the change.[97] It is also possible that a part of the tryptophan is directly hydroxylated to 7-hydroxytryptophan. If dihydroxyanthranilic acid (XVIII) is an intermediate there would be reason to suspect the possible occurrence amongst tryptophan metabolites of 3 : 4-dihydroxykynurenine, and the corresponding dihydroxykynurenic acid. No evidence for these compounds has yet been reported.

Other Metabolic Pathways.—Various aspects of tryptophan metabolism have not been considered in this Review, in particular the tryptophanase reaction by which tryptophan is split to indole, pyruvic acid, and ammonia : this reaction occurs almost exclusively in bacteria, and has been reviewed by Happold.[109] The formation of urinary pigments from tryptophan has not been discussed : it has been established that in the rat the indole ring of tryptophan plays no part in the formation of the pyrrole ring of porphyrins.[65]

Of considerable interest is the wider problem of the function of tryptophan as a precursor of the pyridine nucleotides, a subject on which as yet little work has been done. Ling *et al.*[110] showed that injection of tryptophan into normal rats produced a rise in the diphosphopyridine nucleotide content of the erythrocytes, but this did not occur in pyridoxine-deficient rats. Nicotinic acid was also converted into diphosphopyridine nucleotide, and on this change pyridoxine had no effect. Elvehjem *et al.*[111] found tryptophan to act as a precursor of pyridine nucleotides in rat liver, but found nicotinamide to have little or no effect. Later they confirmed the effect of tryptophan and showed [112] that in young rats nicotinamide had a sparing effect for the pyridine nucleotides, but that in adult rats it had no effect. The

[109] *Adv. Enzymology*, 1950, **10**, 51.
[110] Ling, Hegsted, and Stare, *J. Biol. Chem.*, 1948, **174**, 803.
[111] Williams, Feigelson, and Elvehjem, *ibid.*, 1950, **187**, 597.
[112] Williams, Feigelson, Shahinian, and Elvehjem, *ibid.*, 1951, **189**, 659.

same authors then produced evidence [113] that pyridoxine-deficiency has no effect on the conversion of tryptophan into liver pyridine nucleotides. Thus the position is not clear and it is not yet possible to fit the results into the accepted pathways of tryptophan metabolism.

Summary.—It is now clear that tryptophan is the precursor of nicotinic acid and its derivatives in animals and in many moulds, bacteria, insects, and higher plants. The general metabolic pathway seems to be the same in both higher and lower forms of life. The most probable pathway is: tryptophan (I) → formylkynurenine (XIX) → kynurenine (V) → 3-hydroxykynurenine (X) → 3-hydroxyanthranilic acid (XII) → nicotinic acid (XVI). Between tryptophan and formylkynurenine there lies an unknown intermediate which may be 2 : 3-dihydro-2 : 3-dihydroxytryptophan (XXV), but is not, as previously supposed, β-3-oxindolylalanine (2-hydroxytryptophan) (VIII). The stages between hydroxyanthranilic acid and nicotinic acid have yet to be established. 3 : 4-Dihydroxyanthranilic acid (XVIII) and quinolinic acid (XV) may well be intermediates, and the change must also involve one or more acyclic compounds such as (XIII) or (XIV). Kynurenic acid (III), xanthurenic acid (VII), and anthranilic acid (XXIX) appear to be metabolites formed in side reactions from the kynurenines, as are possibly the N^{α}-acetyl derivatives (XXVI) and (XXVII). The properties of the enzyme systems tryptophan peroxidase-oxidase and formylase, which together convert tryptophan into kynurenine, and of kynureninase which removes the side chains from kynurenine and hydroxykynurenine, have been extensively studied. Pyridoxine has been shown to be essential for the function of kynureninase, and in the absence of pyridoxine the requisite splitting of the side chain is greatly decreased, thereby preventing nicotinic acid formation and causing an accumulation of products in which the skeleton of the tryptophan side chain is still present.

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[113] *Proc. Soc. Exp. Biol. Med.*, 1951, **76**, 441.