

## The Relation between Pyridoxin and Tryptophan Metabolism, Studied in the Rat

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(Received 28 December 1951)

It was long considered that the first product of tryptophan degradation in animals was hydroxytryptophan. This hypothesis rested on indirect evidence (for details and references see Dalgliesh, 1951) and it was not till synthetic hydroxytryptophan became available (Cornforth, Cornforth, Dalgliesh & Neuberger, 1951; Kotake, Sakan & Miwa, 1950) that direct evidence could be obtained. The problem was investigated in this laboratory by the feeding of supplementary tryptophan or hydroxytryptophan to normal and pyridoxin-deficient rats and subsequent chromatographic examination of the urines. As already reported (Dalgliesh, Knox & Neuberger, 1951) the complete difference in the urinary pictures obtained after feeding hydroxytryptophan (which actually exists in its tautomeric form and is more correctly described as oxindolylalanine; Cornforth, Dalgliesh & Neuberger, 1951) showed clearly that it is not a normal tryptophan metabolite. This result has now been confirmed by many workers (cf. Dalgliesh, 1951).

These investigations also showed that the urines of pyridoxin-deficient rats fed supplementary tryptophan contained many substances showing a powerful fluorescence in ultraviolet light, which did not occur either in the urine of normal rats with or without a tryptophan supplement in the diet, or in the urine of pyridoxin-deficient rats receiving no tryptophan supplement. These substances therefore represent tryptophan metabolites formed or accumulated as a result of the pyridoxin deficiency and their identification should throw light on both tryptophan metabolism and the function of pyridoxin. This paper describes a qualitative investigation of these substances.

### EXPERIMENTAL AND RESULTS

#### *Animals and diet*

The rats used were Institute stock strain. From the age of 4 weeks they were fed a diet consisting of: casein (unextracted), 10%; fat mixture, 10%; sucrose, 36%; starch, 40%; and salt mixture, 4%. The fat mixture consisted of arachis oil, 80%, and cod liver oil, 20%. The salt mixture consisted of: NaCl, 22 g.; hydrated calcium phosphate, 130 g.; potassium citrate, 125 g.;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 30 g.; ferric citrate, 5 g.; trace mixture, 0.7 g. The trace mixture consisted of: KI, 12 g.; NaF, 10 g.; anhydrous  $\text{MnSO}_4$ , 2 g.;

$\text{Cu}_2\text{I}_2$ , 1 g.; anhydrous potassium alum, 1 g. To each kg. of the above diet was added a vitamin mixture (cf. Reid, Lepkovsky, Bonner & Tatum, 1944) composed of: thiamine, 10 mg.; riboflavin, 10 mg.; calcium pantothenate, 40 mg.; choline, 500 mg.; nicotinic acid, 100 mg.; *p*-aminobenzoic acid, 600 mg.; inositol, 1 g.; biotin, 25  $\mu\text{g}$ .; vitamin K, 20  $\mu\text{g}$ .;  $\alpha$ -tocopherol, 10 mg.

On this regime the rats continued to grow, but only slowly. Their mean weights were: initially, 35 g.; after 3 months, 91 g.; after 6 months, 124 g.; after 9 months, 162 g.; after 13 months, 171 g. Out of seven rats of the original litter, two were killed after 5.5 months and two after 12 months when they developed acute symptoms of acrodynia. After 13 months the remaining animals were lively and well, and the same applied after 8 months to two animals subsequently placed on the diet.

The continuous, though slow, growth suggests that the diet was low in pyridoxin rather than completely deficient. The rats were in general able to adapt themselves to this low intake, which may have been derived from the casein (unextracted) or the intestinal bacteria. The results of the present work show that the amount of available pyridoxin was insufficient to enable the animals to deal normally with supplementary tryptophan in the diet. There were no apparent differences between animals which received a tryptophan supplement frequently and those which received it rarely, nor were there obvious differences between the urinary pictures of animals which developed acrodynia and those which remained well.

The first feeding experiments were carried out after 3 weeks on the diet. Supplementary tryptophan was administered by mixing with the diet at the rate 0.1 g. of L-tryptophan or 0.2 g. DL-tryptophan/rat/day for 1 or 2 days, the urine being collected for the subsequent 48 hr. The greater part of the work was done using L-tryptophan, but the same fluorescent substances were obtained after feeding the DL-isomers.

#### *Paper chromatography*

In the preliminary experiments the ascending technique (Williams & Kirby, 1948) on Whatman no. 1 paper was used at room temperature. It became clear that resolution at low  $R_f$  values was not being obtained and subsequently the descending technique (Consden, Gordon & Martin, 1944) on Whatman no. 4 paper was used, the solvent front being allowed to travel about 60 cm. The mean  $R_f$  values by the two techniques differed slightly, but individual variations between chromatograms were rendered unimportant by running reference substances.

Many solvent systems were examined. Those principally used were: phenol/water or phenol/ammonia as described by Consden *et al.* (1944); 'collidine', a 1:1 mixture of 2:4:6-

collidine and 2:4-lutidine saturated with 2 vol. of water (Dent, 1948); and the organic phase from a freshly prepared mixture of butanol (4 parts), acetic acid (1 part) and water (5 parts) (Partridge, 1946). Of these the last was by far the best in that it gave almost complete resolution of the fluorescent substances on one-dimensional chromatograms. It will be referred to in this paper as butanol/acetic acid.

#### Diagnostic reactions

Five properties or reactions were used for the routine examination of the chromatograms: (1) fluorescence under ultraviolet light, (2) the ninhydrin reaction, (3) Ehrlich's reaction, (4) Ekman's reaction and (5) Pauly's reaction. In addition various special reactions were applied as occasion demanded. The scope of these reactions, as applied to tryptophan metabolites and related compounds, was investigated, with the following results:

(1) *Fluorescence under ultraviolet light.* The light source was a Hanovia fluorescence lamp, model XI, fitted with a Wood's glass filter of which the principal transmission was around 3660 Å., with very low transmission in the visible. Under the conditions of chromatography used in this work negligible fluorescence was shown by ordinary amino-acids (including tryptophan), tryptamine, *p*-aminobenzoic acid, nicotinic acid or amide, and quinolinic acid. On the other hand, many known tryptophan metabolites and related substances showed powerful fluorescence with considerable variation in colour, and these are summarized in Table 1. Kynurenic acid after running in either butanol/acetic acid or

(3) *Ehrlich's reaction.* This is of particular value in that it reveals both the indole type of structure, and also amino compounds. Moreover, the colours obtained with different types of structure vary widely and characteristically, as do the times taken for the colours to appear.

Table 1. *Fluorescence on paper of some reference substances under ultraviolet light*

Substance	Colour of fluorescence
Kynurenine	Strong pale blue
<i>N</i> α-Acetylkynurenine	Strong pale blue
<i>o</i> -Aminoacetophenone	Strong pale blue
<i>N'</i> -Formylkynurenine	Weak medium blue
<i>N'</i> -Acetylkynurenine	Weak medium blue
3-Hydroxykynurenine	Green
Kynurenic acid	Weak dark blue
Xanthurenic acid	Strong blue
Anthranilic acid	Purple
Acetylthranilic acid	Purple
3-Hydroxyanthranilic acid	Blue-purple

After running in an acid or neutral solvent, the reagent was applied as a 2% (w/v) solution of *p*-dimethylamino-benzaldehyde in 5% HCl, and the chromatogram was allowed to hang at room temperature. After running in a basic solvent difficult to remove, such as collidine, it was found advisable either to increase the concentration of HCl in the spraying reagent or to expose the chromatogram to HCl fumes prior to spraying, in order to avoid formation of an objectionable pink background.

Table 2. *Colours produced on paper by reaction between reference substances and Ehrlich's reagent*

Substance	Immediate colour	Colour after 2 hr.	Colour after 24 hr.
Tryptophan	—	Violet	Green
<i>N</i> α-Acetyltryptophan	—	Blue	Blue
Indole	Violet	Carmine	Carmine
Skatole	Violet after about 2 min.	Violet	Blue
Indolylpropionic acid	—	Blue	Blue
Indolylacetic acid	—	Blue	Blue
Tryptamine	—	Violet	Grey-violet
Oxindolylalanine	Very pale yellow	Pale yellow	Pale yellow
Oxindole	—	Pale yellow	Pale yellow
Kynurenine	Orange	Orange	Orange
<i>N</i> α-Acetylkynurenine	Orange	Orange	Orange
<i>o</i> -Aminoacetophenone	Orange	Orange	Orange
3-Hydroxykynurenine	Orange pink (slight delay in appearance)	Pink	Pink
Anthranilic acid	Deep yellow	Deep yellow	Deep yellow
Acetylthranilic acid	—	—	Yellow
3-Hydroxyanthranilic acid	Very pale yellow	Pale yellow	Orange
Aniline	Deep yellow	Deep yellow	Deep yellow
<i>p</i> -Aminobenzoic acid	Deep yellow	Deep yellow	Deep yellow
Aliphatic amino-acids and amines	—	—	Pale yellow after some days
Urea	Bright yellow	Bright yellow	Bright yellow
Allantoin	Weak pale yellow	Pale yellow	Pale yellow

collidine leaves a weak blue fluorescent spot on the origin. In the absence of reference standards the actual description of a colour in this and other colour tests must be regarded to some extent as subjective.

(2) *The ninhydrin reaction.* This was carried out in the usual way. The only point calling for comment is the orange colour given by certain *N'*-acylated kynurenes and derivatives of α-amino-β-benzoylpropionic acid, discussed later in the paper.

The results obtained are summarized in Table 2. In general, indole derivatives give violet or blue colours appearing only after a delay and sometimes changing in colour later. Derivatives of *o*-aminoacetophenone with an unsubstituted amino group give an orange colour immediately, whereas 3-hydroxykynurenine gives a pink (or orange-pink) colour more slowly. Aromatic amines give an immediate deep-yellow colour, whereas aliphatic amines give very pale yellow colours only visible after a long delay.

Urea gives an immediate strong bright yellow colour (cf. citrulline: Dent, 1948), and should therefore as far as possible be removed from urine preparations.

(4) *Ekman's reaction.* Aromatic amino groups were revealed by spraying the chromatogram, after diazotization, with ethyl-1-naphthylamine as a coupling reagent (Ekman, 1948; Hellmann, 1951) and this will be referred to subsequently as Ekman's reaction. Both Ekman and Hellmann carried out diazotization by spraying with dilute  $\text{NaNO}_2$  in HCl. In the present work diazotization was carried out by exposing the lightly damped paper to nitrous fumes generated in a beaker from  $\text{NaNO}_2$  and HCl. Aromatic amines give a magenta colour increasing in intensity on standing, but the reaction also gives much additional information. On exposure to nitrous fumes (and before spraying with the coupling reagent) tryptophan immediately gives a yellow colour, becoming browner on standing, which is presumably due to formation of the nitroso derivative. A yellow colour is also given by *o*-aminophenols such as 3-hydroxykynurenine and 3-hydroxyanthranilic acid, and after spraying with the coupling reagent and allowing to stand the colour becomes a deep brown. The initial yellow colour is in this case presumably due to benzoxadiazole formation.

(5) *Pauly's reaction.* This was carried out in the usual way. In the presence of excess nitrite tryptophan gave a yellow spot becoming brown, as with Ekman's reagent. *o*-Aminophenols gave a brown spot increasing in intensity on standing.

(6) *Ammoniacal silver nitrate.* *o*-Aminophenol derivatives such as 3-hydroxykynurenine and 3-hydroxyanthranilic acid gave a brown-black spot immediately on spraying. On being allowed to dry at room temperature red-brown spots also appeared with kynurenine, *o*-aminoacetophenone, xanthurenic acid, 8-hydroxyquinoline, and tryptophan.

(7) *Phosphate reagent.* (Hanes & Isherwood, 1949.) Comparison of radioautographs of chromatograms carrying  $^{32}\text{P}$  with 'phosphate' spots as revealed by this reagent showed that artifacts can occur. In particular it was found that after spraying with the perchloric-molybdate solution, and heating, tryptophan gives a blue spot (before  $\text{H}_2\text{S}$  treatment).

#### Behaviour of reference substances

The fluorescence and behaviour to various reagents of many of these substances are described above. Below are summarized some  $R_F$  values not otherwise mentioned in the text, together with certain comments on reactivity, etc. The  $R_F$  values should be taken as approximate, as many depend on single or a few determinations where this was adequate to show by direct comparison non-identity with one of the metabolites. Butanol/acetic acid is abbreviated as B/A; collidine as C; ascending chromatograms as (a), and descending as (d).

(A) *Derivatives of indole.*  $N^\alpha$ -Acetyltryptophan: B/A (a), 0.91; C(d), 0.70. Indolylpropionic acid: B/A (a), 0.93; C(d), 0.84. Indolylacetic acid: C(d), 0.69. Tryptamine: B/A (d), 0.66; C(d), 0.70 (weak) and 0.78 (strong). Skatole: C(d), 0.96. Indole: B/A (a), 0.98; C(d), 0.93.

(B) *Derivatives of o-aminoacetophenone.* DL-Kynurenine and DL-3-hydroxykynurenine are both resolved on paper chromatograms. In both cases the  $R_F$  value of the D-isomer is about 0.9 times that of the L-isomer (Dalglish, 1952). In a B/A (d) chromatogram a solvent run of 60 cm. results in

complete separation of the kynurenine isomers, but hydroxykynurenine, with a lower  $R_F$ , may appear as a dumb-bell shaped spot.

$N'$ -Formylkynurenine (B/A (d), 0.41) and  $N'$ -acetylkynurenine (B/A (d), 0.49) give an orange colour with Ehrlich's reagent only after some while, when hydrolysis of the acyl group has presumably occurred. Both give an orange colour with ninhydrin, as does also a precursor in kynurenine synthesis,  $\alpha$ -amino- $\beta$ -*o*-nitrobenzoylpropionic acid. Neither are resolved on paper chromatograms, nor is  $N^\alpha$ -acetylkynurenine. *o*-Aminoacetophenone moves with or close to the solvent front.

(C) *Derivatives of anthranilic acid and miscellaneous substances:* Anthranilic acid: B/A (d), 0.93; C(d), 0.76. 3-Hydroxyanthranilic acid: B/A (a), 0.89; C(d), 0.84. Acetyl-anthranilic acid: B/A (a), 0.93; C(d), 0.80. Urea: B/A (a), 0.50; C(d), 0.52; butanol/water, 0.29. Allantoin: B/A (d), 0.30; C(d), 0.60. *p*-Aminobenzoic acid: B/A (d), 0.79; C(d), 0.69.

#### Preliminary experiments

The metabolite mixture readily became oxidized, and a rapid isolation procedure was therefore sought. In the first experiments the urine was submitted to the electrolytic desalting procedure of Consden, Gordon & Martin (1947), and after concentration was examined by paper chromatography. In butanol/acetic acid five strongly fluorescent spots were observed under ultraviolet light. Two of these spots appeared at medium  $R_F$  and showed a green and a strong pale-blue fluorescence, and another pair of spots with similar fluorescence appeared at high  $R_F$ . The spots of medium  $R_F$  were recognized, as described later, as L-3-hydroxykynurenine and L-kynurenine, and in Table 3 some  $R_F$  values of the metabolites (described as *E* and *F*) are compared with those of authentic materials. Tryptophan also appeared on the chromatograms, and could be readily demonstrated with Ehrlich's reagent and subsequent exposure to HCl fumes as described by Tabone, Robert, Thomassey & Mamounas (1950).

Table 3.  $R_F$  values of some metabolites and reference substances

Substance	$R_F$ in butanol/acetic acid, ascending†	$R_F$ in collidine, descending*	$R_F$ in butanol/water, ascending†
Metabolite <i>E</i>	0.39	0.59	0.18
3-Hydroxy-L-kynurenine	0.39	0.60	0.18
Metabolite <i>F</i>	0.48	0.51	0.23
L-Kynurenine	0.48	0.51	0.23

\* Whatman no. 4 paper.

† Whatman no. 1 paper.

Electrolytic desalting of the urine was not satisfactory. The darkening during treatment showed that degradation occurred, the proportions of the metabolites varied with the duration of the experiment, and relatively large amounts of non-fluorescent ninhydrin-positive substances remained, together with urea. The latter gives a powerful Ehrlich's reaction and obscures an important region of the chromatograms.

*Ether and butanol extraction of the urines.* Urine adjusted to pH 3 was extracted with ether, giving small amounts of the faster-moving fluorescent metabolites. Continuous extraction for some hours gave larger amounts of material, but also much urea. The urines were therefore adjusted to pH 7, treated with urease at 37°, readjusted to pH 3-4, and extracted as before. The procedure was reasonably satisfactory, but much more time-consuming than precipitation procedures. Butanol was unsuitable for extractions due to simultaneous extraction of appreciable amounts of salts.

complete resolution of these substances. In the remainder of this paper this solvent mixture is assumed to be used unless otherwise stated.

It was then found that much superior results could be obtained more simply by precipitation with mercuric acetate in acid solution, and the following procedure was adopted: the urines were diluted with an equal volume of 50% (v/v) acetic acid, and allowed to stand at 0° for 1-1.5 hr. The precipitated material, which besides protein and extraneous matter contained appreciable amounts of kynurenic and xanthurenic acids, was spun down, and to the supernatant

Table 4. Summary of colour reactions obtained after descending chromatography in butanol/acetic acid on Whatman no. 4 paper

(Spots down to J represent tryptophan metabolites. For remainder see text. A dash represents absence of colour.)

Spot	Mean $R_f$	Fluorescence under ultraviolet illumination	Ninhydrin	Ehrlich's reagent	Ekman's reagent	Pauly's reagent	Probable identity
A	0.07	Blue-green	Reddish purple	Pink after 1 hr. or more	Yellow on diazotizing turning brown slowly	Brown	Glycuronide of 3-hydroxy-L-kynurenine
B	0.12	Blue-purple	—	—	—	—	—
C	0.15	Blue-green	Reddish purple	Pink after 1 hr. or more	Yellow on diazotizing turning brown slowly	Brown	Sulphate of 3-hydroxy-L-kynurenine
E	0.35	Green	Brown-purple	Pink after a few min.	Yellow on diazotizing turning brown slowly	Brown	3-Hydroxy-L-kynurenine
F	0.42	Strong pale blue	Purple	Immediate orange	Magenta after short delay	—	L-Kynurenine
X	0.47	Blue-purple	—	—	—	—	—
G	0.52	Weaker pale blue	—	—	—	—	—
H	0.65	Blue-purple	—	—	—	Red	Kynurenic and xanthurenic acids
I	0.77	Green	—	Pink after a few min.	Yellow on diazotizing turning brown slowly	Brown	$N^\alpha$ -Acetyl-3-hydroxy-L-kynurenine
J	0.82	Strong pale blue	—	Immediate orange	Magenta after short delay	—	$N^\alpha$ -Acetyl-L-kynurenine
K	0.89	Blue-purple	—	Immediate yellow	Magenta rapidly	—	—
Tryptophan	0.49	—	Grey-purple	Violet after 1 hr.	Yellow on diazotizing	Yellow-brown	—
EA 1	0.27	—	—	Immediate yellow	Magenta	—	—
EA 2	0.61	—	—	Immediate yellow	Magenta	Brown	—

*Mercury precipitation of the urines.* The conditions first tried were those described by Cole (1942a) for the precipitation of tryptophan. The urines of normal rats with or without a tryptophan supplement, and of pyridoxin-deficient rats without a tryptophan supplement, gave very slight precipitates under those conditions, whereas the urines of pyridoxin-deficient rats fed tryptophan gave a copious precipitate. The final yellow-green solution was found on chromatography to contain a wealth of fluorescent substances which were labelled consecutively from A to J (D being a region of weak fluorescent background without outstanding spots). Butanol/acetic acid was by far the best mixture for chromatography and gave complete or almost

was added an excess of mercuric acetate in 25% (v/v) acetic acid. A copious yellow-green or green precipitate was formed, which was spun down after 1.5-2 hr. The supernatant, if left for 24 hr., deposited further material. The composition of this was found to be qualitatively similar to that originally precipitated, and as only about 10% more material was obtained on the longer precipitation the supernatant was normally discarded. The residue was suspended in water and treated with  $H_2S$  for 1-2 hr. until the relatively slow precipitation of  $HgS$  was complete. Excess  $H_2S$  was removed by bubbling  $N_2$  through the mixture, and after centrifugation the yellow-green supernatant was filtered, and concentrated *in vacuo*.

The material contained all the spots *A*–*J* obtained by the  $\text{HgSO}_4$  precipitation procedure and also contained: (1) an additional fluorescent substance, *X*, running rather faster than kynurenine, (2) appreciable amounts of non-fluorescent ninhydrin-positive material, (3) urea and (4) in later experiments another fast-running fluorescent substance, *K*. The properties of the various substances are summarized in Table 4. Spots *B* and *C* tended to overlap, and where this occurred the fluorescence of the mixed spot resembled that of kynurenine. Overlapping was sometimes also observed between *F* and *X* and between *J* and *K*. Tryptophan also underlies *X* but does not fluoresce.

It should be noted that some chromogenic substances do occur in the urine fractions of control normal and pyridoxin-deficient rats isolated by the above procedure, but these differ from substances *A*–*J* and are in general present in comparatively small quantities. One substance of interest was, however, noted in the urines of normal rats receiving supplementary tryptophan. This gave a violet-blue colour with Ehrlich's reagent after 1–2 hr., but was not fluorescent and gave no ninhydrin reaction. It was readily extracted by ether from urine at pH 4.5, was extracted from the ether by bicarbonate, and could be re-extracted into ether on acidification. It had an  $R_f$  value of 0.90 in butanol/acetic acid (ascending) and 0.71 in collidine (descending) and appeared to be a simple tryptophan derivative less hydrophilic than tryptophan itself. Its behaviour closely resembled that of  $N^\alpha$ -acetyltryptophan, but the two did not appear to be identical. The substance may be the same as the ether-soluble metabolite of Sarett & Goldsmith (1949).

#### Spectroscopic investigations

The individual substances were eluted from the chromatograms with 0.02M phosphate buffer, pH 7.3, and their spectra determined between 230 and 380  $m\mu$ . using a Beckman spectrophotometer, model DU.

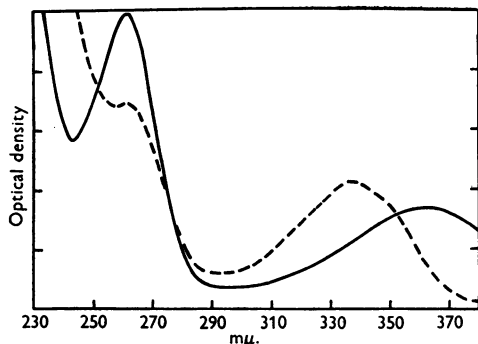


Fig. 1. Absorption spectra of substances *B* (---), and *C* (—) after elution from the chromatograms with buffer solution.

Fig. 1 shows the spectra of substances *B* (broken line) and *C* (full line). The spectrum of *C* is typical of that given by six of the fluorescent substances, *A*, *C*, *E*, *F*, *I* and *J*, and the positions of the maxima observed for these substances are shown in Table 5. It was observed, however, that the further the substance travelled on the chromatogram, the lower the second maximum appeared. This suggested that interaction with paper constituents was occurring. As *F* was already

known to be kynurenine, authentic kynurenine was examined on chromatograms and the same depression of the second maximum, with movement to slightly lower wavelength, was observed. Interaction with paper constituents is also indicated by the resolution observed for DL-kynurenine and DL-hydroxykynurenine, a result which could not

Table 5. Absorption maxima of eluted substances

Substance	Position of first maximum (m $\mu$ .)	Position of second maximum (m $\mu$ .)
<i>A</i>	264	364
<i>C</i>	262	362.5
<i>E</i>	264	368
<i>F</i>	263	355
<i>I</i>	265	365
<i>J</i>	265	355

occur if the chromatography only involved partition between the optically inactive solvent phases. The ready formation of a complex between L-kynurenine and sucrose, used for the preparative resolution of kynurenine (Butenandt & Weichert, 1944) is also relevant in this connexion. It will be noticed that the second maxima of *A*, *C*, *E* and *I* lie at about 365  $m\mu$ ., whereas those of *F* and *J* lie at about 355  $m\mu$ . The first four substances also appear pale yellow on the untreated chromatogram.

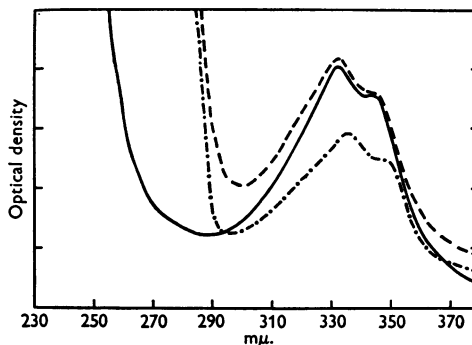


Fig. 2. Absorption spectra of eluted substances *X* (----) and *H* (- - -) compared with a 1:1 mixture of kynurenic and xanthurenic acids (—).

Fig. 2 shows the spectra of substances *X* and *H* and the general resemblance between these and the calculated spectrum of a 1:1 mixture of kynurenic and xanthurenic acids. Both *X* and *H* appear to absorb strongly below 290  $m\mu$ . In the case of *X* this is known to be due to the presence of tryptophan, which underlies *X* on the chromatogram, and it is probable that other indole-type compounds are responsible for the similar absorption of *H*.

The significance of these spectra is considered in detail when the identity of the individual substances is discussed. At the same time various known compounds were examined spectroscopically, and the following are the results obtained ('buffer' refers to the mixture already described): *Kynurenine*: in buffer showed maxima at 257  $m\mu$ . ( $\epsilon=6750$ ) and 361  $m\mu$ . ( $\epsilon=4350$ ) (Fig. 3). *3-Hydroxykynurenine*: in buffer showed maxima at 268.5  $m\mu$ . ( $\epsilon=9840$ ) and 370  $m\mu$ . ( $\epsilon=4240$ ). In water the peaks were unchanged in position

and had  $\epsilon$  values of 7800 and 4000 respectively (Fig. 3). In 0.1N-HCl the maximum at 370 m $\mu$ . had almost disappeared ( $\epsilon=740$ ) and a new maximum appeared at 315 m $\mu$ . ( $\epsilon=2650$ ); the lower wavelength maximum had moved to 251.5 m $\mu$ . ( $\epsilon=8750$ ) (Fig. 3). *Kynurenic acid*: in buffer showed maxima at 332 m $\mu$ . ( $\epsilon=9800$ ) and 344 m $\mu$ . ( $\epsilon=7920$ ) (Fig. 4). In 0.1N-HCl the specimen was too insoluble to determine

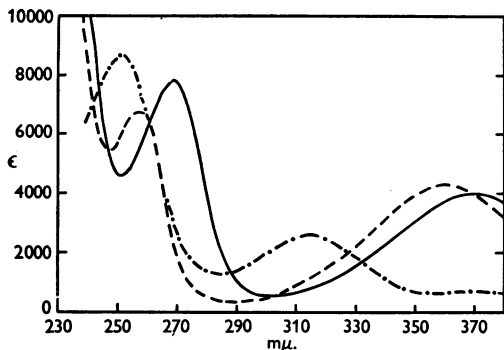


Fig. 3. Absorption spectra of 3-hydroxykynurenine in water (—), kynurenine in buffer (---) and 3-hydroxykynurenine in 0.1N-HCl (-·-·-).

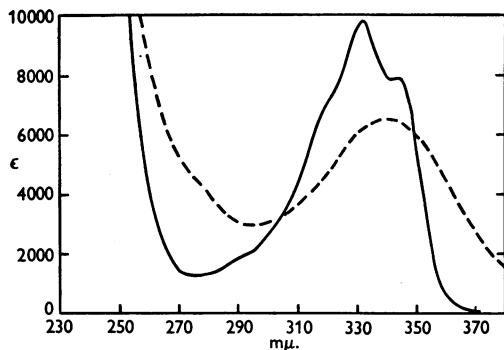


Fig. 4. Absorption spectra of kynurenic acid (—), and xanthurenic acid (---), in buffer.

accurate  $\epsilon$  values, but showed a maximum at 310 m $\mu$ . descending to a flat area centred on 325 m $\mu$ . In 0.1N-NaOH the twin peaks had coalesced to one flat maximum from 320–325 m $\mu$ . ( $\epsilon=6350$ ). *Xanthurenic acid*: in buffer showed a wide maximum centred at 340 m $\mu$ . ( $\epsilon=6500$ ) (Fig. 4). In 0.1N-HCl  $\epsilon$  values were again not determined due to insolubility, but the spectrum showed an inflexion at 315 m $\mu$ . and a maximum at 353 m $\mu$ . superimposed on appreciable general absorption. In 0.1N-NaOH maxima at 282 m $\mu$ . ( $\epsilon=5300$ ) and 327 m $\mu$ . ( $\epsilon=5300$ ) were again superimposed on general absorption. *Anthranilic acid*: in buffer showed maxima at 241 m $\mu$ . ( $\epsilon=6400$ ) and 309 m $\mu$ . ( $\epsilon=2900$ ). *3-Hydroxyanthranilic acid*: in buffer showed an inflexion at about 240 m $\mu$ . and a maximum at 314 m $\mu$ . ( $\epsilon=3240$ ). In 0.1N-HCl showed a maximum at 297 m $\mu$ . ( $\epsilon=3120$ ), agreeing with the results of Nyc & Mitchell (1948).

*Effect of autoxidation on the spectra.* The spectra of hydroxykynurenine and hydroxyanthranilic acid were examined at varying times after making up the solutions to

see whether oxidation during isolation and the chromatographic procedures might have produced changes in the spectra of the metabolites. Little change in the spectrum of a hydroxykynurenine solution was observed during the first few hours. The solution then slowly became more yellow and in the spectral region examined this was accompanied by an increase in general absorption with reduction in  $\epsilon$  values, but no change took place in the position of the maxima. Thus for hydroxykynurenine at 268.5 m $\mu$ . the  $\epsilon$  value of 7800 in fresh solution had dropped to 6460 after 4 days. Hydroxyanthranilic acid showed similar results.

#### Experiments with $^{32}\text{P}$ -labelled phosphate

Hanes's phosphate reagent showed the presence of a phosphate-containing substance sufficiently close to *B* and *C* to raise the question of identity. In addition, further spots were observed corresponding to tryptophan and to substance *Eh 2*. To settle this question tryptophan was fed in the usual manner, together with 0.2 ml. of a phosphate solution containing 16  $\mu\text{c}$ . of  $^{32}\text{P}$ . Chromatograms were examined by the radioautograph technique and by means of the mechanical scanning technique previously mentioned (Dalgliesh *et al.* 1951). Only one phosphorus-containing compound was observed, of  $R_f$  0.14. The outline did not correspond exactly with that of *B* or *C*, and non-identity was conclusively proved by the subsequent observation that whereas *B* and *C* were adsorbed by deactivated charcoal, the phosphorus compound was not. The two other spots observed with Hanes's reagent were artifact spots (see above). The urinary phosphorus compound was compared with inorganic phosphate with which it was not identical (distances run on a chromatogram were 9.0 and 14.5 cm. respectively). Further experiments showed that when the urine of normal rats was submitted to the mercuric acetate precipitation procedure a phosphorus compound was isolated, whether or not supplementary tryptophan had been fed. In this case the phosphorus compound was ninhydrin-positive, thereby differing from the compound in the urine of pyridoxin-deficient rats. The substances have not been further examined.

#### Purification with deactivated charcoal

The work of Syngé & Tiselius (1949) suggested that deactivated charcoal might be of considerable value in separating the tryptophan metabolites from contaminating substances, and this proved to be the case. Charcoal (British Drug Houses Ltd.) deactivated by 4% (w/w) stearic acid by the procedure of these authors gave excellent results, it being found unnecessary to carry out a preliminary purification of the charcoal.

The supernatant obtained by centrifugation, after precipitation of HgS and removal of excess H<sub>2</sub>S, was shaken with the deactivated charcoal, filtered, and the residue well washed with water. The colourless filtrate contained (a) all the ninhydrin-positive non-fluorescent substances (presumably simple amino-acids) in the *A-E* region, (b) the phosphate compound in the *B-C* region, and (c) urea. The charcoal was then eluted with 5% (w/v) phenol and the yellow eluate concentrated in the usual way. All the fluorescent substances (and also tryptophan) were present with no apparent change in their relative proportions. Direct charcoal adsorption from the urine was also examined. After acidification with acetic acid and centrifugation in the usual way the supernatant was shaken with the

deactivated charcoal, filtered, and washed with water. The filtrate in this case was yellow. On elution with phenol as before the eluate, also yellow, was found to contain all the usual metabolites. This isolation procedure is much quicker than any other examined, but as there is some increase in background fluorescence it was preferred to use the mercuric acetate procedure followed by charcoal treatment.

#### Ion-exchange columns

Various resins were examined to see whether a rapid selective separation could be obtained. Zeo-Karb 225 was found satisfactory for this purpose, as it adsorbed all substances except *B* and *C*. The ionizing groups of this resin are solely of the sulphonic acid type, and substances *B* and *C* must therefore be strongly acidic. Resins relying for their activity on carboxyl groups were of no value.

*B'* of  $R_f$  0.27 formed from *B* on mild hydrolysis (see below) together, surprisingly, with hydroxykynurenine, *E*. As considerable heating occurred during the experiment it seemed likely that *B'* and *E* were degradation products, and this was confirmed in an experiment in which the contents of the anode compartment were removed every 10 min. and replaced by fresh electrolyte. In this case the anode compartment contained *B* and *C* with only small amounts of *B'* and *E*. By increasing the concentration of acetic acid in the electrolyte the tendency of substances *A*, *I* and *J* to move towards the anode could be reduced and even reversed owing to the increasing part played by the positively charged form of an aromatic amino group.

To get a more complete picture of the relative mobilities of the various substances the following procedure was adopted: electro-dialysis was carried out as before, using 0.01N-acetic

Table 6. *Distribution of the metabolites on electro-dialysis*

Compartment	Substances present										
4		<i>B</i>	<i>C</i>	( <i>B'</i> )*	( <i>E</i> )		<i>G</i>		( <i>H</i> )		
3	4'	( <i>A</i> )	<i>B</i>	<i>C</i>	( <i>B'</i> )*	( <i>E</i> )	<i>G</i>		<i>H</i>		
	3'	( <i>A</i> )	( <i>B</i> )	( <i>C</i> )			<i>X</i>		<i>H</i>	<i>I</i>	<i>J</i>
	2'	<u><i>A</i></u>	( <i>B</i> )	( <i>C</i> )			<i>X</i>		<i>I</i>	<u><i>I</i></u>	<u><i>J</i></u>
	1'	<i>A</i>						<i>Eh2</i>	<u><i>I</i></u>	<u><i>J</i></u>	<i>K</i>
2				<i>Eh1</i>	<i>E</i>	<i>F</i>	Try.			<u><i>K</i></u>	
1				( <i>Eh1</i> )	<u><i>E</i></u>	<u><i>F</i></u>	<u>Try.</u>			( <i>K</i> )	

\* *B'* represents the substance formed from *B* on mild hydrolysis (see text).

Try. = tryptophan.

#### Electrophoresis and electro-dialysis experiments

The apparatus of Gordon, Gross, O'Connor & Pitt-Rivers (1952) was used to investigate the electrophoretic behaviour of the metabolites and known reference substances on filter-paper strips. Whatman MM paper was used, with solutions of acetic acid of varying strength as the electrolyte, and an applied potential of 240 V. The metabolites separated into four groups. Group I moved towards the cathode and kynurenine, hydroxykynurenine and tryptophan were found to travel in a similar manner. Group II moved slightly towards the cathode in 2% acetic acid but stayed close to the origin in 0.01N-acetic acid, *N*<sup>α</sup>-acetylkynurenine behaved similarly. Group III moved towards the anode, kynurenine and xanthurenic acid behaving similarly. Group IV consisted of bands, which appeared to correspond in properties to *B* and *C*, moving fast to the anode.

In view of these favourable results electro-dialysis was investigated using the apparatus and types of diaphragm described by Syngé (1951). All compartments were filled with 25 ml. of the electrolyte, the specimen being in compartment 2 (cathode compartment=1). The applied potential was 240V., and the experiments continued till most of the colour had migrated from compartment 2. The contents of each compartment were then removed, concentrated, and examined in the usual way. Using 0.01N-acetic acid as electrolyte the cathode compartment was found to contain kynurenine (*F*), hydroxykynurenine (*E*) and tryptophan; compartment 2 contained the same substances together with *Eh1* and *Eh2* (see below) and *K*. Compartment 3 contained *A*, small amounts of *B* and *C*, *X* and substances *G* to *K*. The material in the anode compartment was dark in colour and had obviously undergone decomposition. The main constituents were the substance

acid as electrolyte, the contents of the anode compartment being frequently replaced to reduce decomposition. When most of the colour had migrated from compartment 2 the contents of each compartment were removed and numbered 1-4. The contents of cell 3 (containing the greatest number of the metabolites) were then placed in cell 2 and the procedure repeated using this time 8% (v/v) acetic acid as electrolyte, to give a new distribution, the contents of the compartments being labelled 1'-4'. Effectively this arrangement is equivalent to an eight-compartment cell. The contents of each compartment are indicated in Table 6, brackets indicating small quantities, and underlining indicating that the greater part of that particular substance appeared in that compartment. The substances as written from left to right correspond with the downward order in which they appear on a chromatogram. It should be noted: (a) that the simple amino-acids kynurenine (*F*), hydroxykynurenine (*E*) and tryptophan can be fairly clearly separated from the other metabolites; (b) that *A*, *I* and *J* are of similar acidity and appreciably more acidic than the amino-acids; and (c) that *B* and *C* are strongly acidic and fairly readily hydrolysed.

#### Other separative procedures

Solvent extraction experiments on the crude urine preparation, attempts to use ether-buffered Celite columns, and attempted countercurrent distribution between butanol and water or butanol and 0.01N-HCl gave no useful results, though the latter demonstrated the hydrophilic nature of substances *A*, *B* and *C*.

#### Hydrolysis experiments

A urine preparation was heated in a sealed tube with 5N-HCl for 2 hr. at 100°. Removal of the acid and chromatography of the residue showed that substances *A*, *B*, *C*, *X*, *G*,

*I* and *J* had disappeared, whilst *E*, *F* and *H* remained. In further experiments the urine preparations were applied as a streak to the chromatogram and after development and drying the corresponding spots were cut out, eluted and taken to dryness. Where complete separation did not occur, the eluate was again run, this time in phenol/water, and after development and drying the spots were again cut out, eluted and concentrated. Hydrolysis was carried out under varying conditions. The following  $R_F$  values were noted in phenol/water: *A*, 0.15; *B*, 0.18; *C*, 0.31; *X*, 0.24; L-kynurenine, 0.82.

#### Investigation of blood plasma

A pyridoxin-deficient rat was given 0.5 g. DL-tryptophan in the diet, and next day was killed by anaesthetization and withdrawal of the blood from the heart after heparin injection. The blood (approx. 10 ml.) was diluted with an equal volume of trichloroacetic acid solution (10% w/v) and after 45 min. the mixture was centrifuged. The supernatant was precipitated with mercuric acetate in the usual way, only a slight whitish precipitate being obtained. Chromatography showed that none of the fluorescent urinary metabolites was present.

#### Feeding experiments with L-kynurenine

When the tryptophan supplement to the diet of pyridoxin-deficient rats was replaced by 0.1 g. L-kynurenine sulphate the urine was found to contain all the fluorescent metabolites observed after feeding L-tryptophan. This makes it most unlikely that any of these substances represent an intermediate stage between tryptophan and kynurenine.

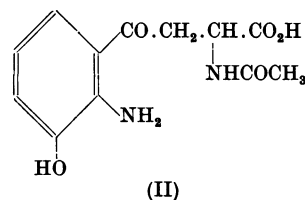
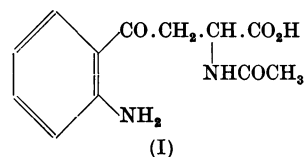
### IDENTITY OF THE METABOLITES

*Substances E, F and H.* Substances *E* and *F* showed in all respects the same behaviour as the L-isomers of 3-hydroxykynurenine and kynurenine. Thus they agreed in  $R_F$  values (e.g. Table 3), colour reactions (as in Table 4), spectroscopic behaviour, were resistant to hydrolysis and moved in the same manner on electrophoresis.

The spot *H* contains such kynurenic and xanthurenic acids as are not precipitated in the initial acid treatment of the urines. Known specimens of these two substances were found to be unresolved on chromatograms and to show the same  $R_F$  and fluorescence as *H* (the fluorescence of xanthurenic acid is more intense than that of kynurenic acid, and makes the dominant contribution). The spectrum of *H* shows the typical twin peak of kynurenic acid (Figs. 2 and 4), xanthurenic acid only having a broad maximum in this region. As is to be expected *H* is unaffected by hydrolysis. Xanthurenic acid is already known to occur in the urine of tryptophan-fed pyridoxin-deficient rats (Lepkovsky, Roboz & Haagen-Smit, 1943).

*Substances I and J.* The great similarity between the pair of substances *E* and *F* and the pair *I* and *J* strongly suggested that *I* and *J* were analogous derivatives of *E* and *F*. It will be seen from Table 4 that the reactions of *E* and *I* to Ehrlich's, Ekman's

and Pauly's reagents were the same, as were the colours shown on fluorescence, and there was similar agreement between substances *F* and *J*. But whereas *E* and *F* gave a ninhydrin reaction, *I* and *J* did not. This suggested that *I* and *J* were derivatives of hydroxykynurenine (*E*) and kynurenine (*F*) respectively in which a substituent was attached to the aliphatic amino group. The similarity of the spectra of *E* and *I* and of *F* and *J* gave further support, as it is known (Knox & Mehler, 1950; Dalgliesh, 1952) that  $N^\alpha$ -acylkynurenines have the same type of spectrum as kynurenine itself. Hydrolysis with 0.1N-HCl at 95° for 1 hr. caused slight hydrolysis of *I* to *E*, but the greater part was unchanged, whereas hydrolysis with N-HCl for 1.5 hr. at 95° caused almost complete hydrolysis of *I* to *E*, and similarly of *J* to *F*. Moreover, both *E* and *I* gave an immediate brown-black spot with ammoniacal silver nitrate, showing that the *o*-aminophenol system was intact. It has been shown by Yanofsky & Bonner (1950) that a mutant strain of *Neurospora*, in which the further metabolism of kynurenine was impaired, accumulated a substance identified as  $N^\alpha$ -acetylkynurenine (*I*). This was therefore synthesized (Dalgliesh, 1952) and its behaviour was found to agree with that of substance *J* in every respect.

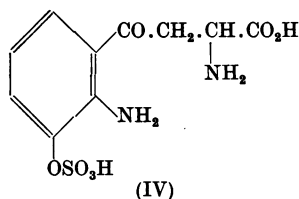
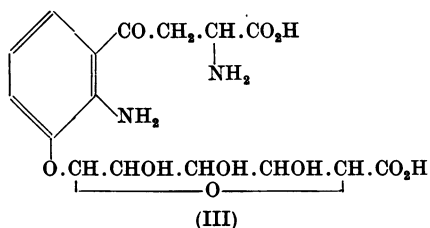


In view of the high  $R_F$  values given by *J* confirmation for this identification was desirable and was obtained, as has already been reported (Dalgliesh *et al.* 1951) by simultaneous feeding of tryptophan and sodium [ $\alpha$ - $^{14}$ C] acetate when the activity appeared in large amounts in substances *I* and *J*. (It should be mentioned here that the lesser activity observed on the chromatograms at low  $R_F$  is likely to have been due to 'background' substances which are not tryptophan metabolites. At the time the experiment was done the purification with de-activated charcoal which removes these substances had not been worked out.) Substance *J* is therefore almost certainly  $N^\alpha$ -acetyl-L-kynurenine (only L- and no D-kynurenine being formed on hydrolysis), and by analogy there is a high probability that substance *I* is  $N^\alpha$ -acetyl-L-3-hydroxykynurenine



(II), though in this case synthetic material has not been available for comparison. The behaviour on electro-dialysis is in accord with these formulations.

*Substances A and C.* Both these substances, like *E* and *I*, appear yellow on the untreated chromatogram and on hydrolysis give L-3-hydroxykynurenine. Substance *A* moves at the same speed on electrophoresis or electro-dialysis as the  $N^\alpha$ -acetyl compounds (I) and (II), and it is reasonable to infer that *A* also has one carboxyl group in excess of the basic groups present (neglecting the aromatic amino groups, which are of very low basicity). The ninhydrin reaction shows that the aliphatic amino group is intact and the residue conjugated with hydroxykynurenine therefore probably contains one carboxyl group (or one basic and two carboxyl groups, etc.). The very hydrophilic nature of *A*, shown both in the counter-current distributions and by its low  $R_f$ , suggests a polyhydroxy compound. The delay in appearance of a colour with Ehrlich's reagent indicates that some hydrolysis is necessary before reaction occurs. The similarity of the spectrum of *A* to that of kynurenine shows that the aromatic amino group is not acylated as  $N'$ -acylkynurenines have markedly different spectra (cf. Dalglish, 1952). An  $N'$ -alkylkynurenine derivative might show the type of spectrum observed, but an alkyl group would be unlikely to be lost on hydrolysis. A substance which might be expected to show the observed properties of *A* is a glycuronide such as (III). This would be expected to show greater resistance to oxidation than hydroxykynurenine, as



is in fact observed. Thus if a chromatogram is left exposed to the air both *E* and *I*, in which the *o*-aminophenol system is intact, turn brown, whereas *A* and *C* are unchanged in colour. Moreover, unlike *E* and *I*, neither *A* nor *C* show immediate reduction of ammoniacal silver nitrate.

A striking property of substance *C* is its high acidity, shown both in the experiments with ion-exchange resins, and on electro-dialysis. The acidity

is such as to suggest an inorganic acid grouping to be responsible, and as the experiments with  $^{32}\text{P}$ -labelled phosphate excluded a substituted phosphoric acid the phenolic sulphate (IV) was considered to be the most likely structure. Strong support for the formulation of *A* and *C* as (III) and (IV) respectively was obtained in hydrolysis experiments.

Hydrolysis of *A* with 0.1N-HCl for 1 hr. at 95° left it almost completely unchanged, though a trace of hydroxykynurenine was detectable. *C*, however, was almost entirely hydrolysed under these conditions, with formation of L-3-hydroxykynurenine. When a portion of the hydrolysate of *C* was tested with  $\text{BaCl}_2$  a white precipitate was obtained, and no such precipitate was obtained from the hydrolysed eluate of substance *E* used as a control. This supports the formulation (IV) for *C*, which is a readily hydrolysable sulphate; no substance other than hydroxykynurenine, sulphate and a trace of unchanged *C* could be detected in the hydrolysate. Hydrolysis of *A* with N-HCl at 95° for 1.5 hr. still left the material mainly unchanged, though a somewhat larger amount of hydroxykynurenine was formed than in the previous case. After hydrolysis with 6N-HCl in a sealed tube at 100° for 1 hr. some of *A* still remained unchanged, but the greater part was hydrolysed to hydroxykynurenine. In this case a second hydrolysis product was also observed at  $R_f=0.22$ , which showed a weak blue fluorescence and gave an orange ninhydrin reaction. The same material was found to be formed from hydroxykynurenine itself under similar conditions and it is probably a substance formed by reaction of the *o*-aminophenol grouping with soluble components of the paper. It is interesting in this connexion to recall that weak blue fluorescence and an orange ninhydrin colour were found to be shown by  $N'$ -acylated kynurenines, and that a yellow or orange ninhydrin colour has also been reported to be given by the unsubstituted phenacylglycine,  $\text{Ph.CO.CH}_2\text{CH}(\text{NH}_2)\text{CO}_2\text{H}$  (Fraser & Raphael, 1950; Wiss, Viollier & Waldi, 1951).

These hydrolytic results are in agreement with the formulation of *A* and *C* as the *O*-glycuronide and *O*-sulphate respectively of L-3-hydroxykynurenine and may be compared with other recent results for similar phenolic derivatives (Porteous & Williams, 1949; Parke & Williams, 1951; Berenbom & Young, 1951). Confirmation for the presence of glycuronic acid was obtained by applying Tollens's reaction according to the procedure of Cole (1942*b*). Unhydrolysed *A* gave a negative test, but after hydrolysis with 5N-HCl in a sealed tube at 100° for 1 hr. the test was positive.

*Substances B and X.* Both *B* and *X* show a blue-purple fluorescence, that of *X* being weaker. Neither substance shows any reaction with the usual reagents and evidence on their constitution is therefore mainly derived from spectroscopic and hydrolytic data.

The spectrum of *X* (Fig. 2) has the typical twin peak of quinoline derivatives with maxima at 336 and 348  $\text{m}\mu$ , which may be compared with that of kynurenic acid (Fig. 4) showing maxima at 332 and

344  $\mu$ . under the same solvent conditions, and kynurenine (4-hydroxyquinoline) which has maxima at 316 and 328  $\mu$ . (Butenandt, Karlson & Zillig, 1951). Substance *X* is completely hydrolysed to kynurenic acid on heating with *N*-HCl for 1.5 hr. at 95°. The residue conjugated with the kynurenic acid was not further investigated.

The spectrum of *B* (Fig. 1) shows a broad maximum at 337  $\mu$ . A similar maximum is shown by xanthurenic acid at 340  $\mu$ . under the same solvent conditions, whereas anthranilic acid, the only other likely substance which the fluorescence at all resembles, has a maximum at 310  $\mu$ ., at which point *B* shows a minimum. (The small peak in the spectrum of *B* at about 260  $\mu$ . is probably due to contamination by *C*.) *B* is strongly acidic, moving rapidly to the anode on electrodialysis and being unadsorbed by Zeo-Karb 225. After hydrolysis with 0.1 *N*-HCl for 1 hr. at 95° most of *B* is destroyed, and as with *C*, sulphate ion is detectable in the hydrolysate. However, only traces of xanthurenic acid are formed and the material is instead transformed to a substance *B'* with the same colour of fluorescence as *B* but a higher  $R_f$  (0.27). After hydrolysis with *N*-HCl at 95° for 1.5 hr. *B'* is still the main product, but hydrolysis with 5 *N*-HCl in a sealed tube for 1 hr. at 100° gives xanthurenic acid. These results indicate that in *B* xanthurenic acid is conjugated with sulphate and with some other residue, which was not identified.

*Substances G, K and minor components.* Substances *A, B, C, E, F, I* and *J* are powerfully fluorescent substances which appear consistently, as do *X* and *H* though their fluorescence is less intense. Substance *G*, however, varies considerably, being fairly strong in some preparations and very weak in others. Whether this is due to variations in the urine, or in the isolation is not known. The colour of fluorescence somewhat resembles that of kynurenine, but is weaker and whiter, *G*, like *B* and *X*, does not react with the normal reagents used in this work. It was not further investigated.

Substance *K* may be a tryptophan metabolite, but its status has yet to be clarified. Urine of pyridoxin-deficient rats which had received no tryptophan supplement was found to contain a substance which had the same  $R_f$  as *K*, and gave an immediate strong yellow Ehrlich's reaction, but which was not fluorescent. The fluorescent material *K* did not appear in the urines until some time after the establishment of pyridoxin-deficiency, and as the use of DL-tryptophan started at about the same time it is possible that it is a metabolite of D-tryptophan. It is possible to separate *K* from the non-fluorescent Ehrlich-positive substance by electrodialysis, and after separation *K* also shows an immediate strong yellow colour with Ehrlich's reagent.

Substance *Eh 1* is not present in large amounts, is non-fluorescent and occurs in the urines when no tryptophan has been fed. Substance *Eh 2* has only been observed in any quantity after feeding DL-tryptophan, and may therefore also be a metabolite of the D-isomer. In general after feeding DL- rather than L-tryptophan 'background' substances increase, and appreciably more tryptophan (presumably mainly the D-isomer) is excreted unchanged. After feeding the DL-compound a narrow band with typical kynurenine-type fluorescence appears on the leading edge of the hydroxykynurenine spot, suggesting a small conversion of D-tryptophan to D-kynurenine. Such a transformation is known to occur in the rabbit (Kotake & Ito, 1937).

## DISCUSSION

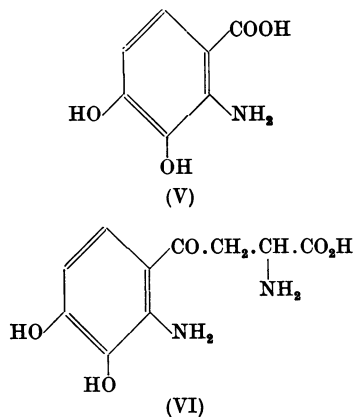
The most striking feature of the various metabolites which have been recognized is that all still contain the carbon atoms originating from the alanine side chain of tryptophan. In the normal conversion of tryptophan to nicotinic acid these carbon atoms are known from isotope experiments to be lost (Heidelberg, Gullberg, Morgan & Lepkovsky, 1949; Hundley & Bond, 1949), and this immediately suggests that pyridoxin is concerned in tryptophan metabolism at the stage at which removal of the side chain occurs. This chemical evidence agrees with evidence from enzyme studies. Kynurenine was shown by Kotake & Nakayama (1941) to be converted to anthranilic acid by an enzyme kynureninase occurring in cat liver and kidney. Subsequent work has shown that kynureninase, which equally converts 3-hydroxykynurenine to 3-hydroxyanthranilic acid, occurs in all animal species tested, and requires pyridoxal phosphate as a co-enzyme (Braunshtein, Goryachenkova & Pashkina, 1949; Wiss & Hatz, 1949; Wiss, 1949; Wiss & Fuchs, 1950; Dalgliesh *et al.* 1951). As pyridoxin is known not to affect the conversion of tryptophan to kynurenine (Braunshtein *et al.* 1949) or of 3-hydroxyanthranilic acid to nicotinic acid (Henderson, Weinstock & Ramasarma, 1951) the site of action of pyridoxin becomes clear, and the accumulation of metabolites still retaining the carbon atoms of the original tryptophan side chain gives strong support to the enzymic evidence.

The occurrence of 3-hydroxykynurenine derivatives conjugated through the phenolic group as the glucuronide (III) and sulphate (IV) indicates that the rat is dealing with an abnormally accumulated metabolite by well established detoxication mechanisms for phenolic substances. These two derivatives are therefore unlikely to be of importance in normal tryptophan metabolism. The occurrence of the *N*<sup>α</sup>-acetyl derivatives (I) and (II) may, however, be of greater significance. Although acetyla-

tion is a recognized detoxication mechanism, occurring, e.g., with aromatic amino groups, acetylation of the aliphatic amino group of naturally occurring amino-acids has been rarely recorded. As already pointed out (Dalglish, 1951) if  $N^\alpha$ -acetylkynurenine were the normal substrate for hydroxylation of the aromatic ring it would be possible for the animal to direct the metabolic changes to hydroxyanthranilic acid at the expense of the formation of anthranilic and kynurenic acids which are not precursors of nicotinic acid in animals.

Certain stages in tryptophan metabolism are still obscure. The first oxidation product of tryptophan, which is in its turn further oxidized to formylkynurenine, is still unidentified. No evidence for such a compound in the urines was obtained in the present work, suggesting that oxidation to formylkynurenine is a rapid process. It is of interest that neither were appreciable amounts of formylkynurenine detected. Formylkynurenine would not be detectable as such on the chromatograms, in which it would be masked by kynurenine, but it would be detectable spectroscopically in the eluted material. Moreover, treatment of the urine preparations with formylase caused no appreciable drop in ultraviolet absorption at 320  $m\mu$ . with a corresponding rise at 360  $m\mu$ . (cf. Knox & Mehler, 1950). The result is to be expected in view of the known high activity of formylase in the intact animal.

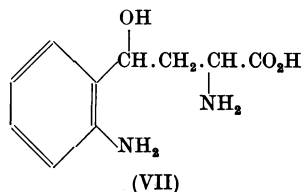
The intermediate stages in the conversion of 3-hydroxyanthranilic acid to nicotinic acid are also still obscure. Opening of the benzene ring must occur, and it has been suggested that 3:4-dihydroxyanthranilic acid (V) forms a step in the chain



(Makino, Itoh & Nishi, 1951). If this were the case it might by analogy be expected that (V) would be formed by the action of kynureninase on the dihydroxykynurenine (VI) and that (VI) or derivatives thereof would accumulate in the urine in pyridoxin deficiency. It would be expected that

(VI) and its derivatives would be more hydrophilic than the corresponding derivatives of hydroxykynurenine, which they would otherwise resemble. No substances with the expected properties were detected, and this suggests that (VI) is not a major tryptophan metabolite. Dihydroxyanthranilic acid (V) cannot, however, be similarly excluded but it is probable that if (V) is an intermediate then introduction of the second phenolic group occurs after loss of the alanine side chain.

It seems probable that tryptophan can be hydroxylated in the benzene ring. For example, the recognition of the vasoconstrictor principle of ox serum, serotonin, as 5-hydroxytryptamine (Rapport 1949; Hamlin & Fischer, 1951) suggests that 5-hydroxylation can occur. No evidence was obtained in the present work to suggest the occurrence in any appreciable quantity of a substance such as 5-hydroxykynurenine, and this and similar pathways therefore probably play only a minor quantitative role in tryptophan metabolism. The finding of Inagaki (1933) that rabbits metabolize *o*-aminoacetophenone by oxidizing part to anthranilic acid and reducing part to *o*-aminophenylmethylcarbinol suggests the possibility of the occurrence of carbinols such as (VII) corresponding to kynurenine and hydroxykynurenine.



But here again no evidence for compounds with the expected properties was found.

## SUMMARY

1. On administration of tryptophan to rats on a low pyridoxin diet numerous metabolites appear in the urine, and the probable identity of the majority of these has been established. A convenient method for the group isolation of these substances is described, involving precipitation by mercuric acetate in acid solution and selective adsorption on deactivated charcoal.

2. The scope of various reagents, in particular Ehrlich's and Ekman's reagents, has been studied, and chromatographic, chemical, spectroscopic and some other properties of the metabolites are recorded.

3. Besides the previously known metabolites L-kynurenine, kynurenic acid and xanthurenic acid, the occurrence of L-3-hydroxykynurenine as a tryptophan metabolite has been demonstrated for the first time in animals.

4.  $N^{\alpha}$ -Acetyl derivatives of both L-kynurenine and L-3-hydroxykynurenine are excreted and it is suggested that these compounds may be normal metabolic intermediates. In addition L-3-hydroxykynurenine is excreted with the phenol group conjugated as the glycuronide and sulphate. These are probably detoxication products.

5. The occurrence of conjugated derivatives of both kynurenine and xanthurenic acids has been demonstrated.

6. The fact that all these metabolites retain the carbon atoms of the original alanine side chain of tryptophan provides strong chemical evidence that pyridoxin participates in tryptophan metabolism at

the stage at which this side chain is split from the rest of the molecule.

7. Alternative metabolic pathways for tryptophan are discussed and it is suggested that 3:4-dihydroxykynurenine is unlikely to be a tryptophan metabolite of major importance.

I should like to express my thanks to Dr A. Neuberger, F.R.S., and Dr W. E. Knox for many valuable discussions and for making available their collections of tryptophan metabolites; also to Prof. A. Butenandt for supplying synthetic hydroxykynurenine; and to Mr A. J. Tilley, Mrs B. Higginson and Miss L. Langford for technical assistance and care of the animals.

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