nine is excreted on giving tryptophan to rats with a simple thiamine deficiency.

3. It is therefore concluded that thiamine is concerned in the conversion of tryptophan into formylkynurenine.

4. Similar experiments combining biotin and pyridoxine deficiencies suggest that biotin is unlikely to be concerned in any stage in the conversion of tryptophan into hydroxykynurenine.

5. Considerable advantages were found in giving vitamins by stomach-tube rather than by mixing them in the diet.

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Excretion of Conjugated 2-Amino-3-Hydroxyacetophenone by Man, and its Significance in Tryptophan Metabolism

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In a survey of aromatic metabolites in the urine of patients with various leukaemias and anaemias it was noticed that several of these urines contained appreciable amounts of a substance with a strong pale-blue fluorescence. This paper describes its identification.

METHODS

Aromatic metabolites were isolated from the urines by adsorption on deactivated charcoal and subsequent phenol elution, as described in detail elsewhere (Dalglish, 1955a). Preparative chromatograms were run by the descending technique on Whatman 3 MM paper and other chromatograms similarly on Whatman no. 1 paper. Paper electrophoresis was carried out for 45–60 min. on Whatman no. 1 paper soaked in 0.4% (v/v) aqueous acetic acid with an applied potential gradient of 50 v/cm. Ultraviolet absorption spectra of eluates were determined with a Unicam spectrophotometer model SP. 500. The reagents used for determining reactive groups have been described elsewhere (Dalglish, 1955a).

The following solvents were used for chromatography: the organic phase of a *n*-butanol-glacial acetic acid–water mixture (4:1:5 by vol.); ‘Phenol liquefactum’ B.P.–water, 100:15 (v/v) (‘neutral phenol’); *n*-pentanol–pyridine–water (7:7:6 by vol.); aqueous KCl (20%, w/v); aqueous sodium formate (5%, w/v).

Persulphate oxidation of o-aminoacetophenone (cf. Boyland & Sims, 1954). To *o*-aminoacetophenone hydro-

chloride (70 mg.) in 1.5 ml. water was added 0.9 ml. *N*-NaOH (approx. 2.2 equiv.) and sufficient acetone to give a clear solution. A solution of potassium persulphate (110 mg., 1 equiv.) in 10 ml. water was added, and the mixture set aside overnight. The brown reaction product was then diluted, filtered and acidified with acetic acid, and the products were adsorbed on deactivated charcoal. This was then eluted with phenol and the products were purified and examined by the procedures used for urinary metabolites.

RESULTS

The unknown substance had a fluorescence very similar in colour to that of kynurenine. It moved on butanol–acetic acid chromatograms at a medium *R_F*, slightly faster than, but usually overlapping, indican, and in its turn slightly slower than, but overlapping, a non-fluorescent substance, positive to Ehrlich’s reagent (*p*-dimethylaminobenzaldehyde in dilute HCl), which will be the subject of a future communication. Preparative chromatography successively in butanol–acetic acid, neutral phenol and again in butanol–acetic acid, the appropriate areas being cut out and eluted at each stage, gave an extract in which no other substances could be detected chromatographically. The ultraviolet spectrum of the final eluate showed maxima at about 260 and 360 mμ., characteristic of a

derivative of *o*-aminoacetophenone. The substance gave an orange colour with Ehrlich's reagent, as is to be expected for an *o*-aminoacetophenone with an unsubstituted amino group, but it did not give the ninhydrin reaction and could be separated from kynurenine on chromatography. On attempting to diazotize and couple (Ekman's reaction) a yellow colour appeared on diazotization (spraying with dilute HCl and nitrite). This behaviour recalled conjugates of hydroxykynurenine (Dalglish, 1952) and strongly suggested a 2-amino-3-hydroxyacetophenone skeleton.

a glucuronide. The hydrolysed material moved as a weak base. An HCl hydrolysate gave a marked precipitate with barium chloride solution. The conjugating group was therefore probably sulphate.

General considerations (see under Discussion) made it likely that the hydrolysed material was 2-amino-3-hydroxyacetophenone itself. An authentic sample of this substance was very kindly given by Professor L. Musajo (cf. Musajo, Spada & Casini, 1950) and hydrolysate and authentic substance were compared. The two behaved identically

Table 1. R_f values of the urinary metabolite and related substances

	Developing solvent*				
	Butanol-acetic acid	Neutral phenol	Pentanol-pyridine-water	20% KCl	5% Na formate
Urinary metabolite	0.42†	0.36	0.58	0.72	0.80
Product of persulphate oxidation of <i>o</i> -aminoacetophenone	0.42	0.36	0.58	0.72	0.79
Hydrolysis product of urinary metabolite	0.85	0.88	0.94	0.47	0.62
Authentic 2-amino-3-hydroxyacetophenone	0.85	0.88	0.95	0.48	0.61

* All on Whatman no. 1 paper by the descending technique. For composition of solvents see under Methods.

† R_f values for some other substances in this solvent under comparable conditions are: kynurenine, 0.40; tryptophan, 0.44; indican, 0.34. In mixtures these values may be modified by mutual displacement.

The substance was stable in neutral solution, or in dilute acetic acid (it was not decomposed over several weeks in urines preserved with acetic acid, and was not hydrolysed on butanol-acetic acid chromatograms). It was readily hydrolysed by mineral acid. When a solution was made normal with respect to HCl and allowed to evaporate overnight in a vacuum desiccator over soda, conversion into a new material occurred. This ran fast on butanol-acetic acid chromatograms, had the typical greenish fluorescence of 3-hydroxykynurenine, but was separable from *N*^α-acetyl-3-hydroxykynurenine. It was a strong reducing agent, reducing ammoniacal silver nitrate immediately, as is to be expected for a substance containing an unsubstituted *o*-aminophenol group. It also gave the expected orange-pink colour with Ehrlich's reagent.

It therefore seemed very probable that the metabolite was 2-amino-3-hydroxyacetophenone or a related substance conjugated on the phenolic group as the sulphate, phosphate or glucuronide. On paper electrophoresis in 0.4% acetic acid, conditions under which carboxyl dissociation is largely inhibited, the unhydrolysed material moved as a strong acid (indican and indoleacetic acid used for comparison; indican moved as a strong acid and indoleacetic acid remained close to the origin under the same conditions). This suggested a phenolic sulphate or phosphate, rather than

on paper chromatography in a variety of solvents, as shown in Table 1. The fluorescent material originally present in the urines was therefore considered to be 2-amino-3-hydroxyacetophenone *O*-sulphate, and this was confirmed by partial synthesis.

Aromatic amines react with alkali persulphates to give *o*-aminophenylsulphates (Boyland & Sims, 1954). Treatment of *o*-aminoacetophenone with potassium persulphate readily gave a product giving the same colour reactions as the urinary metabolite and travelling identically in several chromatographic solvents (Table 1) as well as on paper electrophoresis. Besides this major product there was also a minor product, very similar in chemical behaviour but differing in R_f values, together with free aminohydroxyacetophenones. In an investigation of the reaction of persulphates with anthranilic acid (S. F. Contractor & C. E. Dalglish, unpublished), which was made to investigate possible application of this reaction to large-scale synthesis of hydroxykynurenine and related compounds, it was found that besides the major product, 3-hydroxyanthranilic acid sulphate (considered by Boyland & Sims to be the only phenolic sulphate produced), the isomeric 5-hydroxyanthranilic acid sulphate was also formed, together with free 3- and 5-hydroxyanthranilic acids. The minor product in the reaction of persulphate with *o*-aminoacetophenone may therefore

well be 2-amino-5-hydroxyacetophenone *O*-sulphate, but it has not been further investigated. As only a small amount of *o*-aminoacetophenone was available, the reaction could not be carried out on a sufficiently large scale to make practicable isolation of the products in the crystalline state.

DISCUSSION

The only known likely source of a derivative of 2-amino-3-hydroxyacetophenone is tryptophan, which is metabolized via 3-hydroxykynurenine (I, Fig. 1; see Dalgliesh, 1955*b*, for collected references). Possible changes in the side chain of this substance are shown in Fig. 1. Aminohydroxyacetophenone (VI) might arise by decarboxylation

being formed. This reaction requires pyridoxal phosphate as coenzyme, as does the transamination of hydroxykynurenine leading to xanthurenic acid. It seems very probable that these reactions involve formation of a Schiff's base which can tautomerize, as follows (where Ar represents the 2-amino-3-hydroxyphenyl group and Py·CHO represents pyridoxal phosphate: cf. Metzler, Ikawa & Snell, 1954; Longenecker & Snell, 1955; Dalgliesh, 1955*b*):

The latter form of the Schiff's base (which *in vivo* may be coordinated with a metal and protein) is

Fig. 1. Possible routes for formation of 2-amino-3-hydroxyacetophenone from 3-hydroxykynurenine. Ar represents the 2-amino-3-hydroxyphenyl residue.

of the corresponding substituted acetic acid (II). But neither of the routes for formation of the latter substance generally applicable in amino acid metabolism is likely in the present case. Formation of the acetic acid derivative from the corresponding pyruvic acid (III) is unlikely, as the pyruvic acid is known to cyclize spontaneously to xanthurenic acid (4:8-dihydroxyquinoline-2-carboxylic acid) (Musafo, Spada & Bulgarelli, 1950). Similarly, formation via the corresponding amine (IV) and aldehyde (V) is unlikely, as the aldehyde would also be expected to undergo spontaneous cyclization. Thus the related kynurenamine is almost quantitatively excreted as quinoline derivatives by the rat (Butenandt & Renner, 1953). The only other likely route for formation of the acetophenone is by a direct hydrolytic scission of the hydroxykynurenine side chain. Direct scission on the other side of the methylene carbon is the normal route for hydroxykynurenine metabolism by the enzyme kynureninase, hydroxyanthranilic acid and alanine

analogous in electron distribution to β -keto acids and β -diketones. Direct scission of the hydroxykynurenine side chain to give the acetophenone derivative is thus analogous to the formation of acetone from acetoacetic acid. Excretion of the acetophenone derivative may therefore be due to a small spontaneous decomposition of the hydroxykynurenine-pyridoxal phosphate complex, or the reaction may be enzymically accelerated. The fact that the acetophenone derivative is excreted as the phenolic sulphate, a typical 'detoxication' product, suggests that the reaction is unlikely to be one that is metabolically useful.

Hydroxyaminoacetophenone was first observed in pathological urines. It is also excreted by at least some normal persons, though in amounts so small as to be readily overlooked. Too many at present unknown factors exist in the isolation procedure to allow determination of excretion values, but it appears most unlikely that normal excretion by man is as much as 1 mg./24 hr. The

relationship between the increased excretion and the fundamental lesion in the pathological cases, and the range of pathological cases in which this increased excretion occurs, remain to be determined.

SUMMARY

1. A metabolite excreted in small amount in some normal human urines, and in appreciably larger amounts in certain pathological urines, is shown by degradative and synthetic evidence to be 2-amino-3-hydroxyacetophenone *O*-sulphate.

2. Reasons are given for considering this substance to arise by hydrolytic scission of the side chain of hydroxykynurenine.

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Studies on the Acetylerase of *Sclerotinia laxa*

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This paper deals with the hydrolysis of phenolic esters by enzyme preparations from *Sclerotinia laxa* Aderh. & Ruhl., one of the fungi causing the brown-rot diseases of fruits. The investigations were undertaken on account of the apparent role of a fungal enzyme in the mode of fungistatic action of esters of 2:3-dichloro-1:4-naphthaquinone (Byrde & Woodcock, 1952, 1953).

Macdonnell, Jang, Jansen & Lineweaver (1950), in the course of studies of pectin and acetyl-esterases of fruits of *Citrus* spp., showed also that a pectin esterase of fungal origin readily hydrolysed phenyl acetate and phenyl propionate at pH 6.8, and, to a lesser extent, at pH 4.5. Subsequently Jermyn (1953) demonstrated that three or four components with esterase activity could be separated by paper chromatography from cultures of *Aspergillus flavus-oryzae*. In further studies on the enzymes of this fungus, Crewther & Lennox (1953) showed that a pH of 6.0 was the optimum for esterase activity, and demonstrated that there was little or no activity in cultures under 6 days old.

Amongst the more numerous workers on esterases of non-fungal origin, Aldridge (1953), in a study of serum esterases, classified them into two types, on the basis of substrate specificity and their inhibition by diethyl *p*-nitrophenyl phosphate (paraoxon). He defined type A esterases as those hydrolysing *p*-nitrophenyl acetate more readily than the corresponding butyryl ester, while relatively resistant to inhibition by paraoxon.

Type B esterases, on the other hand, hydrolysed the butyryl derivative more rapidly than the acetate, and were inhibited by concentrations of paraoxon as low as 10^{-7} – 10^{-8} M.

The effect of nuclear substitution on the hydrolysis of phenyl acetate by a wheat-germ esterase was studied by Gawron, Grelecki & Duggan (1953), who suggested that electromeric factors might account for the differing rates of hydrolysis. Electron-withdrawing substituents, especially in the *meta* position, increased the hydrolysis rate, but electron-donating substituents decreased the rate.

EXPERIMENTAL

Source of enzyme

The enzyme used in most of the experiments was a crude preparation from cultures of *S. laxa* grown on autoclaved acidified potato plugs, the medium used in this laboratory for routine production of spores. The mycelium was scraped from the surface of the cultures and macerated with buffer solution or water, according to the method subsequently used for the estimation of enzyme activity. The resultant pulp was filtered under suction and the crude filtrate stored at -4° . The enzyme was roughly concentrated by successive freezing and thawing and removal of ice crystals, resulting in a preparation of moderately high activity.

Preparations of higher activity could be obtained by fractionation with ammonium sulphate in the presence of sodium oxalate, followed by dialysis, as described by Jansen, Jang & Macdonnell (1947).