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TRANSPORT AND FURTHER METABOLISM OF INTERMEDIATE COMPOUNDS OF THE TRYPTOPHAN–NICOTINIC ACID RIBONUCLEOTIDE PATHWAY BY RAT SMALL INTESTINE

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SUMMARY

1. The everted sac technic has been used to study the intestinal transport of several intermediate compounds of the tryptophan–nicotinic acid ribonucleotide pathway.

2. L-Kynurenine and to a lesser extent, 3-hydroxy-L-kynurenine, were transported against a concentration gradient, whereas there was no active transport of D-kynurenine, 3-methoxy-DL-kynurenine, N^{α}-acetyl-L-kynurenine or kynurenic acid.

3. Paper chromatography of the serosal fluids showed that small quantities of L-kynurenine, 3-hydroxykynurenine and 3-methoxykynurenine were acetylated during incubation, and that some deacetylation of N^{α}-acetyl-L-kynurenine took place. Two other products of 3-hydroxykynurenine were most probably the two isomers of 3-hydroxykynurenine-*O*-sulfate.

INTRODUCTION

L-Tryptophan is metabolized to nicotinic acid ribonucleotide in the liver *via* a number of intermediate compounds (Fig. 1). Changes in the activity of the enzymes concerned or in the levels of their cofactors, result in an abnormal excretion of these metabolites in urine, and such disturbances of tryptophan metabolism have been described in association with a variety of diseases[1,2]. Another factor which may possibly influence the urinary excretion of tryptophan metabolites is their mode of transport across cell membranes. The present report describes an *in vitro* study of the transport of some of these metabolites by the rat small intestine, and of their further metabolism by the intestine.

MATERIALS AND METHODS

L-Tryptophan was obtained from Mann Research Laboratories. L- and D-kynurenine sulfates[3], N^{α}-acetyl-L-kynurenine[4], 3-hydroxy-DL-kynurenine[5], 3-methoxykynurenine[5] and kynurenic acid[6] were prepared in this laboratory essentially as previously described, and their purity was checked by paper chromatography. They were

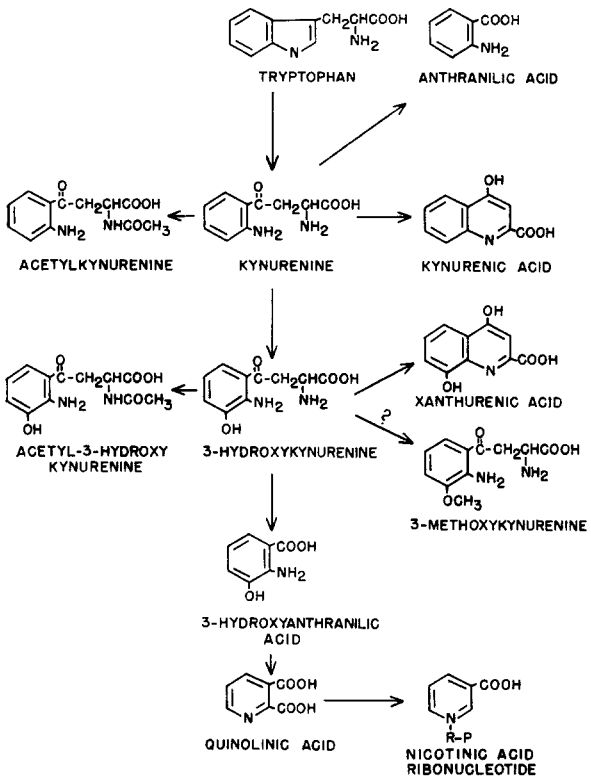

Fig. 1. The metabolism of L-tryptophan to nicotinic acid ribonucleotide.

dissolved in Krebs-bicarbonate solution (pH 7.4) to give a concentration of 2 mM. 3-Hydroxykynurenine was also studied at a concentration of 4 mM.

Sprague–Dawley female rats weighing 180–200 g were studied in the non-fasting state. They were killed by a blow on the head, and the small intestines were removed and everted as described by WILSON AND WISEMAN[7]. Six sacs were made from each intestine, the first starting 10 cm from the pylorus. Each sac, approx. 10 cm long, was filled with 1 ml of Krebs-bicarbonate solution containing 17 mM glucose and the metabolite being tested ('serosal fluid'). The filled sac was immersed in 20 ml of the same solution ('mucosal fluid') in a glass-stoppered 125-ml flask[8], and incubated at 37° for 1 h with constant shaking at a rate of 80 oscillations per min in an atmosphere of O_2–CO_2 (95:5, v/v). After incubation the sac was removed, the outside was blotted dry, and the serosal fluid collected and weighed to determine the water transfer across the intestinal wall. The empty sac was dried in an oven at 100° for 4 h and then weighed to obtain the dry weight. The concentrations of glucose and the test metabolite were measured in the mucosal and serosal fluids.

Glucose was determined by a D-glucose oxidase method (Worthington Biochemical Corp.) and L-tryptophan by the method of SPIES AND CHAMBERS[9]. The concentrations of the metabolites were obtained by ultraviolet spectrophotometry. A study of the absorption spectra of mucosal and serosal fluids from sacs incubated in Krebs-bicarbonate solution without added metabolite showed that no interfering

material leaked from the intestinal wall during the incubation period. The absorption maxima were read from the spectra of suitably diluted mucosal and serosal fluids (1:20 or 1:40), determined over a range of 225–530 mμ with a Bausch and Lomb Spectronic 505 spectrophotometer (Fig. 2). The absorbances of L-kynurenine and D-kynurenine were read at 365 mμ, acetyl-L-kynurenine at 360 mμ, 3-hydroxy-DL-kynurenine and 3-methoxy-DL-kynurenine at 370 mμ, and kynurenic acid at 332 mμ. In each case the original Krebs-bicarbonate solution containing the metabolite was also examined to provide a standard.

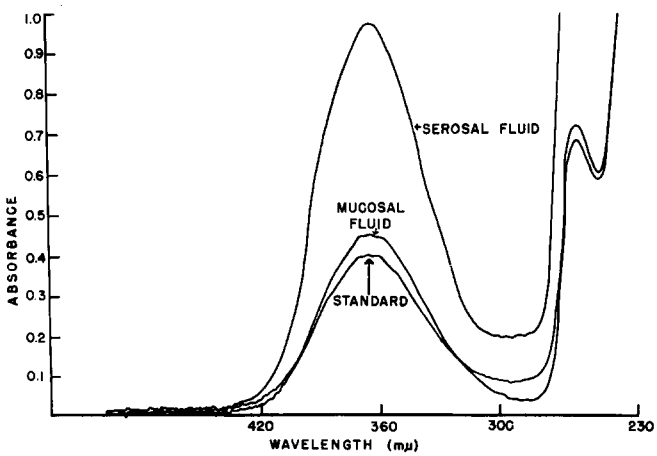

Fig. 2. The absorption spectrum of L-kynurenine in mucosal and serosal fluids after incubation and of the standard solution in Krebs-bicarbonate buffer (pH 7.4). Before incubation the L-kynurenine concentrations in the mucosal and serosal fluids and in the standard solution were identical (2 mM). The intestinal sac was incubated at 37° for 1 h, after which the serosal fluid concentration, indicated by the absorbance at 365 mμ, was more than twice that of the mucosal fluid showing that active transport of the metabolite had taken place. The slight increase in mucosal fluid concentration was due to evaporation which took place when the flask was being gassed at the beginning of the incubation period.

The rate of transport was calculated from the difference between the post- and preincubation concentrations in the serosal fluid, multiplied by the volume of serosal fluid after incubation, and the final result was expressed in μmoles of metabolite transported per 100 mg dry tissue per h.

Ascending chromatograms of the serosal and mucosal fluids were run on Whatman No. 1 paper. The solvents used were methanol–*n*-butanol–benzene–water (2:1:1:1, by vol.) *plus* 1 % glacial acetic acid[10], *n*-butanol–acetic acid–water (12:3:5, by vol.), and 20 % KCl (ref. 11). The completed chromatograms were examined under ultraviolet light, and after spraying with Ehrlich's reagent (2 % (w/v) *p*-dimethylaminobenzaldehyde in 1.3 M HCl) or a modified Ekman's reagent (0.25 % $NaNO_2$ in 0.1 M HCl followed by 0.25 % *N*-1-naphthylethylene diamine·HCl). Flasks containing the metabolites under investigation dissolved in Krebs-bicarbonate solution, but without intestinal sacs, were also oxygenated and incubated at 37° for 1 h, and the contents were then examined by paper chromatography.

3 or 4 rats were used to study each compound. In all cases viability of the sacs was confirmed by the active transport of glucose from the mucosal to serosal fluid.

RESULTS

L-Tryptophan, L-kynurenine (prepared as the sulfate) and 3-hydroxykynurenine were transported across the intestinal wall against a concentration gradient. Sacs from the middle and lower jejunum and upper ileum showed a greater rate of transport of all three compounds than did those from the ends of the intestine (Fig. 3), and so in Table I only the results from the four middle sacs have been included. This shows that L-tryptophan and L-kynurenine were transported at similar rates, whereas 3-hydroxykynurenine was poorly transported at a concentration of 2 mM and not at all when the concentration was increased to 4 mM.

D-Kynurenine, acetyl-L-kynurenine, 3-methoxy-DL-kynurenine and kynurenic acid were not transported against a concentration gradient.

The chromatographic data are summarized in Table II. Paper chromatograms

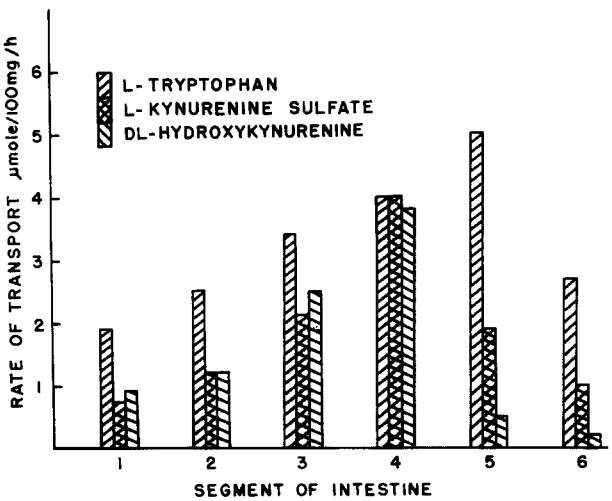

Fig. 3. Relationship between the segment of small intestine used and the rate of metabolite transport. All three metabolites were studied at an initial concentration of 2 mM. Segment 1 started 10 cm from the pylorus and Segment 6 was terminal ileum.

TABLE I

INTESTINAL TRANSPORT OF TRYPTOPHAN METABOLITES

The sacs were incubated for 1 h at 37°. The data for the middle 4 sacs from each rat have been included in the results given, which are expressed as the mean ± S.D.

Metabolite	*No. of rats studied*	*Rate of transport (µmoles/100 mg per h)*	*Ratio of glucose (Concn. serosal/mucosal flu*
L-Tryptophan	4	4.10 ± 0.53	2.10 ± 0.49
L-Kynurenine sulfate	4	3.47 ± 0.93	3.22 ± 0.83
D-Kynurenine sulfate	3	0	3.04 ± 0.87
3-Hydroxy-DL-kynurenine (2 mM)	4	2.04 ± 0.41	2.94 ± 0.50
3-Hydroxy-DL-kynurenine (4 mM)	2	0	2.18 ± 0.06
3-Methoxy-DL-kynurenine	3	0	2.63 ± 0.53
N^{α}-Acetyl-L-kynurenine	3	0	3.07 ± 0.30
Kynurenic acid	3	0	2.04 ± 0.06

TABLE II

PAPER CHROMATOGRAPHY OF THE SEROSAL FLUIDS FROM TRANSPORT STUDIES

Ascending technic with methanol–*n*-butanol–benzene–water (2:1:1:1, by vol.) *plus* 1% acetic acid solvent. 20 and 50 μl applied to paper.

Metabolite under investigation	*Compound on chromatogram*	R_F	*Ultraviolet light*
L-Kynurenine	L-Kynurenine	0.48	Blue
	? Acetyl-L-kynurenine	0.82	Blue
D-Kynurenine	D-Kynurenine	0.37	Blue
Acetyl-L-kynurenine	Acetyl-L-kynurenine	0.82	Blue
	? L-Kynurenine	0.46	Blue
3-Hydroxy-DL-kynurenine	3-Hydroxy-L-kynurenine	0.37	Green
	3-Hydroxy-D-kynurenine	0.30	Green
	? Acetyl-3-hydroxy-L-kynurenine	0.80	Green
	? 3-Hydroxy-L-kynurenine-*O*-sulfate	0.16	Blue
	? 3-Hydroxy-D-kynurenine-*O*-sulfate	0.12	Blue
3-Methoxy-DL-kynurenine	3-Methoxy-L-kynurenine	0.47	Blue
	3-Methoxy-D-kynurenine	0.42	Blue
	? Acetyl-3-methoxy-L-kynurenine	0.86	Blue
Kynurenic acid	Kynurenic acid	0.58	Blue

of mucosal and serosal fluids containing D-kynurenine or kynurenic acid showed only spots corresponding to these metabolites. After incubation of the flasks containing L-kynurenine a spot with the same R_F value as acetyl-L-kynurenine was found consistently on chromatograms of the serosal fluids. The R_F values of this spot in *n*-butanol–acetic acid–water and 20% KCl solvents were also the same as those of acetyl-L-kynurenine and it gave identical color reactions with Ehrlich's and Ekman's reagents (Table III). Chromatography of the serosal fluid from experiments with acetyl-L-kynurenine revealed the presence of small quantities of L-kynurenine.

Chromatograms of the serosal and mucosal fluids from studies of 3-hydroxy-DL-kynurenine transport showed several features of interest. A larger spot of 3-hydroxy-L-kynurenine than that of the D-isomer was always seen on the serosal fluid chromatograms, whereas with the mucosal fluids the two spots were of equal size. Three other spots were always present on chromatograms of the serosal fluids. One of these was considered to be acetyl-3-hydroxy-L-kynurenine and the others, which were much more prominent under ultraviolet light, the two isomers of 3-hydroxykynurenine-*O*-sulfate (Table III). Some decomposition of 3-hydroxykynurenine to xanthommatin occurred in the mucosal fluid during incubation. This was noted also when solutions were incubated without the presence of an intestinal sac, indicating that the formation of xanthommatin was not due to enzymic activity in the intestinal mucosa.

The serosal fluids from experiments with 3-methoxy-DL-kynurenine showed an extra spot on chromatograms developed in methanol–*n*-butanol–benzene–water *plus* 1% acetic acid solvent. This was probably the acetyl derivative as its R_F value corresponded closely with those of acetyl-L-kynurenine and acetyl-3-hydroxy-

TABLE III

FURTHER CHROMATOGRAPHIC IDENTIFICATION OF PRODUCTS FROM METABOLITES UNDER TEST

Two-dimensional ascending paper chromatography with *n*-butanol–acetic acid–water (I) as the first solvent, followed by 20% KCl (II).

	R_F		*Reaction with*	
	*I**	*II*	*Ehrlich's reagent*	*Ekman's reagent*
Authentic L-kynurenine	0.33	0.72	Orange	Violet
? L-Kynurenine	0.32	0.72	Orange	Violet
Authentic N^{α}-acetyl-L-kynurenine	0.84	0.81	Orange	Violet
? N^{α}-Acetyl-L-kynurenine	0.84	0.80	Orange	Violet
N^{α}-Acetyl-3-hydroxy-L-kynurenine**	0.75	0.74	Pink	Nil
? N^{α}-Acetyl-3-hydroxy-L-kynurenine	0.77	0.75	Pink	Nil
3-Hydroxykynurenine-*O*-sulfate**	0.11	0.80	Pink after 1 h	Nil
? 3-Hydroxy-L-kynurenine-*O*-sulfate ? 3-Hydroxy-D-kynurenine-*O*-sulfate	0.12***	0.81***	Nil	Nil

* *n*-Butanol–acetic acid–water (12:3:5, by vol.) is equivalent to the organic layer of a 4:1:5 mixture as used by DALGLIESH[11,15].

** Data given by DALGLIESH[11,15].

*** Isomers were not separated in these solvents.

kynurenine, but as neither authentic material nor published data about this compound was available, further investigation was not carried out.

DISCUSSION

The present study has shown that L-kynurenine, unlike the D-isomer, is actively transported against a concentration gradient by the rat small intestine. The greater size and intensity of the 3-hydroxy-L-kynurenine spot as compared with that of 3-hydroxy-D-kynurenine on paper chromatograms of the serosal fluids indicates that active transport of this metabolite is also confined to the L-isomer. In this respect these basic amino acids behave like their parent compound, L-tryptophan, and most other amino acids[12]. N^{α}-Acetyl-L-kynurenine and kynurenic acid were not transported against a concentration gradient, which is in accord with the finding of COHEN AND HUANG[13] that the presence of an α-amino and a carboxyl group is essential for the transport of various analogs of tryptophan.

It is of interest that substitution at the 3-position of the benzene ring impairs the capacity for intestinal transport. 3-Hydroxykynurenine at a concentration of 2 mM was actively transported, but less so than L-kynurenine. It was thought that this might have resulted from only the L-isomer of the racemic mixture being transported against the concentration gradient so that the effective concentration was only 1 mM. However, when the concentration of 3-hydroxykynurenine in the incubation medium was increased to 4 mM there was complete loss of transport despite normal activity of the intestinal sacs with respect to water and glucose transfer.

Further, 3-methoxy-DL-kynurenine, which like 3-hydroxykynurenine retains both the α-amino and carboxyl groups of kynurenine, showed no evidence of active transport.

The demonstration that L-kynurenine and 3-hydroxy-L-kynurenine are actively transported by the intestinal mucosa raises the question of the manner in which these metabolites are handled by the renal tubule. In general, there are marked similarities between the transport of amino acids by the intestine and the kidney, and so it seems likely that L-kynurenine and 3-hydroxy-L-kynurenine are reabsorbed from the glomerular filtrate. The capacity for such reabsorption would influence not only the urinary levels of these tryptophan metabolites, but also those which are derived from them as reabsorption would allow recirculation and further exposure for the biosynthesis of nicotinic acid ribonucleotide from L-tryptophan.

Paper chromatography showed that small quantities of L-kynurenine, 3-hydroxykynurenine and probably 3-methoxykynurenine were acetylated during incubation with the intestinal sacs. Some deacetylation of acetyl-L-kynurenine also appeared to take place, although the amount of L-kynurenine produced was very small and its transfer to the serosal fluid was not sufficient to be demonstrated during the spectrophotometric study of acetylkynurenine transport. WHALER[14] found that the acetyl derivatives of several amino acids, but not acetyltryptophan, are deacetylated by the intestinal mucosa, and considered the possibility of a transacetylating enzyme being present in rat small intestine. The present results indicate that both acetylation of L-kynurenine and deacetylation of acetyl-L-kynurenine can take place, and are consistent with the existence of a single transacetylase system.

DALGLIESH reported the presence of 3-hydroxykynurenine-*O*-sulfate in the urine of pyridoxine-deficient rats[15]. A comparison with his data indicates that the rat small intestine can form the two isomers of this compound from 3-hydroxy-DL-kynurenine, and that they appear in the serosal fluid during intestinal transport *in vitro*. Conjugation of an amino acid to form the sulfate ester does not appear to have been described previously in studies of intestinal transport, although glucuronide synthesis occurs in the intestine, and UDP glucuronyltransferase activity has been demonstrated throughout the gastrointestinal tract[16]. TAPLEY *et al.*[17] found that monoiodotyrosine forms a glucuronide which is present in the serosal fluid after this amino acid has been incubated with rat intestinal sacs. In addition to 3-hydroxykynurenine-*O*-sulfate, DALGLIESH[15] identified 3-hydroxykynurenine glucuronide in urine, but the higher polarity of this compound distinguished it from the sulfate ester, and a product having the chromatographic characteristics of the glucuronide conjugate was not seen in the present study.

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