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Enzyme activities of tryptophan metabolism along the kynurenine pathway in various species of animals

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Abstract

The purpose of this study was to investigate variations in the enzyme activities of the kynurenine pathway in various mammals (rabbit, mouse, rat, guinea-pig). Liver tryptophan 2,3-dioxygenase, small intestine indole 2,3-dioxygenase, liver and kidney kynurenine 3-monooxygenase, kynureninase, kynurenine-oxoglutarate transaminase, 3-hydroxyanthranilate 3,4-dioxygenase and aminocarboxymuconate-semialdehyde decarboxylase were analysed. Small intestine superoxide dismutase activity and free and total serum tryptophan were also measured. Liver tryptophan 2,3-dioxygenase was present as both holoenzyme and apoenzyme only in rat, while in the other species only holoenzyme activity was observed. Also, small intestine indole 2,3-dioxygenase activity was more abundant in rat than in the other animals studied. The highest activity of small intestine superoxide dismutase was found in rat, and the lowest in rabbit. Liver and kidney kynurenine 3-monooxygenase activity was very elevated and higher in mouse, followed by rat; rabbit showed the lowest activity. Kynureninase activity appeared to be much lower among the enzymes of the kynurenine pathway. However, guinea-pig showed higher activity in both liver and kidney in comparison with other species. With regard to kynurenine-oxoglutarate transaminase, all species examined here presented more abundant enzyme activity in kidney, the value being similar between rat and mouse. Guinea-pig was the animal with the lowest activity. 3-Hydroxyanthranilate 3,4-dioxygenase showed the highest activity of all the enzymes evaluated in the study, but with different levels in liver and kidney, varying among species. The most elevated activity of aminocarboxymuconate-semialdehyde decarboxylase was present in kidney of guinea-pig, and the lowest in rabbit. Serum concentrations of tryptophan were higher in rat, followed by mouse, rabbit and guinea-pig. In conclusion, the present study demonstrates that the enzyme activities of the kynurenine pathway are very active in tissues of the four species of mammals investigated. The proposed method of in vitro enzyme determination represents a valid alternative to study of the tryptophan metabolic route.

Keywords: Tryptophan metabolism; Kynurenine pathway enzymes; Animal species

1. Introduction

Every day a normal diet in man supplies about 600–900 mg of tryptophan. Besides being partly incorporated into proteins (30% of dietary tryptophan), in man tryptophan is metabolised along various pathways, yielding biologically important compounds [1–4]. Scheme 1 summarizes the metabolic pathways of this amino acid.

The major metabolic pathway is the kynurenine pathway leading to the biosynthesis of nicotinic acid (niacin) (Scheme 2): 60 mg of tryptophan yields 1 mg of niacin. The sequence of steps that takes place in vivo along this pathway, named kynurenine pathway in order to distinguish it from others, has now been elucidated through the efforts of many researchers. Along this route, only small amounts of tryptophan metabolites are excreted daily by normal human subjects fed a free diet.

The sum of metabolites corresponds to 6.04 mg, about 1–2% of tryptophan ingested with diet [1]. Human subjects convert the largest part of dietary tryptophan to non-aromatic compounds along the glutarate

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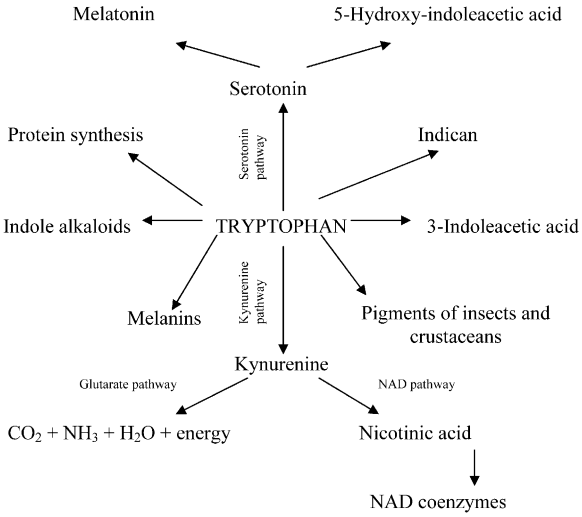


Scheme 1.

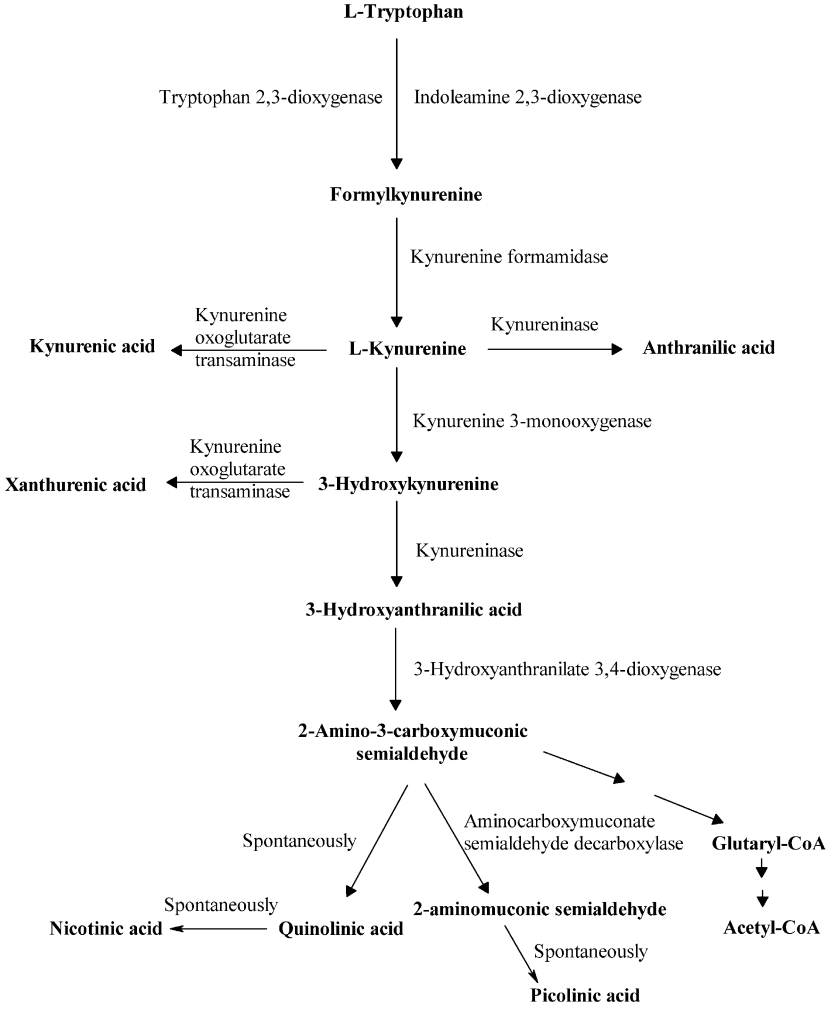
pathway. However, in some diseases and in cases of vitamin deficiency, this pattern can change quantitatively [1].

Along the serotonin pathway, about 1–2% of ingested tryptophan is metabolised to give 5-hydroxy-indoleacetic acid. In patients with carcinoid, however, as much as 60% of dietary tryptophan may be converted to 5-hydroxy-indoleacetic acid [3,5].

From tryptophan, by the action of intestinal bacteria, 3-indoxysulphate (indican) is formed, which is excreted in relatively large amounts in the urine of healthy subjects, corresponding to about 3% of dietary tryptophan [6]. The excretion of indican may increase in various diseases (Hartnup's disease, schizophrenia, phenylketonuria, malabsorption, pernicious anemia) [3].

Tryptophan is also a precursor of 3-indoleacetic acid, normally excreted in the urine of human subjects up to 100 μmol per day (about 3–4% of dietary tryptophan) [3,7]. It is formed by both tissue enzymes and bacteria in the intestine. This amount may rise more than 10 times in 24 h in various pathological conditions (such as idiopathic sprue) [3].

The involvement of tryptophan in the biosynthesis of melanins has been demonstrated by our laboratory [8–



Scheme 2.

10]. How much tryptophan is necessary for this route is not known.

Very little tryptophan is excreted unmodified in urine (0.5%). The kynurenine pathway therefore represents the major metabolic route through which tryptophan is totally degraded along the glutarate pathway, yielding energy (Schemes 1 and 2). This pathway is altered in a great number of diseases [1–3].

The administration of a loading dose of L-tryptophan is widely used to reveal abnormalities in tryptophan metabolism, by determining the urinary metabolites of the kynurenine pathway [11,12]. In humans, the enzyme activities of this route were rarely measured, due to the impossibility of obtaining isolated organs or tissues, unless after surgical treatment [13,14] or death [15,16]. Therefore, other mammals may be used to provide useful models for investigating enzyme activities along the kynurenine pathway.

The discovery of the enzyme indoleamine dioxygenase (IDO) [17–19] in many tissues and organs, cleaving tryptophan via kynurenine, has thrown light on the interpretation of results obtained until now in the abnormal metabolism of tryptophan in various pathological conditions [1–3]. Hayaishi [20] has suggested that, when some tissues are invaded by viruses, bacteria or endotoxins, interferon is produced, stimulating the synthesis of prostaglandins, inducers of indoleamine dioxygenase. As a consequence of inflammation, superoxide anion is liberated and used by IDO, causing a marked increase in the conversion of tryptophan to kynurenine.

The catabolism of tryptophan along the kynurenine pathway has aroused considerable interest, not only for the role that some metabolites play in several neuropathological conditions [21–24], but also due to the recent results obtained by Munn and coworkers [25–27], who have shown that cells expressing IDO are able to inhibit T-cell proliferation in vitro.

The purpose of the present study was to investigate if any difference exists in the enzyme activities of the kynurenine pathway among various species of animals and if the mammalian species considered—rabbit, mouse, rat and guinea-pig—are good animal models for investigating tryptophan metabolism in pathological conditions by measuring enzyme activities along the kynurenine pathway.

The following enzymes were determined: liver tryptophan 2,3-dioxygenase [TDO; L-tryptophan:oxygen 2,3-oxidoreductase (decyclizing), EC 1.13.11.11], small intestine indole 2,3-dioxygenase (indoleamine 2,3-dioxygenase) [IDO; indole:oxygen 2,3-oxidoreductase (decyclizing), EC 1.13.11.17], liver and kidney kynurenine 3-monooxygenase [L-kynurenine, $NADPH_2$:oxygen oxidoreductase (3-hydroxylating), EC 1.14.13.9], kynurenine-oxoglutarate transaminase [L-kynurenine: 2-oxoglutarate aminotransferase, EC 2.6.1.7], kynureninase [L-kynurenine hydrolase, EC 3.7.1.3], 3-hydroxyanthranilate 3,4-dioxygenase [3-hydroxyanthranilate:oxygen 3,4-oxidoreductase (decyclizing), EC 1.13.11.6], and aminocarboxymuconate-semialdehyde decarboxylase (picolinic carboxylase) [2 amin-3-(3-oxo-prop-2-enyl)-but-2-enedioate carboxy-lyase, EC 4.1.1.45]. The activity of small intestine superoxide dismutase (SOD, EC 1.15.1.1) and free and total serum tryptophan concentrations were also determined.

2. Materials and methods

2.1. Chemicals

L-Tryptophan, L-kynurenine sulfate, α-ketoglutaric acid, pyridoxal phosphate, 3-hydroxyanthranilic acid, anthranilic acid, haematin hydrochloride, NADPH, catalase, superoxide dismutase (SOD), nitro blue tetrazolium, methylene blue, L-ascorbic acid and xanthine oxidase were purchased from Sigma Chemical Company. Bovine erythrocyte SOD was used as standard. All other chemicals were of analytical R grade.

2.2. Animals

Forty adult male animals were considered: 10 New Zealand rabbits (3–4 kg body weight), 10 Wistar rats (300–350 g body weight), 12 mice (24–27 g body weight) and eight guinea-pigs (450–460 g body weight). All animals were maintained on a standard diet with water ad libitum, and were sacrificed by cervical dislocation. Liver, kidney, small intestine and blood were removed immediately after death, weighed, cut into small pieces, and homogenized (20% homogenate with 50 mM sodium phosphate, pH 7.2) at 4 °C in a Sorwall omnimix homogenizer for liver and kidney and a Potter–Elvehjem homogenizer with a Teflon pestle for intestine. Blood was collected in a non-heparinized tube, allowed to clot at room temperature for 30 min, and then centrifuged at $4000 \times g$ for 20 min.

2.3. Enzyme assays

Enzyme activities were expressed as nmol of product formed per min per mg of protein (specific activity) and as nmol of product formed per min per g of wet tissue.

2.3.1. Tryptophan 2,3-dioxygenase

TDO activity was determined in the supernatant of liver homogenate, prepared as reported by Bertazzo et al. [28] either in the absence (holoenzyme activity) or presence (total enzyme activity) of added haematin (2 μM) during the linear phase of kynurenine formation, according to the method of Feigelson and Greengard [29], slightly modified [30].

2.3.2. Indole 2,3-dioxygenase

IDO activity was measured in small intestine according to the method of Shimizu et al. [31], slightly modified in our laboratory. After homogenization, intestine was lyophilized and then centrifuged at $10\,000 \times g$ for 20 min. The resulting product was determined spectrophotometrically at 335 nm ($\varepsilon = 3500\ M^{-1}\ cm^{-1}$), the isosbestic point of formylkynurenine and kynurenine.

2.3.3. Kynurenine 3-monooxygenase

Enzyme activity was assayed spectrophotometrically in liver and kidney mitochondria, obtained according to Mawal and Deshmuth [32] by decreasing the optical density at 340 nm for a period of 5 min, following the method of Hayaishi [33].

2.3.4. Kynureninase

Kynureninase activity was measured in liver and kidney supernatants [28] according to the Saran method [34] by measuring the formation of anthranilic acid fluorometrically.

2.3.5. Kynurenine-oxoglutarate transaminase

Enzyme activity was measured in both liver and kidney supernatants by a modification of the method of Mason [35], as reported by Bertazzo et al. [28].

2.3.6. 3-Hydroxyanthranilate 3,4-dioxygenase

Enzyme activity was determined spectrophotometrically as reported by Mehler [36]. The increase in absorbance at 360 nm was monitored for 15 s at 24 °C. A molar extinction coefficient of $47\,500\ M^{-1}\ cm^{-1}$ for the reaction product was used [37].

2.3.7. Aminocarboxymuconate-semialdehyde decarboxylase

Activity was measured in the conditions reported above for the 3-hydroxyanthranilate 3,4-dioxygenase assay. Since the molar extinction coefficient of α-amino-β-carboxymuconic-ε-semialdehyde is approximately 4.5×10^{-4} M [38], in our assay conditions one unit of enzyme activity corresponds to the disappearance of 1 μmol of the substrate per minute.

2.3.8. Superoxide dismutase

SOD activity was measured in the supernatant obtained from small intestine according to the method of Beauchamp and Fridovich [39]. Bovine erythrocyte SOD was used as standard. Inhibition of 50% was achieved at 0.0068 μg superoxide dismutase/ml, an amount defined as one unit of this enzyme in the conditions of assay.

2.4. Protein analysis

Protein was measured by the method of Lowry et al. [40] using bovine serum albumin as a standard.

2.5. Tryptophan determination

Free and total (free + protein-bound) tryptophan was assayed spectrofluorimetrically in serum, as reported by De Antoni et al. [41] according to the method of Denkla and Dewey [42].

3. Results

Table 1 shows the activities of liver TDO and small intestine IDO. TDO activity was present only as holoenzyme in rabbit, mouse and guinea-pig, whereas rat liver also contained TDO-apoenzyme. In addition, rat presented higher activity of TDO in comparison with the other species. Also, IDO activity was more abundant in rat than in the other animals.

Table 1 also shows the activity of small intestine SOD. Among the species of animals considered, the highest activity was found in rat and the lowest in rabbit.

Tables 2 and 3 show the activities of kynurenine 3-monooxygenase, kynureninase, kynurenine-oxoglutarate transaminase, 3-hydroxyanthranilate 3,4-dioxygenase and aminocarboxymuconate-semialdehyde decarboxylase, in liver and kidney, respectively.

Kynurenine 3-monooxygenase activity was very elevated and was higher in mouse, followed by rat in both liver (Table 2) and kidney (Table 3); rabbit showed the lowest activity. While mouse and rat kidney was richer than liver, in rabbit liver had higher enzyme content in comparison with kidney. In guinea-pig, enzyme activity in liver was similar to that in kidney.

Kynureninase activity appeared to be much lower among the enzymes of the kynurenine pathway. However, guinea-pig showed higher activity in both liver and kidney in comparison with other species of animals. Activities in rabbit, rat and guinea-pig were similar in both organs, whereas in mouse activity was markedly higher in liver than in kidney.

With regard to kynurenine-oxoglutarate transaminase, all species showed more abundant enzyme activity in kidney, the value being similar between rat and mouse. Guinea-pig showed the lowest activity.

3-Hydroxyanthranilate 3,4-dioxygenase showed the highest activity among all the enzymes evaluated in the study. Comparing enzyme content in liver of different species, guinea-pig presented both activities (specific activity, and activity per g of fresh tissue) higher than in the other species; rabbit showed the lowest (Table 2). In kidney, specific activity was higher in rabbit, followed by guinea-pig. However, considering the activity per g of

Table 1
Liver TDO, intestine IDO and intestine SOD in various mammalian species

Species	Liver holo-TDO		Small intestine IDO		Small intestine SOD
	Specific activity (nmol/min/mg protein)	Activity/g fresh tissue (nmol/min/g tissue)	Specific activity (nmol/min/mg protein)	Activity/g fresh tissue (nmol/min/g tissue)	Units/mg of protein
Rabbit	0.041±0.002	9.51±0.69	0.151±0.013	16.35±1.33	0.57±0.07
Rat	0.20±0.01*	38.00±1.12**	0.73±0.08	73.01±4.33	4.79±0.61
Mouse	0.067±0.003	10.15±0.67	0.112±0.008	3.02±0.23	1.58±0.32
Guinea-pig	0.07±0.004	12.06±0.79	0.141±0.006	12.00±0.10	1.37±0.11

Data are expressed as means±SEM. Apoenzyme+holoenzyme: * 0.28±0.01 and ** 54.17±1.41.

fresh tissue, guinea-pig showed higher activity than rabbit (Table 3). In rat and above all in mouse, the activity of this enzyme was higher in liver (Table 2) than in kidney (Table 3).

The highest values of aminocarboxymuconate-semialdehyde decarboxylase were found in kidney of guinea-pig (Table 3) and the lowest in rabbit, where no difference was observed between liver (Table 2) and kidney (Table 3). Rat too showed higher activity in kidney than in liver.

Table 4 shows serum concentrations of tryptophan. The values, reported as μg/ml of serum, were higher in rat, followed by mouse. In rabbit and guinea-pig, serum concentrations were similar and lower than those of the other two species. The free fraction was about 10% of total serum tryptophan.

4. Discussion

Study of tryptophan metabolism along the kynurenine pathway has patho-physiological relevance, as suggested for example by the fact that this route is rapidly induced by IFN-γ in human mononuclear cells [43,44].

Mammals are very useful animal models for studying this catabolism in pathological conditions, by measuring urinary excretion of metabolites after a load of L-tryptophan or by determining enzyme activities along the kynurenine pathway. In some animals, repeated doses of tryptophan cause death, due to the toxicity of the amino acid as a consequence of the absence of TDO-apoenzyme [30,45–52]. To obviate this disadvantage, one useful method of investigating the abnormal metabolism of tryptophan is to measure enzyme activities in isolated organs and tissues.

The purpose of our study was therefore to investigate whether the enzymes involved in this metabolic route showed species differences in their activities in rabbit, rat, mouse and guinea-pig, in order to establish their role as models for the study of tryptophan metabolism, also extended to pathological conditions. Results show that the tryptophan→nicotinic acid pathway is very active in mammals, although the single enzyme activities vary among species.

Liver TDO is present only as a holoenzyme in rabbit, mouse and guinea-pig, supporting the results obtained by Hvitefelt and Santti [47], Badawy and Evans [45,46] and our laboratory [28,30,53–55]. Rat, also possessing TDO-apoenzyme, presents markedly higher activity of

Table 2
Enzyme activities of kynurenine pathway in liver of various animal species

Enzymes in liver		Animal species			
		Rabbit	Rat	Mouse	Guinea-pig
Kynurenine 3-monooxygenase	Specific activity	1.131±0.045	2.18±0.16	2.39±0.06	1.5±0.05
	Activity/g fresh tissue	151.42±5.81	583.29±42.85	656.55±18.99	388.72±13.85
Kynureninase	Specific activity	0.019±0.001	0.056±0.002	0.037±0.001	0.094±0.006
	Activity/g fresh tissue	4.08±0.23	11.33±0.62	10.69±0.26	14.40±0.72
Kynurenine-oxoglutarate transaminase	Specific activity	0.337±0.024	0.347±0.021	0.223±0.016	0.138±0.010
	Activity/g fresh tissue	60.11±4.62	53.00±3.83	48.12±2.36	23.09±0.93
3-Hydroxyanthranilate 3,4-dioxygenase	Specific activity	10.826±0.476	19.87±0.73	17.14±0.92	32.78±0.97
	Activity/g fresh tissue	1916±65	5454±158	4803±256	8186±200
Aminocarboxymuconate-semialdehyde decarboxylase	Specific activity	0.377±0.023	0.628±0.085	0.549±0.021	0.645±0.035
	Activity/g fresh tissue	56.25±2.43	161.63±9.45	153.90±5.86	184.00±16.77

Data are means±SEM and are expressed as specific activity (nmol/min/mg protein) or activity per g of fresh tissue (nmol/min/g tissue).

Table 3
Enzyme activities of kynurenine pathway in kidney of various animal species

Enzymes in kidney		Animal species			
		Rabbit	Rat	Mouse	Guinea-pig
Kynurenine 3-monooxygenase	Specific activity	0.519±0.020	2.61±0.26	4.13±0.36	1.506±0.09
	Activity/g fresh tissue	47.00±3.86	694.73±59.81	901.50±73.50	377.35±21.64
Kynureninase	Specific activity	0.020±0.001	0.051±0.003	0.026±0.006	0.095±0.005
	Activity/g fresh tissue	3.84±0.21	10.01±0.31	1.43±0.04	14.46±0.57
Kynurenine-oxoglutarate transaminase	Specific activity	0.645±0.047	1.125±0.033	1.098±0.055	0.315±0.013
	Activity/g fresh tissue	87.82±6.91	153.28±7.01	135.15±4.97	34.04±2.22
3-Hydroxyanthranilate 3,4-dioxygenase	Specific activity	45.519±2.551	15.07±1.45	5.03±0.20	33.20±1.74
	Activity/g fresh tissue	6516±156	3053±286	1108±59	8307±344
Aminocarboxymuconate-semialdehyde decarboxylase	Specific activity	0.371±0.014	0.861±0.043	0.605±0.024	1.120±0.090
	Activity/g fresh tissue	60.75±1.67	197.80±17.78	132.62±6.40	280.56±21.56

Data are means±SEM and are expressed as specific activity (nmol/min/mg protein) or activity per g of fresh tissue (nmol/min/g tissue).

Table 4
Serum tryptophan (free and total) in various species of animals. Data are expressed as means±SEM

Species	Serum tryptophan (μg/ml)	
	Free	Total
Rabbit	1.68±0.12	15.27±2.54
Rat	4.4±0.4	40.6±1.16
Mouse	3.44±0.25	26.85±0.22
Guinea-pig	1.57±0.09	14.88±0.73

TDO in comparison with the other species of mammals examined. Intestine IDO also shows higher activity in rat, as does small intestine SOD. The latter enzyme was determined for the first time in the intestine of animals. It is known that SOD activity decreases concomitantly with increased IDO activity [56].

Most of the kynurenine produced by the action of TDO and IDO enzymes is hydroxylated by kynurenine 3-monooxygenase to 3-hydroxykynurenine. The activity of this enzyme is especially high in mouse, kidney being richer than liver, and in rat (Tables 2 and 3), thus explaining the abnormal urinary excretion of xanthurenic acid observed by us [54,55,57,58] after treatment with a load of L-tryptophan.

As regards kynureninase, which hydrolyses kynurenine and 3-hydroxykynurenine to give anthranilic acid and 3-hydroxyanthranilic acid respectively, the highest activity was found in both liver and kidney of guinea-pig, but only in mouse did this activity appear to be more elevated in liver in comparison with kidney, although in all four mammals it was low compared with the other enzyme activities of the kynurenine pathway.

Kynurenine-oxoglutarate transaminase activity is higher in rat and mouse than in rabbit and guinea-pig. In all four species, activity is markedly higher in kidney. The high activity in rat and mouse fits the high urinary excretion of the products of transamination, xanthurenic and kynurenic acids, after a load of tryptophan [54,55,57,58].

Furthermore, 3-hydroxyanthranilate 3,4-dioxygenase, which transforms 3-hydroxyanthranilic acid to quinolinic acid, then converted spontaneously to nicotinic acid [59], showed the highest activity among the enzymes of the kynurenine pathway, but with great differences among the species of mammals. In rabbit, in which specific activity is higher than that of the other species, kidney is richer than liver, whereas in guinea-pig there is no difference between liver and kidney. Instead, in mouse, activity is higher in liver than in kidney. Considering activity per gram of fresh tissue, guinea-pig has higher values in both liver and kidney, compared with other animal species. In contrast with the high activity of this enzyme, aminocarboxymuconate-semialdehyde decarboxylase activity, which converts 3-hydroxyanthranilic acid to picolinic acid [38], is markedly lower, demonstrating that the preferred route of tryptophan metabolism is via NAD in all species studied.

5. Conclusions

The present study demonstrates that the enzyme activities of the kynurenine pathway are very active in tissues of the four species of mammals investigated. Study of tryptophan metabolism in vivo through urinary metabolite determination after repeated administration of tryptophan is limited by toxic events. The proposed method of in vitro enzyme determination represents a valid alternative to study of the tryptophan metabolic route.

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