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Enzyme activities along the tryptophan-nicotinic acid pathway in alloxan diabetic rabbits

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Abstract

Recent data from our laboratory have indicated that the rabbit is a suitable animal model for the study of enzyme activities of the tryptophan-nicotinic acid pathway. We report here the pattern of tryptophan metabolism in rabbits made diabetic with alloxan treatment, and hypercholesterolemic with a high-cholesterol diet. A group of rabbits with only hypercholesterolemia was also considered. The enzymes assayed were: liver tryptophan 2,3-dioxygenase (TDO), intestine indoleamine 2,3-dioxygenase (IDO), liver and kidney kynurenine 3-monooxygenase, kynurenine-oxoglutarate transaminase, kynureninase, 3-hydroxyanthranilate 3,4-dioxygenase and aminocarboxymuconate-semialdehyde decarboxylase. TDO showed a reduction of specific activity in liver of diabetic-hyperlipidemic and hyperlipidemic rabbits compared to controls. Intestine IDO activities and liver and kidney kynurenine monooxygenase were unchanged with respect to controls. Kynurenine-oxoglutarate transaminase and kynureninase activities were reduced in the kidneys, but not in the liver, of diabetic-hyperlipidemic rabbits. The main finding was the reduction of 3-hydroxyanthranilate 3,4-dioxygenase activity (expressed as activity per g of fresh tissue) in the liver and kidneys of diabetic-hypercholesterolemic and hyperlipidemic rabbits compared to controls. Conversely, aminocarboxymuconate-semialdehyde decarboxylase activity was significantly higher in diabetic hypercholesterolemic rabbits in comparison with control and hypercholesterolemic rabbits. These data demonstrate that also in diabetic rabbits there is an alteration of tryptophan metabolism at the level of 3-hydroxyanthranilic acid → nicotinic acid step. Also dyslipidemia seems to be involved in enzyme activity variations of the tryptophan metabolism along the kynurenine pathway.

Keywords: Tryptophan metabolism; Kynurenine-pathway enzyme; Alloxan-diabetic rabbit

1. Introduction

The enzyme activities of the tryptophan-nicotinic acid pathway, also called kynurenine pathway (Scheme 1), were studied in several mammals and it was found that the animals have various capacities of converting tryptophan to NAD [1,2]. The study of these enzymes in the liver of different species showed that above all the activity of aminocarboxymuconate-semialdehyde decarboxylase varied considerably in different species, and was about 30 times higher in cat than in rat [3–5]. Therefore, in cat the high activity in aminocarboxymuconate-semialdehyde decarboxylase causes 2-amino-3-carboxymuconic semialdehyde, the intermediate of the action of 3-hydroxyanthranilate 3,4-dioxygenase, to enter the glutarate pathway before it cyclizes spontaneously to quinolinic acid, precursor of nicotinic acid [4,6]. Aminocarboxymuconate-semialdehyde decarboxylase activity is increased in the liver of alloxan-treated rats [6] and also in the kidneys of pancreotectomized rats in respect to the controls [7]. McDaniel et al. [8] reported that in alloxan-treated diabetic rats there is a decreased urinary excretion of nicotinic acid ribonucleotide metabolites. These findings have been related to the increase in aminocarboxymuconate-semialdehyde decarboxylase in the liver of these animals [6]. Aminocarboxymuconate-semialdehyde decarboxylase competes for substrate with a spontaneous reaction, shunting 3-hydroxyanthranilic acid to picolinic acid instead of quinolinic acid.

Mehler et al. [9] observed that in livers of diabetic rats, no decrease was observed in the 3-hydroxyanthranilate 3,4-dioxygenase activity, and concluded that the failure of diabetic rats to form nicotinic acid ribonucleotide did not depend on a

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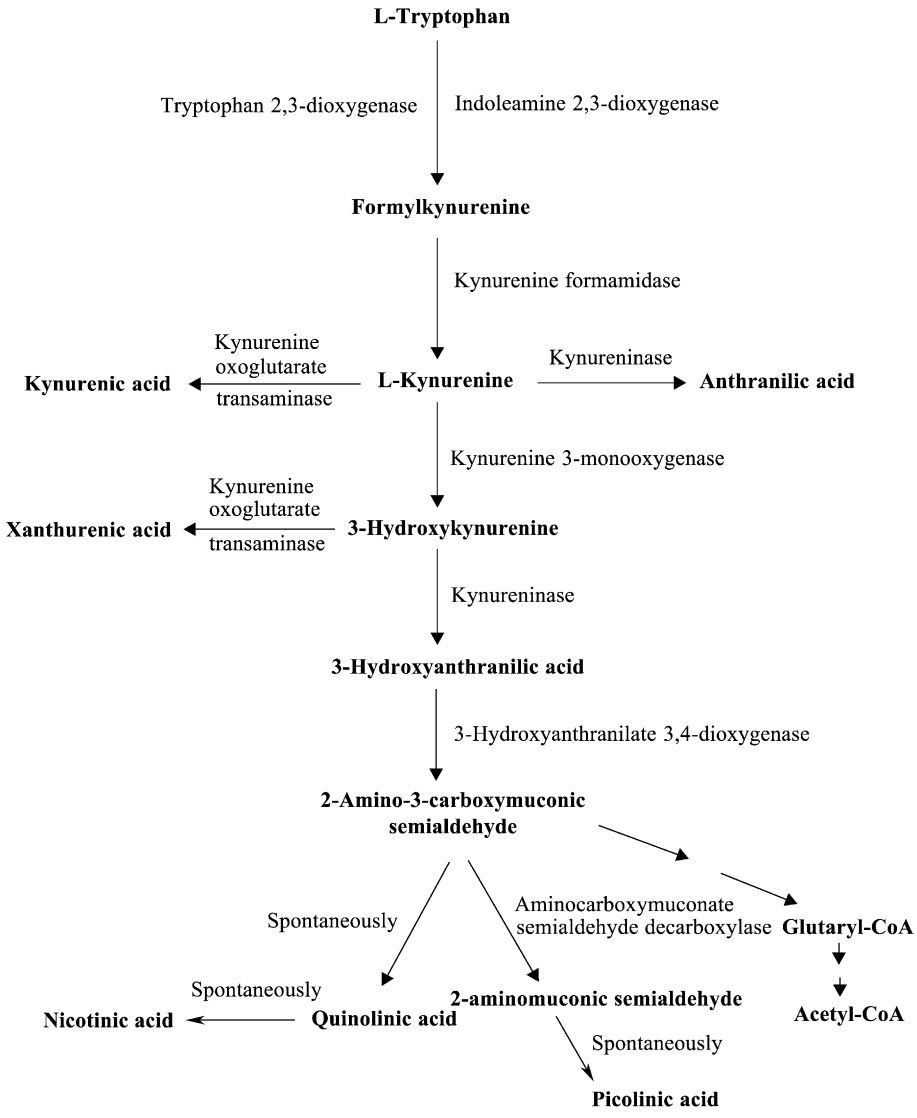

Scheme 1.

decrease in the enzymes which convert 3-hydroxyanthranilic acid to nicotinic acid ribonucleotide, but is due to a decrease in the formation of quinolinic acid, probably caused by an increase of aminocarboxymuconate-semialdehyde decarboxylase activity. Ikeda et al. [4] obtained similar results, showing that the transformation of 3-hydroxyanthranilic acid to nicotinic acid in liver and kidney was inversely related to the activity of aminocarboxymuconate-semialdehyde decarboxylase, converting 2-amino-3-carboxymuconic semialdehyde to 2-amino-muconic semialdehyde which cyclizes spontaneously to picolinic acid. However, before cyclization to picolinic acid, 2-amino-muconic semialdehyde may be oxidized to give acetyl-CoA with a metabolic sequence called "the glutarate pathway" by Ichiyama et al. [10].

Therefore, in diabetes there are some abnormalities of tryptophan metabolism, as some authors reported [11–14]. Schor and Frieden [15] found that in alloxan diabetic rats another enzyme activity of the tryptophan-nicotinic acid pathway increases. In fact, liver tryptophan peroxidase-oxidase, that is tryptophan 2,3-dioxygenase (TDO), a key enzyme in the metabolism of tryptophan, shows an 8- to 10-fold increase after diabetes is induced with alloxan. Schor and Frieden [15] found also that insulin induces TDO in vivo in controls as well as in adrenalectomized rats. This activity in rat liver increases also after pancreatectomy [7].

Liver TDO exists as holoenzyme and apoenzyme in some animals, as rat, mouse, pig, chicken, turkey, and in man. In other species of animals, such as guinea pig, rabbit, cat, gerbil, frog, hamster, sheep and ox, the apoenzyme is absent [16]. Animals lacking the apoenzyme, such as rabbit, have a deficient kynurenine pathway and are sensitive to tryptophan toxicity [16]; therefore these species were considered as a poor model for studying tryptophan metabolism in pathological conditions.

The activity of liver TDO is generally regulated by three mechanisms: hormonal induction by glucocorticoids

[17,18], substrate activation by tryptophan [19] and cofactor activation by haem [20]. TDO converts tryptophan into formylkynurenine. The active holoenzyme does not require the addition of exogenous haematin for its activity in vitro, whereas apoenzyme, the haem-free predominant form, does.

In a previous paper [21], we demonstrated that rabbit, even though lacking TDO-apoenzyme, can metabolise tryptophan along the kynurenine pathway being that the enzymes of this route are very active. In fact, in rabbit there is another enzyme able to degrade tryptophan to kynurenine, as TDO does, named indoleamine 2,3-dioxygenase (IDO) that shows a high activity in the intestine [22,23].

In this study, we analysed the enzyme activities, along the kynurenine pathway, of TDO, IDO, kynurenine 3-monooxygenase, kynurenine-oxoglutarate transaminase, kynureninase, 3-hydroxyanthranilate 3,4-dioxygenase and aminocarboxymuconate-semialdehyde decarboxylase, in rabbits made diabetic by alloxan treatment in order to show possible variations of tryptophan metabolism in a model of dismetabolic disease. Tryptophan levels in serum were also determined.

Hyperlipidemia is often found associated with diabetes [24–28] and it increases the atherosclerotic risk due to abnormal lipoprotein metabolism. In order to mimic the clinical feature of diabetes in humans, alloxan diabetic rabbits were maintained on a high-cholesterol diet. As a comparison, besides the normolipidemic healthy controls, a group of rabbits was fed a high-cholesterol diet.

2. Materials and methods

2.1. Chemicals

L-Tryptophan (Trp), L-kynurenine sulfate (Kyn), α-ketoglutaric acid, pyridoxal phosphate (PLP), 3-hydroxyanthranilic acid, anthranilic acid, haematin hydrochloride, NADPH, catalase, methylene blue and L-ascorbic acid were from Sigma. All other chemicals were analytical R grade.

2.2. Animals

Twenty-one male 4-month-old New Zealand white rabbits, weighing 2.5–3.0 kg, were fed a standard diet (2RB15, Mucedola, Milan, Italy). In eight of these animals, the diet was enriched with 1% cholesterol and 10% corn oil (consisting largely of triglycerides of the unsaturated oleic and linoleic acids, with smaller proportions of palmitic and stearic acids) for 8 weeks. In other five rabbits, after 7 days from the beginning of the hyperlipidemic diet, diabetes was induced by intravenous administration of alloxan monohydrate (100 mg/kg i.v. over a period of 1 min) freshly prepared in saline. Alloxan administration induces an initial hypoglycemia, due to massive insulin release triggered by the cytotoxic effect of the drug on pancreatic beta cells. Excessive decrease of blood glucose was prevented by providing food ad libitum and administering glucose (5% solution, 100 mg/kg s.c., at hours 2, 4 and 6 from the treatment). The cholesterol/high-lipid feeding was further continued for 5 weeks. At the end of treatment, the animals were killed by cervical dislocation and the liver, kidneys, small intestine and blood of each animal were removed separately and immediately after death of the animals. Liver, kidneys and intestine were weighed, cut into pieces and homogenized (20% homogenate) at 4 °C in a Sorvall omni-mix homogenizer for the liver and kidneys and in a Potter-Elvehjem homogenizer with a Teflon pestle for the intestine.

The blood was collected in a non-heparinised tube and allowed to clot at room temperature for 30 min and then centrifuged at $3000 \times g$ for 10 min.

The values of blood glucose were 194 ± 13 mg/dl in diabetic-hyperlipidemic rabbits, compared to 104 ± 6 mg/dl in controls. Serum cholesterol was 5.03 ± 0.67 g/dl in diabetic-hyperlipidemic and 4.50 ± 0.52 g/dl in hyperlypidemic rabbits (vs. 40 ± 4 mg/dl in the controls).

2.3. Assay of enzymes

2.3.1. TDO (Trp: oxygen 2,3-oxidoreductase (decyclizing), EC 1.13.11.11)

The liver homogenate was obtained according to Bertazzo et al [21] and the assay of activity was carried out according to the method of Feigelson and Greengard [29], slightly modified [30].

The activity of holo-TDO was expressed as nmol of kynurenine formed per min per mg of protein (specific activity) and as nmol of kynurenine formed per min per g of wet weight liver.

2.3.2. IDO (indole: oxygen 2,3-oxidoreductase (decyclizing), EC 1.13.11.17)

The activity of indole 2,3-doxygenase was assayed in the intestine according to Shimizu et al. [31] and was expressed as nmol of product formed per min per g of fresh tissue. The specific activity was expressed as nmol of product formed per min per mg of protein.

2.3.3. Kynurenine 3-monooxygenase (L-kynurenine-$NAPH_2$: oxygen oxidoreductase (3-hydroxylating), EC 1.14.13.9)

The activity of kynurenine 3-monooxygenase was determined in the liver and kidney mitochondria as reported by Bertazzo et al. [21] according to the method of Saito et al. [32] and Hayaishi [33].

The enzyme activity was expressed as nmol of 3-hydroxykynurenine formed per min per g of fresh tissue, and the specific activity as nmol of 3-hydroxykynurenine per min per mg of protein.

2.3.4. Kynureninase (L-kynurenine hydrolase, EC 3.7.1.3)

The activity was assayed in the liver and kidney supernatants, obtained as reported by Bertazzo et al. [21], according to the Saran method [34].

The enzyme activity was expressed as nmol of product per min per g of wet weight liver or kidney and specific activity as nmol of product per min per mg of protein.

2.3.5. Kynurenine-oxoglutarate transaminase (L-kynurenine: 2-oxoglutarate aminotransferase, EC 2.6.1.7)

The activity was measured by a modification of the method of Mason [35] in both liver and kidney as reported by Bertazzo et al. [21]. The enzyme activity was expressed as nmol of kynurenic acid formed per min per g of fresh tissue and the specific activity as nmol of kynurenic acid formed per min per mg of protein.

2.3.6. 3-Hydroxyanthranilate-3,4-dioxygenase (3-hydroxyanthranilate: oxygen 3,4-oxidoreductase (decyclizing), EC 1.13.11.6)

The activity was assayed spectrophotometrically in both liver and kidney homogenates as described by Mehler [36]. The assay mixture consisted of 100 μl of the enzyme preparation, 3-hydroxyanthranilic acid (0.07 μmol), 100 μl of phosphate buffer 0.1 M, pH 7.5, and distilled water in a 3 ml final volume. The reaction was started by the addition of the substrate and the increase in absorbance at 360 nm was monitored over 15 s at 24 °C. For the activity determination molar extinction coefficient of 47,500 M^{-1} cm^{-1} for the reaction product (α-amino-β-carboxymuconic acid ε-semialdehyde) was used [37]. The enzyme activity was expressed as nmol of product formed per min per g of wet weight of liver or kidney and specific activity as nmol per min per mg of protein.

2.3.7. Aminocarboxymuconate-semialdehyde decarboxylase (2-amin-3-(3-oxoprop-2-enyl)but-2-enedioate carboxylyase, EC 4.1.1.45)

The activity was determined spectrophotometrically at 360 nm as described by Mehler [36] under the conditions of 3-hydroxyanthranilate 3,4-dioxygenase assay. The enzyme activity was expressed as decrease of the optical density per min of 2-aminomuconic semialdehyde that is converted to picolinic acid.

2.4. Protein determination

Protein was determined by the method of Lowry et al. [38] using bovine serum albumin as a standard.

2.5. Estimation of tryptophan in serum

Tryptophan was measured in duplicate according to the spectrofluorimetric method of Denkla and Dewey [39], using 0.02 ml of the serum.

2.6. Statistical analysis

The data are expressed as mean ± S.E. Statistical analysis between groups was performed using unpaired Student's *t*-test. Differences were considered significant at $P < 0.05$ (two tails).

3. Results

Table 1 shows the values (mean ± S.E.) of specific activity, expressed as nmol of kynurenine formed per min per mg of protein, of liver holo-TDO in the three groups of rabbits examined. This activity significantly decreases in diabetic-hyperlipidemic rabbits ($P < 0.05$) and in hyperlipidemic ($P < 0.005$) in comparison with the healthy controls. Regarding the activity calculated as nmol formed per min per g of wet weight liver, no significant differences were observed between the diabetic-hyperlipidemic rabbits and controls, while the value was lower in the hyperlipidemic group ($P < 0.01$) compared to the controls.

In the same table, the intestine IDO activity is reported and resulted markedly higher than liver TDO activity in all the groups of animals. IDO activities did not show any significant difference in the values from hyperlipidemic and diabetic-hyperlipidemic rabbits in comparison to the controls.

Tables 2 and 3 report the results obtained assaying the activities of kynurenine 3-monooxigenase, kynureninase, kynurenine-oxoglutarate transaminase, 3-hydroxyanthranilate 3,4-dioxygenase and aminocarboxymuconate-semialdehyde decarboxylase in the liver (Table 2) and kidneys (Table 3) of the rabbits.

The specific activity of kynurenine 3-monooxygenase appeared to be double and the activity per g of fresh tissue triple, in the liver with respect to the kidneys, but no significant differences were found in either the liver or kidneys among the three groups of rabbits.

Table 1
Liver TDO and small intestine IDO activities in New Zealand male healthy, hyperlipidemic and diabetic-hyperlipidemic rabbits

Rabbits	Liver TDO oloenzyme		Small intestine IDO	
	Specific activity (nmol product/mg protein/min)	Activity in fresh tissue (nmol product/g tissue/min)	Specific activity (nmol product/mg protein/min)	Activity in fresh tissue (nmol product/g tissue/min)
Controls (8)	0.041 ± 0.002	9.51 ± 0.69	0.151 ± 0.013	15.00 ± 1.30
Hyperlipidemic (8)	0.032 ± 0.001 c	6.97 ± 0.37 b	0.135 ± 0.011	13.48 ± 0.87
Diabetic-hyperlipidemic (5)	0.034 ± 0.002 a	8.07 ± 0.80	0.143 ± 0.020	14.82 ± 1.51

The enzyme activities were measured as described in Section 2 and the values are expressed as means ± S.E. The number of animals are in parentheses. Comparison controls vs. hyperlipidemic or diabetic-hyperlipidemic: a = $P < 0.05$; b = $P < 0.01$; c = $P < 0.005$.

Table 2
Liver kynurenine 3-monooxygenase, kynureninase, kynurenine-oxoglutarate transaminase, 3-hydroxyanthranilate 3,4-dioxygenase and aminocarboxymuconate-semialdehyde decarboxylase activities in male New Zealand healthy, hyperlipidemic and diabetic-hyperlipidemic rabbits

Enzymes	Rabbits					
	Controls (8)		Hyperlipidemic (8)		Diabetic-hyperlipidemic (5)	
	Specific activity (nmol product/mg protein/min)	Activity in fresh tissue (nmol product/g tissue/min)	Specific activity (nmol product/mg protein/min)	Activity in fresh tissue (nmol product/g tissue/min)	Specific activity (nmol product/mg protein/min)	Activity in fresh tissue (nmol product/g tissue/min)
Kynurenine 3-monooxygenase	1.131 ± 0.045	151.42 ± 5.81	1.010 ± 0.053	138.08 ± 7.73	1.026 ± 0.030	137.25 ± 6.63
Kynureninase	0.019 ± 0.001	4.08 ± 0.23	0.018 ± 0.001	3.24 ± 0.08 c	0.018 ± 0.001	3.48 ± 0.10
Kynurenine-oxoglutarate transaminase	0.337 ± 0.024	60.11 ± 4.62	0.268 ± 0.025	49.78 ± 3.75	0.273 ± 0.014	50.18 ± 3.22
3-Hydroxyanthranilate 3,4-dioxygenase	10.826 ± 0.476	1916.25 ± 64.77	9.894 ± 0.640	866.35 ± 138.68 d	10.988 ± 0.932	1049.20 ± 68.38 d
Aminocarboxy-muconate-semialdehyde decarboxylase	0.377 ± 0.023	56.25 ± 2.43	0.370 ± 0.025	46.25 ± 3.02 a	0.702 ± 0.047 d,D	95.00 ± 3.36 d,D

The enzyme activities were assayed as described in Section 2 and the values are expressed as means ± S.E. The number of animals are in parentheses.
Comparison controls vs. hyperlipidemic or diabetic-hyperlipidemic: a = $P < 0.05$; c = $P < 0.005$; d = $P < 0.001$.
Comparison diabetic-hyperlipidemic vs. hyperlipidemic: D = $P < 0.001$.

With regard to kynureninase, similar specific activities were observed in the liver and kidneys among the groups of rabbits, whereas the activity per g of fresh tissue resulted significantly lower ($P < 0.005$) in the liver of hyperlipidemic (Table 2) and in the kidneys of diabetic-hyperlipidemic rabbits ($P < 0.05$) (Table 3) than in the controls.

The specific activity and activity per g of fresh tissue of kynurenine-oxoglutarate transaminase were found higher in the kidneys (Table 3) than in the liver (Table 2) of all three groups of rabbits, which showed similar values in the liver (Table 2). In the kidneys of diabetic-hyperlipidemic rabbits, the values of both activities appeared significantly lower ($P < 0.05$) than in the controls (Table 3). Moreover, the activity per g of fresh tissue appeared lower in the kidneys of hyperlipidemic rabbits ($P < 0.05$) than in controls (Table 3).

Considering 3-hydroxyanthranilate 3,4-dioxygenase in the liver (Table 2), the specific activity was similar in all the groups of animals, whereas the activity per g of fresh tissue resulted markedly lower both in the hyperlipidemic ($P < 0.001$) and diabetic-hyperlipidemic rabbits ($P < 0.001$) than in the controls. However, no difference in the values appeared between hyperlipidemic and diabetic-hyperlipidemic animals. On the contrary, the above enzyme activities assayed in the kidneys (Table 3), expressed as specific activity or activity per g of fresh tissue, showed values

Table 3
Kidney kynurenine 3-monooxygenase, kynureninase, kynurenine-oxoglutarate transaminase, 3-hydroxyanthranilate 3,4-dioxygenase and aminocarboxymuconate-semialdehyde decarboxylase activities in male New Zealand healthy, hyperlipidemic and diabetic-hyperlipidemic rabbits

Enzymes	Rabbits					
	Controls (8)		Hyperlipidemic (8)		Diabetic-hyperlipidemic (5)	
	Specific activity (nmol product/mg protein/min)	Activity in fresh tissue (nmol product/g tissue/min)	Specific activity (nmol product/mg protein/min)	Activity in fresh tissue (nmol product/g tissue/min)	Specific activity (nmol product/mg protein/min)	Activity in fresh tissue (nmol product/g tissue/min)
Kynurenine 3-monooxygenase	0.519 ± 0.020	47.00 ± 3.86	0.469 ± 0.020	44.07 ± 3.49	0.465 ± 0.043	43.51 ± 2.46
Kynureninase	0.020 ± 0.001	3.84 ± 0.21	0.021 ± 0.001	3.04 ± 0.34	0.019 ± 0.002	3.17 ± 0.13 a
Kynurenine-oxoglutarate transaminase	0.645 ± 0.047	87.82 ± 6.91	0.532 ± 0.041	66.56 ± 3.63 a	0.500 ± 0.027 a	65.81 ± 3.37 a
3-Hydroxyanthranilate 3,4-dioxygenase	45.519 ± 2.551	6516.62 ± 156.52 d	20.830 ± 1.130 d	2593.00 ± 104.64 d	9.476 ± 0.467 d,D	1080.00 ± 81.23 d,D
Aminocarboxy-muconate-semialdehyde decarboxylase	0.371 ± 0.014	60.75 ± 1.67	0.387 ± 0.042	49.00 ± 4.40	0.918 ± 0.080 d,D	105.60 ± 9.09 d,D

The enzyme activities were assayed as described in Section 2 and the values are expressed as means ± S.E. The number of animals are in parentheses.
Comparison controls vs. hyperlipidemic or diabetic-hyperlipidemic: a = $P < 0.05$; d = $P < 0.001$.
Comparison diabetic-hyperlipidemic vs. hyperlipidemic: D = $P < 0.001$.

Table 4
Tryptophan levels in serum of male New Zealand healthy, hyperlipidemic and diabetic-hyperlipidemic rabbits

Rabbits	Serum tryptophan (μg/ml)
Controls (8)	15.27 ± 2.54
Hyperlipidemic (8)	14.21 ± 2.65
Diabetic-hyperlipidemic (5)	13.25 ± 1.52

In parentheses are the number of animals considered.

significantly lower ($P<0.001$) in the hyperlipidemic and diabetic-hyperlipidemic rabbits than in the controls. In addition, comparing the two groups of hyperlipidemic and diabetic-hyperlipidemic rabbits, the enzyme activities in the kidneys appeared significantly lower ($P<0.001$) in the diabetic rabbits (Table 3). Besides, 3-hydroxyanthranilate 3,4-dioxygenase activity appeared greatly more elevated both in the liver and kidneys in comparison to all other enzyme activities.

Tables 2 and 3 also report the values of the amino-carboxymuconate-semialdehyde decarboxylase. The activities (expressed as specific activity or activity per g of fresh tissue) in the liver and kidneys of diabetic-hyperlipidemic rabbits were significantly higher ($P<0.001$) than in the controls and hyperlipidemic group. In the liver and kidneys, the specific activities did not show any difference between the controls and hyperlipidemic rabbits, whereas in the liver the activity per g of fresh tissue was lower ($P<0.05$) in hyperlipidemic rabbits than in the controls.

The determination of total tryptophan in serum of all rabbits examined (Table 4) did not show any significant difference among the diabetic-hyperlipidemic (13.25 ± 1.52 μg/ml), hyperlipidemic (14.21 ± 2.65 μg/ml) and healthy rabbits (15.27 ± 2.54 μg/ml).

4. Discussion

Modification of food intake involves a mechanism in the regulation of metabolism of all indispensable amino acids [40]. A diet poor in tryptophan leads to a deficiency in nicotinic acid, as this substance derives from the tryptophan metabolism along the kynurenine pathway. Liver TDO is the first enzyme of this important pathway, although extrahepatic IDO catalyses the same oxidation reaction of tryptophan. The oxidation product of both these enzymes is formylkynurenine which is rapidly hydrolysed into kynurenine by the tissue's formamidase.

In rabbit, we found that TDO exists only as holoenzyme [21], as Badawy and Evans [16] observed; in the present study we detected a decrease of specific activity in the liver of diabetic-hyperlipidemic ($P<0.05$) and hyperlipidemic rabbits ($P<0.01$) compared to the controls. But, no difference exists between diabetic-hyperlipidemics and hyperlipidemics. Therefore, the decrease does not appear to depend on the diabetic state, but it could be related to the hyperlipidemic state. Also the activity per g of fresh liver in hyperlipidemic rabbits shows lower values ($P<0.01$) than in the controls. Instead, Mehler et al. [6] reported that the activity of TDO in the liver of rats made diabetic with alloxan tends to be higher than in the normal liver. Also Schor and Friend [15] found that TDO is markedly increased in alloxan diabetic rats and that insulin can induce formation of the enzyme in vivo by making more energy available to the enzyme-forming system. Ginoulhiac et al. [7] demonstrated that TDO increases in the liver of rats made diabetic by pancreatectomy and that this increase corresponds, in the study of urinary metabolites, to higher elimination of kynurenine than in the controls [41].

The small intestine of rabbits was used to study the IDO activity because it shows the highest activity of this enzyme [23]; IDO catalyses the oxygenative ring cleavage of tryptophan utilizing the superoxide anion [42–45] generated by xanthine oxidase, an abundant enzyme in the intestine [46,47]. Our results show a higher activity of IDO in comparison with TDO (Table 1), and no difference of IDO values in the three groups of rabbits. Therefore, in rabbits, IDO seems to be considered the key enzyme which determines how much tryptophan enters the kynurenine pathway also under physiological conditions.

Most of the kynurenine formed from tryptophan is hydroxylated to 3-hydroxykynurenine by means of kynurenine 3-monooxygenase. Kynurenine and 3-hydroxykynurenine may be metabolised by kynurenine transaminase to kynurenic acid and xanthurenic acid, respectively, or transformed to anthranilic acid and 3-hydroxyanthranilic acid, respectively, by means of kynureninase. 3-Hydroxyanthranilic acid is the last stable intermediate of the tryptophan-nicotinic acid path before splitting into the glutarate pathway.

By studying these enzymes in diabetic-hyperlipidemic and hyperlipidemic rabbits, we found that specific activity and activity per g of fresh tissue of kynurenine 3-monooxygenase are double in the liver in comparison to the kidneys, but no difference in the values is present either between these two groups or in comparison with the controls.

Kynureninase activity is similar in the liver and kidneys. However, in the liver the activity per g of fresh tissue tends to decrease in the hyperlipidemic group compared to the controls ($P<0.01$), but no significant difference exists in comparison to diabetic-hyperlipidemic rabbits. Instead, in the kidneys the decrease of kynureninase activity per g of fresh tissue was significant in diabetic-hyperlipidemics ($P<0.05$ vs. controls) but not in hyperlipidemic rabbits. Kynurenine-oxoglutarate transaminase activity does not show any difference in the liver among the three groups of animals, whereas in the kidneys it decreases in the diabetic and hyperlipidemic animals, but no difference is present between them. In all three groups of rabbits, the activity is higher in the kidneys than in the liver.

Ginoulhiac et al. [7] did not find any difference in these enzyme activities in diabetic rats in comparison to the

controls. Our results, therefore, may be related to a metabolic effect related to the hyperlipidemic status.

From 3-hydroxyanthranilic acid, α-amino-β-carboxymuconic-Σ-semialdehyde is formed, through the 3-hydroxyanthranilate 3,4-dioxygenase [10]; this metabolite is then decarboxylated by aminocarboxymuconate-semialdehyde decarboxylase to form α-amino-muconic-semialdehyde, that in part readily cyclizes to give picolinic acid [10,48]. On the other hand, the α-amino-β-carboxymuconic-Σ-semialdehyde also cyclizes spontaneously to form quinolinic acid [49–51], which then converts to nicotinic acid [1] (Scheme 1).

Ginoulhiac et al. [7] demonstrated that in diabetic rats an alteration of the metabolism of 3-hydroxyanthranilic acid was present and already appeared at the end of the second month after pancreatectomy, and mainly consisted of reduced urinary excretion of N_1-methylnicotinamide, a metabolite of nicotinic acid [7]. Pancreatectomized rats compared with controls also eliminate a smaller amount of nicotinic acid and a higher quantity of kynurenine after a dose of tryptophan (500 mg/kg, orally). Moreover, Tenconi [41] found that after a dose of 3-hydroxyanthranilic acid (50 mg/kg orally or 500 mg/kg by endoperitoneal injection) the urinary excretion of quinolinic acid, nicotinic acid, N_1-methylnicotinamide and 3-hydroxyanthranilic acid itself increases in both the controls and diabetic rats; however, the increase in diabetic rats is less than that observed in control rats [41].

McDaniel et al. [8] showed that rats made diabetic with alloxan excreted less *N*-methylnicotinamide than normal rats when test doses of tryptophan were given; Ginoulhiac et al. [7] investigated on the nature of the enzymatic alteration responsible for the differences found between the diabetic and normal groups of animals and observed a high activity in aminocarboxymuconate-semialdehyde decarboxylase in the liver of alloxanized rats, whereas 3-hydroxyanthranilate 3,4-dioxygenase appeared unchanged. Even after several weeks of insulin administration to the diabetic rats the aminocarboxymuconate-semialdehyde decarboxylase activity remained slightly elevated, while increased excretion of *N*-methylnicotinamide was observed only some days or weeks after reduction in the activity of aminocarboxymuconate-semialdehyde decarboxylase [6].

Our data demonstrate that liver 3-hydroxyanthranilate 3,4-dioxygenase specific activity did not vary among the three groups of rabbits examined, but the activity per g of fresh tissue was significantly lower ($P<0.001$) in hyperlipidemic and diabetic-hyperlipidemic rabbits than in the controls. However, the decrease in activity did not seem to depend on a diabetic state, as there was no difference between hyperlipidemic and diabetic-hyperlipidemic rabbits. In the kidneys, specific activity and activity per g of fresh tissue showed higher values ($P<0.001$) in the controls in comparison to the other two groups. In addition, a significant decrease ($P<0.001$) in 3-hydroxyanthranilate 3,4-dioxygenase activity, both as specific activity and per g of fresh tissue, was also present in the kidneys of diabetic-hyperlipidemic rabbits in comparison to the hyperlipidemic group, demonstrating that the decrease depended on the diabetic state. Regarding aminocarboxymuconate-semialdehyde decarboxylase, its activity per g of fresh liver or kidney was lower in hyperlipidemic rabbits than in the controls ($P<0.05$); however, both the activities in the liver and kidneys were significantly higher ($P<0.001$) in diabetic-hyperlipidemic than in the controls and hyperlipidemic animals.

These studies revealed that also in rabbits made diabetic by alloxan, as in diabetic rats, there was an alteration of tryptophan metabolism at the level of the 3-hydroxyanthranilic acid → nicotinic acid step. Other differences in enzyme activities may be related to the hyperlipidemic status. Changes in tryptophan metabolism did not affect, however, the serum levels of this amino acid.

A large increase in aminocarboxymuconate-semialdehyde decarboxylase activity could have the effect of reducing the formation of nicotinic acid and NAD in diabetes. On the other hand, it has been demonstrated that alloxan, as well as streptozotocin, causes DNA-strand breaks resulting in a stimulation of poly(ADP-ribose) polymerase (PARP) activity [52–55] which uses NAD as a substrate. Alloxan treatment leads to a reduction of intracellular NAD levels [54], causing impairment of pancreatic islet cell function, a fall of ATP levels and cell death [52,55]. Therefore, NAD depletion in alloxan-treated animals could be also a consequence of such process at the level of PARP, although the effect of the drug in extrapancreatic tissue should be demonstrated. In our experimental conditions, probably this effect of the drug disappeared during the prolonged (5 weeks) post-treatment period.

Anyhow, aminocarboxymuconate-semialdehyde decarboxylase activity could be an important regulatory factor controlling the amount of tryptophan that enters the NAD pathway or the glutarate pathway. The detected differences might deserve further research in order to clarify their role in dismetabolic diseases.

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