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Effect of Pyrolidine and its Copper Complex on Rabbit Tyrosinase

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Abstract: The effect of pyrolidine (L) or its copper-complex © on rabbit melanocytes tyrosinase were studied. The results revealed that (L) appeared to be a non competitive inhibitor of tyrosinase for about 30% , while (C) was an activator for about 4.6 fold. In addition, general effects of (L) or (C) on rabbit were studied via serum parameters. The obtained data afforded an additional biological effect of the tested compounds .

Key words: Tyrosinase, pyrolidine, copper-complex, inhibition, activation.

Introduction

Tyrosinase (EC 1.14 . 18.1), the enzyme responsible for catalyzing the initial steps in melanogenesis, i.e., the hydroxylation of tyrosine to dihydroxyphenylalanine (DOPA), and the subsequent oxidation of DOPA to DOPA quinone (Hearing *et al.*, 1978) .

Tyrosinase was used by several groups to investigate the accessibility of the tyrosyl groups on the surface of the proteins and to determine if these tyrosyl groups are in the active site (Cory and Frieden, 1967) . Also, substantial evidence demonstrates a correlation between the decrease in tyrosinase activity and a loss of tumor (Kovacs *et al.*, 1981 and Sukhanov *et al* .,1991). It was reported that, studies on serum from patients with widespread melanoma metastases showed detectable quantities of tyrosinase, while there was no tyrosinase activity in control sera from individuals without malignancies (Agrup *et al.*, 1989).

Kinetic properties of tyrosinase showed two binding sites for aromatic compounds and four gram atoms of copper as integral part per molecule and inhibition of tyrosinase was postulated as an important factor in melanogenesis (Jolley *et al.*, 1974).

Few studies on pyrolidine or its derivatives were carried to find their biological effect such as that of Katoch *et al.* (1999), who reported that pyrolidine derivative (1- [2-hydroxy-3-octadecan-1-oate]propyl-tetramethyl pyrolidine) N-oxyl-3-carboxylate was used as a potential spin probe for membrane structure studies. Meanwhile, N-methyl-N-(1-methyl-4-pyrolidino-2-butyl] acetamide was used to release acetyl choline from rat and that derivative appeared to have agonist effect on the cholinergic receptor, (Sethy *et al.*, 1988). So, the present work aims to study the effect of pyrolidine alone or its copper-complex on rabbits, separately and pursued their biological effects via some serum parameters .

Materials and Methods

Tyrosine was purchased from Sigma Chemical Co. (St. Louis, Mo, USA). Folin-Ciocalteu's phenol reagent was purchased from Merck, Hohenbrunn. Bovine serum albumin was purchased from Fisher Biotech (Fisher Scientific Fair Lawn, N.J., USA) . Pyrolidine and its copper-complex were kindly provided by Prof. Dr. M. Abdel Rahman, Inorganic Laboratory, Faculty of Science, Alexandria University. The work was carried out at Biochemistry Department, Faculty of Science, Alexandria University.

Animals: Nine healthy black rabbits, (weighing 80 ±5 g each) were group housed and maintained under standard laboratory conditions. These were randomly divided into three groups as follows :

The first group (N) served as untreated control group receiving normal diet and corn oil .

The second group (L) was given normal diet and received a daily oral dose of pyrolidine in corn oil (10 mg / ml), for four days.

The third group © was given normal diet and treated daily with oral dose of pyrolidine-copper-complex in corn oil (10 mg per ml) for four days. After that, animals were sacrificed, blood samples were collected separately and each obtained serum was ready for biochemical assays.

Isolation of tyrosinase: According to the method of Hearing *et al.* (1978), fresh tissues (dorsal epiderms),15g, from the examined black rabbit, were dissected and homogenized at 4°C in 0.1M sodium phosphate buffer pH 7.4, using homogenizer type MPW-302 (Mechanika Precyzyina).

The homogenate was centrifuged at 1000 x g for 5 minutes at 4°C using Hettich Zentrifugen EBA 12 R cooling centrifuge, then the supernatant was recovered and melanin granules were sedimented at 10,000 g for 15 minutes. After suspension in phosphate buffer, the melanosomes were washed and centrifuged off. The purified granules were then solubilized for 5 minutes in 0.1% Triton X-100, insoluble particles were sedimented at 10,000 g for 15 minutes, finely the residue was suspended in phosphate buffer as prepared enzyme .

Estimation of protein: Protein determination was carried out according to the method of Lowry *et al.* (1951), using standard curve of bovine serum albumin .

Enzyme assay: The activity of tyrosinase was assayed as previously described method, (Strothkamp and Mason, 1974), by adding 1 ml of phosphate buffer (pH 7.4) and 0.9 ml glass-distilled water to 1 ml of 1 mM tyrosine. The reaction mixture was oxygenated by bubbling oxygen into cuvettes through a capillary tube for 4-5 minutes and cuvettes were transferred to spectrophotometer model 340 (Turner) and the absorbance was recorded at 280 nm for 4-5 minutes to achieve the temperature equilibration and to establish blank rate. Added 0.1 ml of the enzyme preparation, to record the absorbance for 10-12 minutes. The change in the absorbance from the linear portion of the curve, was determined and specific activity was calculated as U/min/mg protein.

Kinetic studies on tyrosinase: The effect of (L) or (C) on tyrosinase activity was carried out using 1 ml of different concentrations of tyrosine (0.125 - 1.5 mM) either in control or treated groups and estimating the enzyme activity as mentioned before. The type of inhibition and inhibition constant K_i were determined according to the method of Lineweaver and Burk (1934).

Biochemical assay: The following serum parameters : alanine aminotransferase (ALT), aspartate aminotransferase (AST), total protein, albumin, bilirubin, urea, creatinine, creatine kinase (SCK) were measured using kits produced by Boehringer Mannheim, Germany .

Results

Studies on pyrolidine alone and the copper-complex of pyrolidine (Fig. 1), showed a distinct effect on the activity of tyrosinase. Tyrosinase was isolated and partially purified from control, (L)-treated and © treated rabbit epiderms each separately starting from the homogenate to the final step as indicated in Tables 1, 2 and 3 .

The examined rabbits treated with (L) showed inhibition of tyrosinase with relative activity (ratio of the specific activity of (L) treated enzyme to that of the control) of about

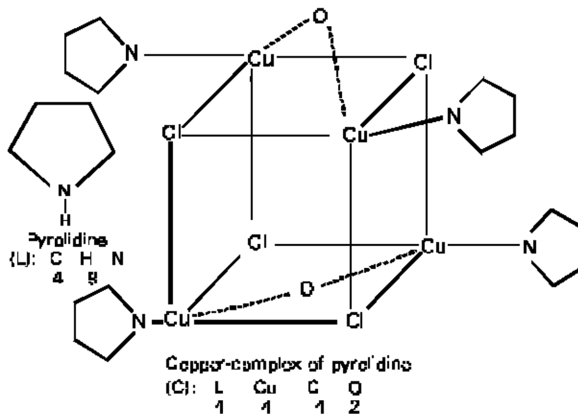

Fig. 1: Compounds tested for lyrosinase activity.

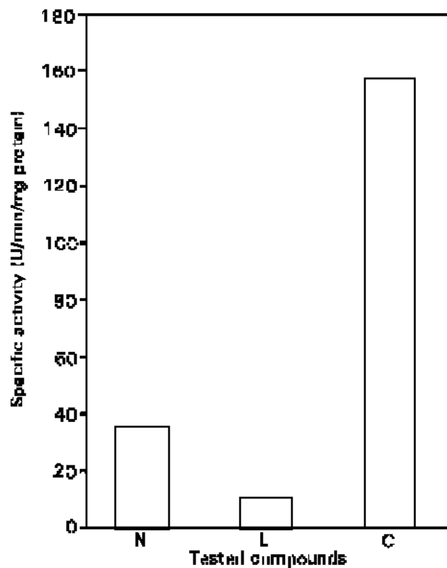

Fig. 2: The specific activity of tyrosinase in absence (N) and presence of (L) and (C), at 1 mM tyrosine.

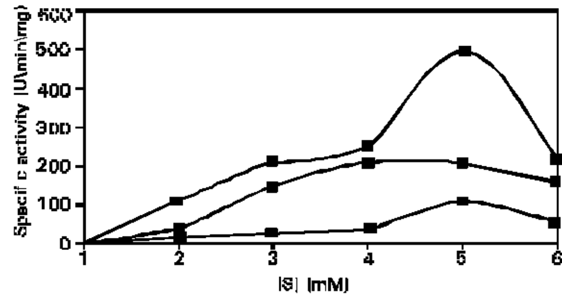

Fig. 3: *In vivo* effect of (L) and © on tyrosinase specific activity. The enzyme was assayed at different concentration of tyrosine.

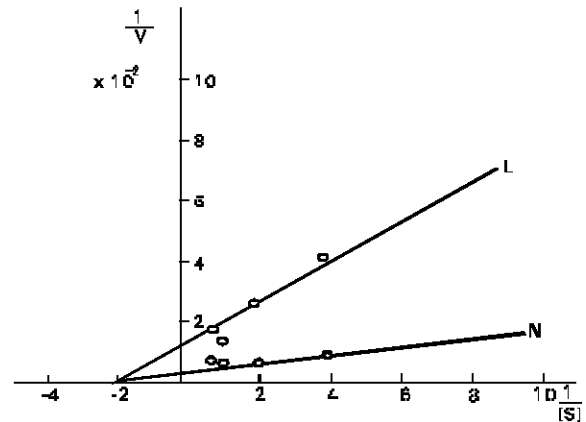

Fig. 4: Lineweaver-Burk plot of tyrosinase in absence (N) and presence of (L) with varying concentrations of tyrosine (mM)

30 %, while a higher relative activity was observed in case of (C)-treated rabbit for about 4.6 fold compared to control as shown in Fig. 2.

The effect of (L) and © on tyrosinase enzyme, using different concentrations of tyrosine, as shown in Table 4, revealed that activation of tyrosinase with compound (C) being effective at low substrate concentrations, with V_{max} 5.05 X 10^2 U/min/mg protein at 1 mM substrate concentration and K_m equals to 0.46 mM, as indicated in Fig. 3.

The Lineweaver-Burk plot revealed that compound (L) was a non-competitive inhibitor of tyrosinase enzyme with K_m equals to 0.5 mM, and inhibition constant K_i equals to 1.88 as shown in Fig. 4.

Table 5 demonstrates the obtained values of the examined parameters measured in the sera of (L)-treated and (C) –treated rabbits, separately compared to control. The results revealed that AST/ALT ratio was 0.97 and 0.75 compared with control 0.74 respectively. Estimation of serum creatine kinase activity of (L)-treated and (C) –treated rabbits showed an increased level to 3000 and 2264 U/L compared to control 1636 U/L. Serum total proteins and serum albumin levels were determined and the obtained data showed a remarkable decrease in A/G ratio to the level of 2.1 and 1.9 as compared to control (3.1) respectively. Also, serum bilirubin was decreased to the level of 0.26 and 0.28 as compared to

Table 1: Purification steps of tyrosinase from control rabbit (N).

Fraction	Total protein (mg)	Total activity U/min	Specific activity U/min/mg	Purification fold
Homogenate	195.0	192.7	0.99	1
Supernatant, first 1000 g	178.6	186.6	1.1	1.1
Supernatant, first 10000g	126.6	189.6	1.5	1.5
Triton X-100 soluble fraction	7.0	121.4	17.3	17.5
Particle bound enzyme	6.0	205.5	34.3	34.6

Table 2: Purification steps of tyrosinase from (L)-treated rabbit.

Fraction	Total protein (mg)	Total activity U/min	Specific activity U/min/mg	Purification fold
Homogenate	335.0	115.5	0.34	1
Supernatant, first 1000 g	237.6	126.5	0.53	1.6
Supernatant, first 10000 g	222.6	136.8	0.62	1.8
Triton X-100 soluble fraction	17.0	105.9	6.2	18.2
Particle bound enzyme	10.8	111.1	10.3	30.3

(L) : Pyrolidine

Table 3: Purification steps of tyrosinase from (C)-treated rabbit.

Fraction	Total protein (mg)	Total activity U/min	Specific activity U/min/mg	Purification fold
Homogenate	124.2	257.9	2.1	1
Supernatant, first 1000 g	106.25	260.0	2.5	1.2
Supernatant, first 10000 g	92.0	272.5	3.0	1.4
Triton X-100 soluble fraction	9.8	161.6	16.5	7.9
Particle bound enzyme	3.2	505.2	157.9	75.2

© : Copper complex

Table 4: Specific activity of isolated tyrosinase enzyme at varying concentrations of tyrosine substrate.

[S]	Sp .Ac. (L)	Sp.Ac. (C)	Sp. Ac. (N)
0.125	9.3	104.2	27.8
0.25	24.7	208.3	138.9
0.50	40.1	255.2	205.6
1.00	111.1	505.2	205.6
1.50	58.6	218.7	161.1

(L) : Pyrolidine , © : Copper complex , (N) : Control, [S] : Substrate concentration

Table 5: Examined serum parameters of (L)-treated and (C)-treated rabbits, compared to control rabbit (N).

Parameter	L	C	N
ALT (U/L)	71	52	65
AST (U/L)	69	39	48
AST/ALT	0.97	0.75	0.74
SCK (U/L)	3000	2264	1636
Total protein (g/dl)	4.25	4.43	4.37
Albumin (g/dl)	2.88	2.92	3.30
A/G	2.1	1.9	3.1
Bilirubin (mg/dl)	0.26	0.28	0.37
Creatinine (mg/dl)	0.68	0.63	0.70
Urea (mg/dl)	66	51	58
Urea/Creatinine	97.1	80.9	82.8

control (0.37 mg/dl) respectively. In addition, estimation of urea and creatinine levels in the sera of (L)-treated and (C)-treated rabbits showed a change in urea/creatinine ratio to 97.1 and 80.9 compared with control 82.8 respectively.

Discussion

The present work deals with investigation of the distinct effect of pyrolidine (L) and its derivative, the copper complex (C) on rabbits .

The data in Table 1 indicated that, partial purification of the crude homogenate of mammalian tyrosinase giving soluble fraction, triton X-100-soluble fraction and partical bound enzyme with specific activity : 1.5, 17.3 and 34.3 U/min/mg protein respectively. All these fractions had different enzymatic activity due to their different ability to solubilize. This result was in agreement with the data of (Burnett, 1971), who studied the tyrosinase enzyme in mammalian tissue extracts, which contain active tyrosinase in both soluble and insoluble, particle bound enzyme. Also, he reported that about 20 % of the total amount of active mammalian tyrosinase is soluble and the remaining 80 % is particle bound and cannot be easily or completely released in soluble form. In addition, he suggested that their were three distinct forms of active mammalian tyrosinase T_1, T_2 and T_3 differing in amino acid compositions, two soluble and one insoluble .

Our study on tyrosinase which isolated from (L)-treated rabbit, as shown in Table 2 revealed a significant decrease in specific activity of the soluble fraction, triton X-100-soluble fraction and particle bound enzyme to the level 0.62, 6.2 and 10.3 U/min/mg protein respectively. That inhibitory effect was probably due to the breakdown of the disulfide linkage (or linkages) within the enzyme. The result was emphasized by Jolley *et al.* (1969), who indicated the importance of a disulfide linkage (or linkages) in the molecular configuration, necessary for enzyme activity and the ability of the enzyme probably to refold partially in an environment favorable for oxidation. In addition, the recorded inhibition of tyrosinase enzyme, may be influenced by the formed anions of the given pyrolidine molecules which spontaneously interacted with the involved copper atoms. The result was agreed with (Duckworth and Coleman, 1970), who showed the presence of two distinct substrate binding sites on enzyme molecule, one with high affinity for aromatic compounds including phenolic substrates, the other for metal-binding agents. The latter site presumably involves enzyme copper. Lineweaver-Burk plot of 1/v against 1/[S] showed that pyrolidine was a non competitive inhibitor of tyrosinase, since no change in the value of K_m in both cases (control and (L)-treated rabbits) .The results ensured the binding of pyrolidine molecules with the copper atoms in the enzyme.

In contrast to the effect of pyrolidine on tyrosinase enzyme, compound (C) showed an increase in specific activity of soluble fraction, triton X-100-soluble fraction, and particle bound enzyme to the level : 3.0, 16.5 and 157.9 U/min/mg protein respectively. This result suggesting that, there was a binding between compound (C) and a specific activator site on the enzyme, facilitates the enzyme binding to substrate and enhancing the rate of interaction to about 4.6 fold compared with control. That activator site on the enzyme was different from the above distinct substrate binding sites as illustrated by (Duckworth and Coleman, 1970) .

On the other hand, analysis of different enzyme activities in the blood sera of (L)-treated and © treated rabbits, compared to control, is a valuable evaluation of many pathological conditions. It was known that alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are important in the diagnosis of heart and liver damage, besides creatine kinase which is the first heart enzyme to appear in blood after a heart attack (Lehninger *et al.*, 1993).

Estimation of AST/ALT ratio in sera obtained from (L)-treated and (C) –treated rabbits compared to control elaborated a non significant increase because AST/ALT ratio was considered as normal ratio between 0.7 – 1.4, while this ratio was increased in hepatotoxicity, becoming more than 2 (Wallach, 1999).

Albumin generally parallels the total protein, except when total protein changes, due to the formation of gamma globulins. This value was used as a marker of disorders of protein metabolism (Wallach, 1999). Estimation of serum total proteins and serum albumin levels in each examined case showed a slight decrease in A/G ratio which may be due to

slight impaired synthesis process in the liver.

Also, there was a slight decrease in serum bilirubin for both treated cases due to the effect of tested compounds on hepatocytes. The results agreed with that of Wallach (1999) in case of other drugs such as barbiturates.

In addition, estimation of urea/creatinine ratio in the examined sera, showed a non significant change in case of (C)-treated rabbits, while a remarkable increase was observed in case of (L)-treated rabbits, probably due to slight renal disorder, since urea/creatinine level was used for the diagnosis of renal insufficiency and is a more specific indicator for renal disease (Wallach, 1999).

However, it was appeared from the obtained data that the activity of creatine kinase SCK, especially in case of (L)-treated rabbits was increased more than that in (C)-treated rabbits, compared with control, probably due to the binding of pyrolidine (L) with serum copper as a ligand, causing a deficient-copper-induced hypertrophy. Our results are in coincidence with the study on rats maintained on copper supplemented or copper-deficient diets, revealing that the relative proportions of type V collagen was reduced in the copper-deficient hearts causing cardiac hypertrophy (Dawson *et al.*, 1982).

Finally, it may be concluded that the tested compounds (L) and (C) have a distinct effect on tyrosinase and a marked change on examined serum parameters reflecting the liver, kidney and heart functions.

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