AMINO ACID DECARBOXYLASES OF *ANCYLOSTOMA CEYLANICUM* AND *NIPPOSTRONGYLUS BRASILIENSIS*

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ABSTRACT

Dialysed homogenates as well as partially purified extracts of *Ancylostoma ceylanicum* and *Nippostrongylus brasiliensis* mediated decarboxylation of all twelve amino acids were examined. Maximum CO_2 was produced by glutamic acid, and minimum by leucine, phenylalanine and valine. γ-Aminobutyric acid and ethanolamine were identified as the decarboxylation products of glutamate and serine respectively. Evidence concerning the presence of true amino acid decarboxylases in the two parasites has also been collected. All the anthelminthics employed in the study were found inhibitory to glutamate decarboxylase, with slight variation in degree of effectivity.

INTRODUCTION

ANCYLOSTOMA CEYLANICUM and *Nippostrongylus brasiliensis* have recently been shown to produce CO_2 from a number of amino acids[1]. Similar information is available for a few other parasites[2,3]. These parasites however mediate active transamination also[1,3,4] and therefore are capable of converting amino acids into the corresponding ketoacids which may be additional source of CO_2. It is not yet clear whether the released CO_2 comes directly from amino acids or indirectly from their transamination products. In order to establish direct decarboxylation of amino acids, transamination must be made non-functional. This can be achieved by removing the required cofactors from the system. Accordingly decarboxylation has been studied with partially purified homogenates. Attempts have also been made to characterize the metabolic products of a few decarboxylases and to examine their susceptibility to certain known and candidate chemotherapeutic agents.

MATERIALS AND METHODS

Chemicals and reagents

U-^{14}C-labelled alanine, arginine, aspartic acid, lysine, glutamic acid, proline, serine and tyrosine; 1-^{14}C-labelled glycine, leucine, phenylalanine and valine; and 5-^{14}C-labelled glutamic acid were obtained from Bhabha Atomic Research Centre, Bombay. A kit of unlabelled amino acids was procured from Loba Chemie, Bombay. Mebendazole was from Cadila Laboratories, Ahmedabad; praziquantel from E. Merck, West Germany; niclosamide from Bayer AG, West Germany; and levamisole from Janssen Pharmaceutica, Belgium, as gift. Compounds 81/470, i.e. methyl-5(6)-[4-(2-pyridyl)]-piperazine carbamoylbenzimidazole-2-carbamate[5]; 82/437 i.e. 2,2′-dicarbomethoxylamino-5,5′-dibenzimidazolyl ketone[6]; and 83/148, i.e. methyl-5(6)-[2,5-dimethyl-3-methoxycarbamylfuranobenzimidazole]-2-carbamate[7] were products of the Medicinal Chemistry Division, CDRI, Lucknow.

Parasites

A. ceylanicum were isolated from golden hamsters with a 18–20-day-old infection and *N. brasiliensis* from rats infected 9 days earlier. The worms were collected in normal saline and thoroughly cleaned.

Preparation of homogenate

A 5% (w/v) homogenate of worms was prepared in 1.15% KCl and centrifuged at 9000 *g* for 30 min. The supernatant was dialysed against several changes of 20 mM phosphate buffer, pH 7.4 and recentrifuged.

Column chromatography

The dialysed supernatant (10-20 mg protein) was loaded on a DEAE-Sephacel column (1 × 40 cm) equilibrated with 20 mM phosphate buffer, pH 7.4. Unadsorbed proteins were washed off the column by running 100 ml of equilibrating buffer. The adsorbed

proteins were eluted with the same buffer containing 0.5 M NaCl. Four ml fractions were collected at a flow rate of 1 ml/min. Fractions of two peaks containing significant amount of protein were pooled, dialysed against buffer, and assayed for decarboxylation.

Assay of decarboxylation

Suitable aliquots of homogenate of purified fractions were shaken at 37°C in 25 ml capped Erlenmeyer flasks containing (in 1.0 ml) 100 μmol phosphate buffer pH 7.4, 10 μmol $MgCl_2$; 1.0 μmol labelled amino acid (sp. activity 1 μCi/5 μmol). The liberated $^{14}CO_2$ was absorbed in 0.1 ml of 1 M methylbenzethonium (hyamine) hydroxide soaked up on filter paper wicks placed in the glass-wells which were suspended into the flasks through the caps[2]. After 2 h the reaction was terminated by injecting 0.1 ml of 1 M perchloric acid. The flasks were then shaken for an additional 30 min to allow the liberated $^{14}CO_2$ to be absorbed by the alkali. Appropriate controls without parasite fractions were run simultaneously. The filter papers containing absorbed $^{14}CO_2$ were counted for radioactivity.

Identification of reaction products

One hundred μl of enzyme preparation (concentrated 20-fold by immersible CX-10 ultrafiltration (Millipore, USA)) was incubated at 37°C with 1 μmol of labelled amino acid (5 μCi), and 200 units of penicillin-G and 200 μg of streptomycin. Total volume of the reaction mixture was 200 μl. After 0.5, 6 and 24 h, a 50 μl aliquot was removed and placed in a boiling water bath for 5 min. Twenty μl was applied on a Whatman paper No. 1 along with standards. The material was chromatographed for 18 h in *n*-butanol–acetic acid–water (4:1:1). After drying the paper in air, radioactive spots were visualized by autoradiography while the unlabelled standards were stained with ninhydrin. The areas corresponding to the standards were cut out from the chromatogram and counted for radioactivity.

Radioactivity counting

The samples were mixed with 10 ml of Bray's solution and counted for radioactivity in a Packard Tricarb scintillation spectrometer. Correction for quenching was applied using an internal standard.

Protein was estimated colorimetrically[8] using bovine serum albumin as standard.

RESULTS

Dialysed homogenates of both *N. brasiliensis* and *A. ceylanicum* catalysed decarboxylation of all the 12 amino acids were examined (table 1). *A. ceylanicum*; in general, produced greater amounts of CO_2 than *N. brasiliensis*. Among the amino acids, glutamate was decarboxylated to the greatest extent while the aromatic amino acids phenylalanine and tyrosine were less susceptible. Passage of the homogenate through DEAE-Sephacel resulted in distribution of proteins between unadsorbed and adsorbed fractions (figure 1). The adsorbed protein could be eluted with buffer containing 0.5 M NaCl. Surprisingly, both fractions decarboxylated all the twelve amino acids with little quantitative differences. This cannot be considered to be due to peak trailing since the separation of proteins into two distinct fractions is clearly evident from figure 1.

Formation of decarboxylation product, as judged by the product vs substrate ratio, increased with reaction time (table 2). For instance, the ethanolamine/serine ratio was very low 0.019 at 0.5 h but rose to 0.298 and 1.08 after 6 and 24 h respectively. Likewise, for [U-^{14}C]glutamate, the ratio of γ-aminobutyrate/glutamate increased from 0.032 at 0.5 h to 1.16 at 24 h. Similar observations were recorded for *A. ceylanicum* also.

All the anthelminthics tested reduced CO_2 liberation from glutamate. The maximum inhibition was by 25 μM Praziquantel of the *A. ceylanicum* enzyme (table 3). For *N. brasiliensis*, on the other hand, compounds 82/437 and 83/148 proved most effective.

DISCUSSION

Liberation of CO_2 from amino acids appears to be a common feature of helminth parasites[1-3,9,10]. However, the question whether this CO_2 arises from direct decarboxylation of amino acids or from keto acids produced during transamination remains to be answered. The present results provide strong evidences in favour of direct decarboxylation of amino acids by *A. ceylanicum* and *N. brasiliensis*. The first convincing clue is obtained from the observation concerning the production of CO_2 in the presence of dialysed and partially purified parasite extracts (table 1). During these steps certain important cofactors are removed from the extracts. For example, an amino group acceptor like 2-oxoglutarate, pyruvate or oxalacetate is required for

Table 1 *Rate of $^{14}CO_2$ production from various amino acids by homogenate and partially purified extract of A. ceylanicum and N. brasiliensis*

	$^{14}CO_2$ produced (nmol/h/mg protein)					
	N. brasiliensis			*A. ceylanicum*		
Amino acid	Homogenate	Unadsorbed fraction	Adsorbed fraction	Homogenate	Unadsorbed fraction	Adsorbed fraction
[U-^{14}C] Alanine	7.5	9.5	11.66	45.97	141.05	43.05
U-^{14}C] Arginine	20.3	12.38	21.28	27.67	48.03	56.18
[U-^{14}C] Aspartate	18.6	24.94	31.29	38.69	107.49	177.31
[U-^{14}C] Glutamate	158.5	218.25	247.1	593.0	781.8	1222.0
[5-^{14}C] Glutamate	5.3	16.34	80.49	81.59	258.01	309.02
[1-^{14}C] Glycine	4.5	11.1	19.14	17.18	29.25	60.76
[1-^{14}C] Leucine	1.84	7.22	11.44	1.34	1.84	12.66
[U-^{14}C] Lysine	15.0	20.93	32.07	12.52	31.01	105.76
[1-^{14}C] Phenylalanine	3.45	2.32	10.26	2.73	4.37	5.35
[U-^{14}C] Proline	18.6	22.70	36.70	46.13	99.03	142.99
[U-^{14}C] Serine	8.5	18.8	28.69	39.8	141.22	−44.85
[U-^{14}C] Tyrosine	0.95	2.73	10.64	5.22	12.16	11.51
[1-^{14}C] Valine	0.89	1.76	2.43	8.77	6.01	18.24

Data are mean of three experiments.

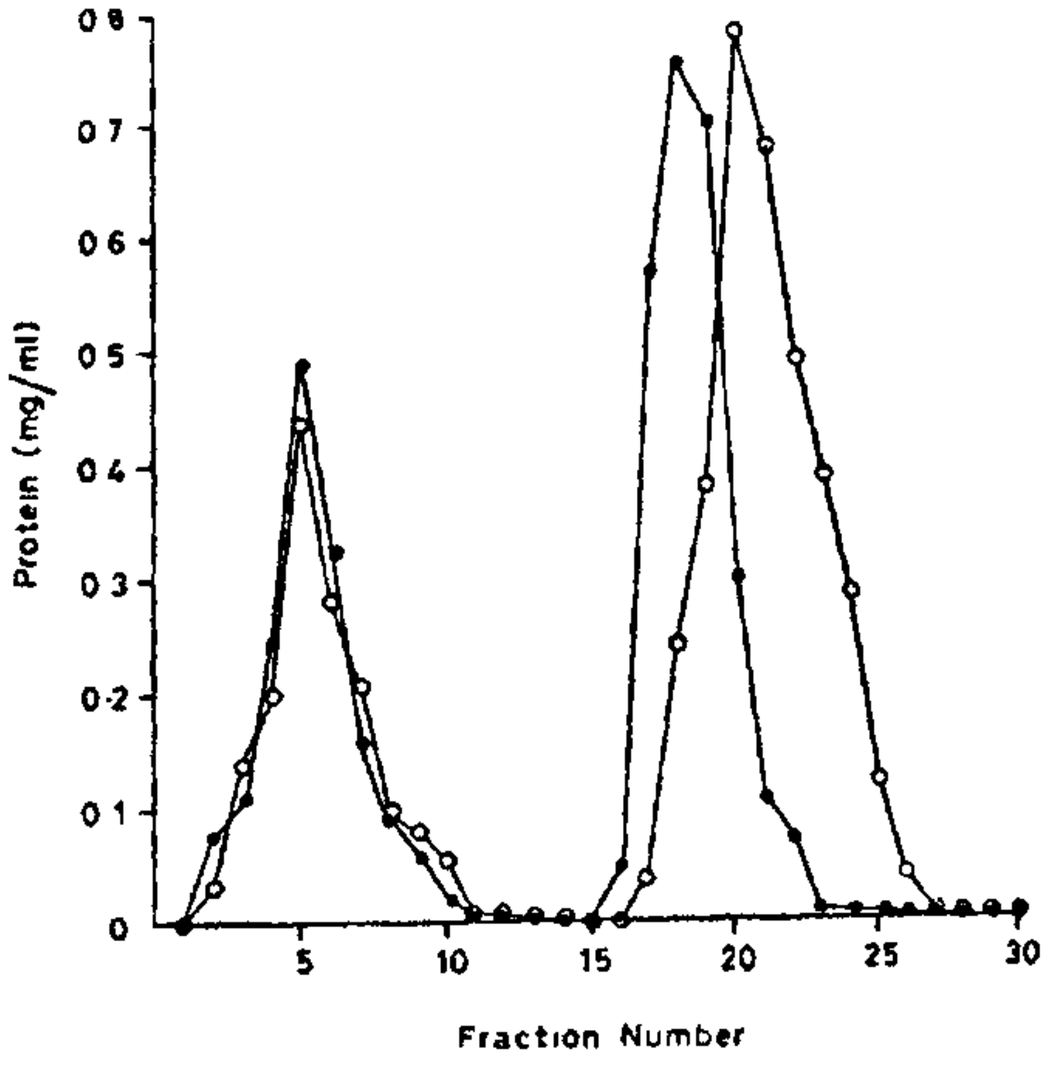

Figure 1. Elution pattern of soluble proteins of *A. ceylanicum* (○) and *N. brasiliensis* (●) on DEAE-sephacel.

transamination. Likewise, NAD, NADP or FAD is needed for dehydrogenases. In the absence of these factors, as in present case, transamination or dehydrogenation reactions cannot proceed. Minor amounts of tightly bound cofactors cannot support these reactions significantly. Hence, CO_2 evolved in presence of dialysed or partially purified extracts may originate directly from amino acids.

Table 2 *Accumulation of decarboxylated metabolites by activity of purified extract of N. brasiliensis and A. ceylanicum*

	Product/substrate ratio after		
Substrate	0.5 h	6 h	24 h
N. brasiliensis			
[5-^{14}C]Glutamate	0.012	0.080	0.31
[U-^{14}C]Glutamate	0.032	0.141	1.16
[U-^{14}C]Serine	0.019	0.298	1.08
A. ceylanicum			
[5-^{14}C]Glutamate	0.048	0.130	0.398
[U-^{14}C]Glutamate	0.032	0.251	1.261
[U-^{14}C]Serine	0.192	0.360	1.683

Data are mean of three experiments.

Table 3 *Effect of anthelminthics on partially purified glutamate decarboxylase of A. ceylanicum and N. brasiliensis*

	Inhibition (%)			
	N. brasiliensis		*A. ceylanicum*	
Anthelminthic agent	10 μM	25 μM	10 μM	25 μM
Mebendazole	11.04	23.65	14.59	27.23
Praziquantel	14.42	20.41	16 41	70.30
Niclosamide	15.25	36.74	28.49	46.17
Levamisole	15.76	34.83	16.25	38 20
81/470	12.81	10.37	12.34	25.70
82/437	34.23	41.26	16.21	49 11
83/148	38.61	44.79	17.18	24.68

Data are mean of 3 experiments.

Identification of ethanolamine, γ-aminobutyrate and 2-aminobutyrate as the reaction products provides further support for the existence of true decarboxylase(s) at least for two amino acids, serine and glutamate. Formation of both γ-aminobutyrate and 2-aminobutyrate suggests release of CO_2 from both carboxyl groups of glutamate. [5-^{14}C]Glutamate, when decarboxylated at the γ-carboxyl group, would yield unlabelled product. Hence labelled reaction product from [5-^{14}C]glutamate can be generated only in presence of α-decarboxylase. Further, generation of labelled CO_2 from [U-^{14}C]glutamate proceeds at nearly four times the rate from [5-^{14}C]glutamate. This can only be explained by liberation of more labelled CO_2 from the labelled γ-carboxyl group in [U-^{14}C]glutamate.

All the anthelminthics examined inhibit glutamate decarboxylase of both parasites. The very little difference in degree of effectivity between these compounds would seem to preclude a specific mode of action against this enzyme. Yet, the inhibition of *A. ceylanicum* enzyme by praziquantel is interesting, as the antischistosomal action of this drug has been attributed to interference with neuromuscular activity of the parasite[11]. Glutamate decarboxylase produces γ-aminobutyrate which is a known neurotransmitter.

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