Effect of salicylate on perfused rat hearts. The percent of phosphorylase a was calculated after 10 min of perfusion. Glycogen content measured as μmoles of glucose (after hydrolysis) per g and lactate production was expressed as μmoles of lactate per h per g. The results are expressed on a fresh weight basis.

Determinations*	Control	Salicylate
Ph. a (per cent)	7.2 ± 3**	23 ± 7
Glycogen content	83.3 ± 8	10.1 ± 4
Lactate production	25 ± 3.2	112.5 ± 14

*6 hearts were used as control and 6 for treatment with sodium salicylate. **S. E. M.

that the enzymes participate in activation of glycogenolysis caused by salicylate.

Experiments to determine the phosphorylase level in the hearts treated by aspirin, which is also a glycogenolytic agent[7], revealed that in fact the phosphorylase a level was increased about 3fold, i.e. from 7.2 ± 3 in the controls to 27 ± 4 in the hearts subjected to the drug. This effect of either sodium salicylate or aspirin on heart phosphorylase might be expected, since the high rate of glycogenolysis found in muscle subjected to the action of these drugs requires the maintenance of the enzyme in the activated form.

Our finding that salicylate increases phosphorylase a activity might be explained through a mechanism involving the uncoupling of oxidative phosphorylation. For example, previous work from our laboratory[8] has shown that DNP results in elevated levels of phosphorylase a of heart and skeletal muscle. This could be explained by a decrease in the ATP/ADP ratio which would increase phosphofructokinase activity and thereby maintain glucose-6-phosphate at a low level in the cell. Since glucose-6-phosphate is known to inhibit phosphorylase b to a conversion, this inhibition would be reduced in the presence of the uncoupling agent resulting in higher phosphorylase a levels. In support of this idea, other experiments with salicylic acid[3] have shown that in the isolated rat diaphragm the content of inorganic phosphate is increased, whereas creatine phosphate and ATP are severely reduced in the presence of the drug.

The results of Segal and Blair[2] of phosphorylase a inhibition in isolated hemidiaphragms is found only 30 min after salicylate administration, a time which, according to the authors, glycogenolysis proceeds at a much slower rate even though glycogen is still present in relatively high amounts.

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Interaction of N-(DL-seryl)N′-(2,3,4-trihydroxybenzyl)-hydrazine with L-Dopa decarboxylase from pig kidney

C. Borri-Voltattorni, A. Minelli and P. Borri

Institute of Biological Chemistry, Faculty of Pharmacy, Institute of Nervous and Mental Diseases, University of Perugia, I-06100 Perugia (Italy), 19 July 1976

Summary. Interaction of seryl trihydroxy-benzyl-hydrazine with a highly purified preparation of Dopa decarboxylase from pig kidney has been studied. This compound was found not to be a powerful inhibitor in vitro. Kinetic and spectral data suggest some possibilities on the binding nature of the inhibitor and substrates.

The N-(DL-seryl)N′-(2,3,4-trihydroxybenzyl)-hydrazine (Ro 4-4602) is a product used, in addition to L-Dopa, in the treatment of Parkinson's disease. This compound is an inhibitor of peripheral Dopa decarboxylase and does not appear to pass the blood-brain barrier at therapeutic doses in Parkinsonism. Its effect on cellular metabolism lies in potentiating the biochemical behaviour of peripherally administred L-Dopa. This pharmacological action, largely investigated from a clinical point of view, reflects the interaction of this compound at the molecular level with peripheral L-Dopa decarboxylase. Such interaction has been studied in vitro measuring the inhibition on crude homogenate of hog or rat kidney[1,2], not yet investigated on a highly purified enzymic preparation.

The present communication deals with the binding of Ro 4-4602 to an homogenous Dopa decarboxylase from pig kidney. The inhibition constants and the effect on the absorption peaks of the enzyme-bound pyridoxal-P give some information on the interaction of this compound with Dopa decarboxylase and may also provide more detailed insights of its active site.

Materials and methods. The enzyme, purified according to Borri Voltattorni et al.[3], appeared to be homogenous in polyacrylamide gel electrophoresis and in the ultracentrifuge. Its coenzyme content was in good agreement with that reported by Christenson et al.[4].

L-m-tyrosine and L-o-tyrosine were synthetized from corresponding racemic forms by the action of D-amino acid oxidase and catalase in a 0.05 M pyrophosphate buffer, pH 8.3 under O_2 stream. The enzymatic degradation of the D-amino acid form was followed measuring at time intervals the formation of the keto acid form with 2,4-dinitrophenyl hydrazine. The end-point of the reaction was achieved when, even after further additions of enzymes, no more keto acid was detected. Then the reaction mixture, brought to pH 3, was poured on a 50 × 8 Dowex column. After elution of the keto acid with 0.2 M pyridine buffer, pH 3.1, the L-amino acid was eluted with 2 M pyridine buffer, pH 5. Ether was added

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Michaelis-Menten constants and inhibition constants of Ro 4-4602 for different substrates for Dopa-decarboxylase

Substrate	$K_m \times 10^4$	$K_i \times 10^4$
L-Dopa	0.68	3.40
L-5HTP	1.64	18.00
L-m-tyrosine	2.10	3.65
L-o-tyrosine	5.70	3.65

to the evaporated solution, and the precipitate was filtered and dried over P_2O_5 under vacuum. The final products do not react at all with D-amino acid oxidase and are fully converted into the keto acid form by L-amino acid oxidase.

The enzyme activity was measured according to Sherald et al.[5], as modified by Charteris et al.[6]. The spectra were determined on a Beckman DB-GT spectrophotometer, connected to a Beckman 10-inch recorder. The absorption of the purified enzyme solution at pH 6.8 in the 300–500 nm region was similar to that previously described[7], with a very constant absorbance ratio at 335 and 420 nm.

Results and discussion. The Ro 4-4602 (a kind gift of Roche S.p.A., Milan) behaved as a competitive inhibitor with respect to L-Dopa and as a 'pure' non competitive inhibitor towards 5-Hydroxy-L-tryptophan (L-5HTP), L-m-tyrosine and L-o-tyrosine (figure 1). K_m and K_i of Ro 4-4602 values measured at pH 6.8 for these substrates are reported in the table. Although the experimental conditions were different, the affinity of Ro 4-4602 was not as high as suggested by Burkard et al.[2]. We used, infact, instead of a crude extract, an homogenous enzyme preparation, a more sensitive assay, a lower temperature and substrates in L-form in order to avoid the inhibition by D-form[8].

The addition of the inhibitor to an enzyme solution at pH 6.8 changed the spectral properties by decreasing the intensity of the 420 nm and the 335 nm peaks (figure 2). The absorbance at 420 nm of the enzyme plus increasing amounts of Ro 4-4602 and the concentration of this compound in a reciprocal plot are reported in the inset of figure 2. The dissociation constant measured in this way was $3.2 \cdot 10^{-4}$ M. This value was in line with K_i for 3 substrates but not for L-5HTP. The lower affinity of Ro 4-4602 observed only in presence of L-5HTP is not easy to explain and might be related to the indolic ring of this substrate.

The different types of inhibition observed with the various aromatic amino acids discloses some possibilities about the binding nature of the inhibitor and substrates. The modified absorption peaks induced by the compound on the enzyme-bound pyridoxal-P suggests its binding to the active site.

On the other hand, the observed 'pure' non-competitive inhibition towards 3 substrates may indicate that the inhibitor and these substrates do not compete for the same site on the enzyme surface, but that they are able to become simultaneously attached to the enzyme. The disagreement between these data can be interpreted in at least two ways. 2 substrate binding sites could exist on the enzyme molecule, one for L-Dopa and the other one common for the other three substrates. The Ro 4-4602 could bind to the enzyme at the same site as L-Dopa. At present, this is just a hypothesis which must be confirmed by further investigations; moreover, it would

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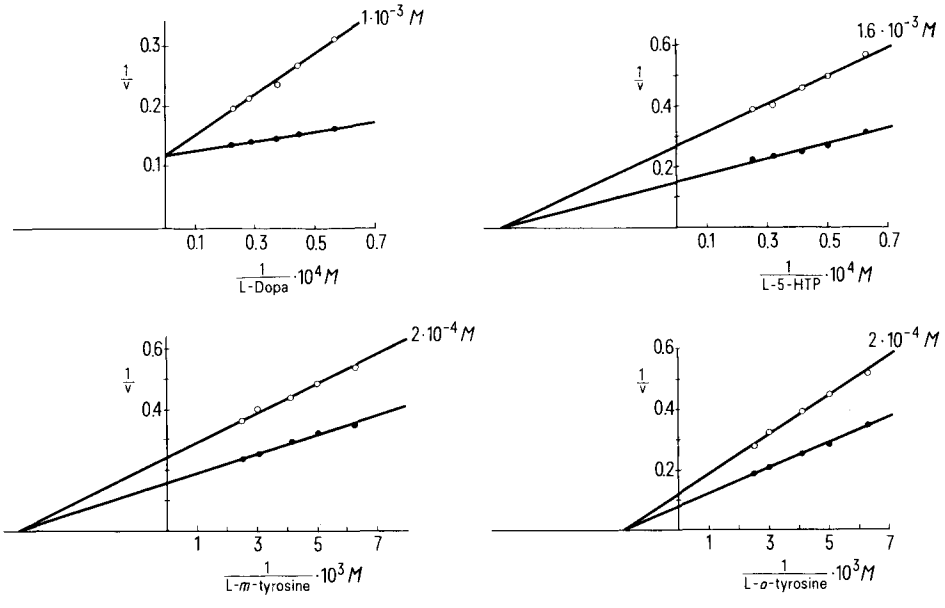

Fig. 1. Inhibition by Ro 4-4602 of decarboxylase activity on various aromatic amino acids presented as double reciprocal plot. The reaction, in a final volume of 250 µl, was carried out in 40 mM phosphate buffer pH 6.8 at 25 °C. Time course of reaction was 5 min for L-Dopa, L-o-tyrosine and L-m-tyrosine; 10 min for L-5HTP. 1 unit of activity is defined as the amount of enzyme which produces 1 nmole amine per min under the specified conditions.

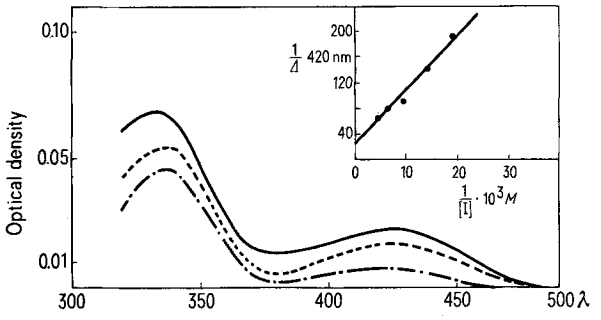

Fig. 2. Spectra in phosphate buffer pH 6.8: ——, enzyme; – – –, enzyme plus $7 \cdot 10^{-5}$ M seryl-trihydroxy-benzyl hydrazine; –·–, enzyme plus $21 \cdot 10^{-5}$ M seryl-trihydroxy-benzyl-hydrazine. The spectra are corrected for the absorption of the compound added. In the inset: absorbance at 420 nm of the enzyme plus seryl-trihydroxy-benzyl-hydrazine as a function of its varying concentrations presented as double reciprocal plot.

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be interesting to check the binding sites specificity and their functional properties.

The other possibility might involve only one substrate binding site and a second intermediate, in addition to the Michaelis complex. The relative magnitudes of rate constants of 2 intermediates could be quite different, depending on the substrates used. As suggested by Krupka and Laidler[9], an apparent non-competitive inhibition may be at play in the sense that the inhibitor becomes attached to the second intermediate and not to the Michaelis complex. It must be pointed out in this connection that, for Dopa decarboxylase, some intermediate substrate-enzyme complexes have already been characterized and their properties have been connected with the structural variations of substrate[10].

Besides these hypotheses, the results show that the trihydroxybenzylhydrazide seryl derivative is not a powerful inhibitor of the aromatic amino acid decarboxylase in vitro. Owing to a large substituent in 1 position of the aromatic ring and the lack of a free hydrazinic group, the requirements for a good interaction at the active site are probably not met. The powerful inhibition observed in vivo therefore probably reflects the interaction between the peripheral decarboxylase and the compound formed by hydrolysis of the seryl hydrazine linkage.

Enzymic basis for the nutritional requirement of arginine in insects[1]

S. Raghupathi Rami Reddy[2] and J. W. Campbell[3]

Department of Biology, Rice University, Houston, Texas 77001 (USA), 4 June 1976

Summary. The fat body of the cockroach, Blaberus cranifera, and the silkmoth, Hyalophora gloveri, has been tested for some enzymes of the urea cycle. While ornithine transcarbamoylase and argininosuccinase activities could not be detected, arginase is present in the fat body of these insects. These results explain the essentiality of arginine in the diet of insects.

The arginine biosynthetic pathway, believed to be present in whole or in part in all organisms, has been successfully exploited for ammonia detoxification during the evolution of ureotelism associated with the invasion of the terrestrial habitat by animals[4]. Terrestrial insects, which are uricotelic, seem to have lost the ability to synthesize arginine, and thus the potential for forming urea as an excretory product. This is indicated by the nutritional requirement for arginine by many insects[5]. However, in some species of insects, arginine in the diet can be replaced by citrulline but not by ornithine[6,7]. These findings suggest that insects, like birds[8,9], can form arginine from citrulline, but are unable to synthesize citrulline from CO_2, NH_3 and ornithine. To find out the biochemical basis for these nutritional results, we have studied the distribution of some of the urea cycle enzymes in the fat body of the cockroach, Blaberus cranifera, and the silkmoth, Hyalophora gloveri. These results are reported here.

Material and methods. The procurement and maintenance of the insects, Blaberus cranifera and Hyalophora gloveri, and the methods of arginase assay (L-arginine amidinohydrolase, E.C. 3.5.3.1) and protein determination have been described earlier[10]. Ornithine transcarbamoylase (Carbamoylphosphate: L-ornithine carbamoyltransferase, E.C. 2.1.3.3) and argininosuccinase (L-argininosuccinate arginine-lyase, E.C. 4.3.2.1) assays were performed in 10% (w/v) fat body extracts essentially by the methods described by Campbell and Speeg[11].

Results and discussion. The distribution of the 3 urea cycle enzymes in the fat body of the cockroach and silkmoth is shown in the table. Ornithine transcarbamoylase (OTCase) and argininosuccinase (ASase) activities could not be detected in the fat body of these insects. The recovery of citrulline added to the OTCase assay system was 104%. There is no inhibitor of OTCase in fat body tissue of Blaberus cranifera, as determined by experiments in which a purified bacterial OTCase was mixed with fat body extracts of this insect and assayed. Further, the methods used for the assay of these enzymes are

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2 Recipient of a Fulbright Travel Grant from the USEFI (New Delhi). Present address: Department of Zoology, University of Poona, Poona, Maharashtra 411 007, India.
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