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The assay of aromatic amino acid decarboxylase using radioactive substrates

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AROMATIC amino acid decarboxylase is a relatively non-specific enzyme occurring in many animal tissues, and it is especially rich in guinea pig kidney. Its substrates include 3 : 4-dihydroxyphenylalanine (DOPA), 5-hydroxytryptophan (5HTP) and histidine, which it converts to the corresponding amines at widely varying rates, the relative activities found by Lovenberg, Weissbach and Udenfriend[1] being 6400, 1000 and 10 respectively. A convenient method of estimating this enzyme and rapidly evaluating its inhibitors has now been devised, using radioactively labelled substrates and separating the amine formed from the unchanged amino acid by chromatography or electrophoresis on paper. For the separation of 5-hydroxytryptamine from 5HTP 10 μl samples of the incubation mixture are spotted in one application on to strips of Whatman No. 1 paper, 10 × 1cm, which are dried rapidly in hot air (about 75°) to stop the reaction and chromatographed in 6 × 1″ boiling tubes containing 5 ml of isopropanol:ammonia (0·880):water = 100: 5: 10 until the solvent front is 6 cm above the origin. The portion beyond the solvent front is then cut off and the remainder divided in two at 2 cm from the origin and the two parts placed in Tri-Carb vials containing 20 ml toluene phosphor, (4·0 g P.P.O., 0·10 g P.O.P.O.P./l). for counting in a Tri-Carb scintillation spectrometer. (When histidine ^{14}C is used as substrate the papers are divided at 1·5 cm from the origin). The origin contains the unchanged amino acid and the other portion the amine formed. By running controls with boiled enzyme, the amounts of amino acid used and amine formed are readily found, and these are usually in good agreement unless the enzyme activity is very low.

DOPA and dopamine are not separated by such a wide margin on paper chromatograms, and oxidation is likely to occur in alkaline solvents, so paper electrophoresis in an acid buffer is found preferable. For this purpose Whatman No. 1 paper 12·5 cm wide is cut into a grid of 10 strips 1 cm wide by 20 cm long, separated by 1 mm gaps to prevent diffusion. The paper is soaked in 0·5 M acetate buffer pH 4·5 which stops the enzyme reaction when the sample is applied. Delay of up to 30 min between applying the sample and carrying out the electrophoresis (at 3 kV for 20 min) does not affect the result. Markers of DOPA and dopamine are run at the same time and stained with alkaline ferricyanide. The corresponding regions of the test papers are then cut out and counted as described above.

When this method was first applied to the assay of DOPA decarboxylase in phosphate buffer with pyridoxal phosphate as coenzyme, the recovery of radioactivity in the spots corresponding to DPOA and dopamine was low and variable. The remainder of the radioactivity was found in a region to the anode side of the DOPA, and its origin was traced to the non-enzymatic reaction of DOPA with pyridoxal phosphate, presumably with formation of the tetrahydroisoquinoline described by Schott and Clark.[2] These authors showed that the presence of an active *m* hydroxy group is necessary for the reaction, so it seemed possible that the use of borate to complex the catechol hydroxyl groups might suppress the reaction with the coenzyme. This has proved to be the case, and when the assay is carried out in borate buffer the amount of radioactivity migrating to the anode is greatly reduced.

For the rapid, preliminary evaluation of inhibitors, guinea pig kidneys are homogenised in water (3 ml/g) and the homogenate centrifuged at 25,000 g (average) for 30 min. The supernatant liquid contains about 20 mg protein/ml and 1 ml is incubated at 37° with 0·5 ml pyridoxal phosphate (1 mg/ml water) for 5–10 min before mixing with the other components. The monoamine oxidase activity of this preparation is low, and for short incubation times the use of an amine oxidase inhibitor is unnecessary. Three concentrations of inhibitor are incubated with each of two concentrations of substrate. A typical incubation mixture is as follows:-

0·1 ml labelled substrate in ·01 N HCl (about 0·5 $\mu c^{14}C$)
0·1 ml unlabelled substrate in buffer
0·1 ml inhibitor in buffer
0·1 ml buffer (0·05 M borax + 0·1 M KH_2PO_4, pH 7·6)
0·2 ml enzyme + pyridoxal phosphate mixture (about 2·3 mg protein)

The final substrate concentrations used are (approx.) DOPA: $1{\cdot}5 \times 10^{-4}$ M and 10^{-3} M; 5HTP $6{\cdot}7 \times 10^{-5}$ M and $2{\cdot}7 \times 10^{-4}$ M. These concentrations are well below the saturation point of the enzyme, so that weak inhibitors should be readily detected. Samples are taken after 4 min incubation at 37° since after this time the velocity of the DOPA reaction falls off rapidly. The 5HTP reaction has a

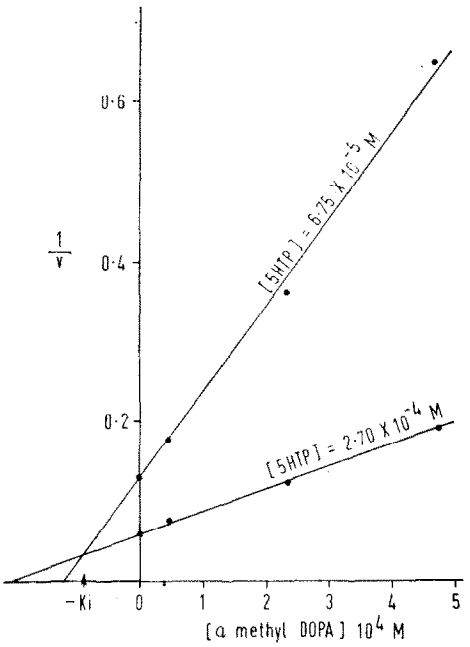

FIG. 1. Competitive inhibition of 5 HTP decarboxylase by α methyl DOPA.

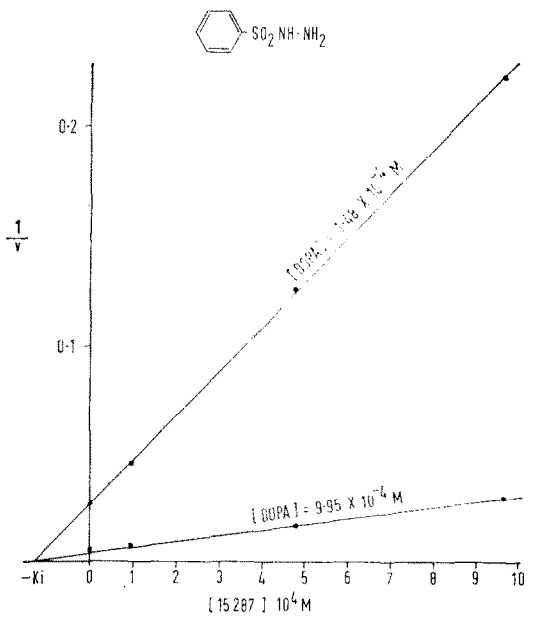

FIG. 2. Non-competitive inhibition of DOPA decarboxylase by phenylsulphonylhydrazide.

constant velocity up to about 8 min. The amount of substrate changed is (counts/min in amine spot/total counts/min) × total substrate, and this is taken as a measure of the initial velocity *v*. *Ki* and the type of inhibition can then be found by plotting (1/*v*) against inhibitor concentration.[3] This method of calculation avoids the need to know the counting efficiency or the exact sample size. Figures 1 and 2 are plots obtained by this method showing the competitive inhibition of 5HTP decarboxylation by α-methyl DOPA and the non-competitive inhibition of DOPA decarboxylation by phenylsulphonylhydrazide.

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The effect of amitriptyline on glycogen phosporylase in cardiac muscle

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INVESTIGATIONS in the peripheral pharmacology of amitriptyline (5-(3-dimethylaminopropylidine)-dibenzo-(a,d)(1-4)-cycloheptadiene) have suggested that this recently introduced antidepressant drug might activate the β- adrenotropic receptor.[1] This conclusion was based on observations made in experiments on the whole animal, on hind limb blood flow and upon the action of amitriptyline on the heart in vivo. In these latter experiments it was demonstrated that amitriptyline potentiated the increase in cardiac output induced by isopropyl nor-adrenaline, but antagonised the increase in output induced by adrenaline.

In view of these observations it was decided to examine the effect of amitriptyline on the activation of glycogen phosphorylase (α-1,4-glucan: orthophosphate glucosyl transferase EC2411) in cardiac muscle. A number of workers have observed that activation of the enzyme phosphorylase depends upon the formation of the substance cyclic 3,5-adenosine monophosphate (cyclic 3,5-AMP).[2] The formation of cyclic 3,5-AMP is promoted by catechol amines; once formed it activates the enzyme phosphorylase kinase which in turn converts "inactive" phosphorylase or phosphorylase-b to "active" phosphorylase or phosphorylase-a. This latter enzyme catalyses the breakdown of glycogen to glucose-1-phosphate. A correlation appears to exist between the increased force of contraction brought about by catechol amines or sympathetic stimulation and the biochemical event, that is, the activation of phosphorylase.[3, 4] This suggestion is further strengthened by the observation that the β-adrenergic blocking agent dichlorisopropyl nor-adrenaline blocks both these actions,[3, 4] and suggests that phophorylase activation may be associated with the β-adrenotropic receptor. Robson and Stacey[5] have proposed that the enzyme catalysing the formation of cyclic 3·5-AMP may be synonymous with the β-receptor.

It was therefore of interest to investigate the action of amitriptyline on heart muscle phosphorylase both alone and in combination with catechol amines.