Ph.D. BIOCHEMISTRY

ABSTRACT

By Gerald Alan Lancaster

PURIFICATION OF HOG KIDNEY DOPA DECARBOXYLASE

This thesis outlines a method for purifying dopa decarboxylase from 300 g of hog kidney by heat treatment, ammonium sulfate fractionation and chromatography on DEAE-Sephadex, hydroxylapatite and Sephadex G150. This method yields a product of high purity as shown by gel electrophoresis in three different systems as well as different gel concentrations. The purified material has a specific activity of 21.1 μmoles of 5HT produced per mg protein per h.

The purified enzyme has 7 times the activity towards L-DOPA as it does towards 5HTP. This ratio does not change during purification. 5HTP and DOPA act as mutual competitive inhibitors with K_I's similar to their K_M's suggesting that they bind at the same site on the enzyme. Activity towards ortho- and meta-tyrosine and erythro-DOPS was also detected in the purified enzyme. Decarboxylase activity was not detected towards para-tyrosine or threo-DOPS.

The molecular weight of the purified enzyme found by gel filtration was $8.5\text{-}9.0 \times 10^4$. Dodecylsulfate polyacrylamide gel electrophoresis gave two bands. The sum of the molecular weights of these bands was $8.8\text{-}9.4 \times 10^4$. As the method used breaks disulfide bonds it is probable that dopa decarboxylase consists of two peptides of unequal length.

By direct measurements on the purified enzyme, it was found that 1 PLP is present per mole of enzyme. Additional PLP does not appreciably stimulate activity towards most substrates suggesting that the purified material is holoenzyme.

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I. INTRODUCTION

Dopa decarboxylase was discovered by Holtz, Heise and Lüdtke in mammalian kidney as early as 1938. Its possible importance in the synthesis of adrenaline was recognized soon after by Blaschko (1939) when he showed that the enzyme prepared from guinea pig kidney was not active on tyrosine or N-methyltyrosine. Clark, Weissbach and Udenfriend (1954) found decarboxylase activity towards 5-hydroxytryptophan (5HTP) in guinea pig kidney, and suggested that the enzyme is responsible for the production of the important biogenic amine, serotonin (5HT). Investigation of the enzyme has branched out in several directions. Its occurrence in nature has been extensively documented. Its substrate specificity, cofactor requirements and inhibitors have all been looked into. A multiplicity of assays is available for its measurement. More recently, purification from a number of sources has been attempted.

1. Occurrence in Nature

a) Liver, kidney, intestine

After the initial report by Holtz et al. for guinea pig, pig and rabbit kidney, Holtz and his coworkers extended the range of tissues in which the enzyme is contained, including guinea pig gut (Holtz, Credner and Reinhold, 1939) and the intestines and livers of guinea pigs, rabbits, pigs, sheep, goats, chickens, rats, mice, cattle and frogs (Holtz, Reinhold and Credner, 1939). More recently Klingman, Kardaman and Haber (1964) have found differences in the activity of the enzyme along the length of the rat gastrointestinal tract, the

values ranging from 1.1 μmole dopamine (DA)/g/h* at the pyloric antrum, to 8.9 in the small intestine, and 3.1 in the cecum. In 1942 Holtz and Credner detected the enzyme in the mammalian pancreas. Blaschko (1942) summarized the activities of the enzyme in the livers and kidneys of a variety of mammals. For example, pig kidney has an activity of 13-89 μmoles DA/g/h; guinea pig kidney is more active with 83-324 μmoles/g/h, whereas guinea pig liver had 29-42. Human kidney has an activity corresponding to 9-42 μmoles DA/g/h. The enzyme is present in frog liver (1.3-3.6 μmoles DA/g/h), but not in frog kidney. Huang, Tannebaum and Hsia (1960) studied the effect of age on rat kidney enzyme. They found no activity in foetal kidney, 0.42 μmoles 5HT/g/h for 2-5 day old rats, and 2.68 μmoles 5HT/g/h for adult rats.

b) Adrenal medulla

Langemann (1951) noted the presence of dopa decarboxylase in bovine adrenal medulla; the activity was 35-40 μmoles DA/g/h as compared to 11 μmoles DA/g/h for bovine kidney and liver. Westermann (1957) showed that the enzyme is present in the adrenal medulla of most of the common mammals.

c) Nervous tissue

Holtz and Westermann (1956) studied the enzyme in bovine nervous tissue. Most of the activity is found in post-ganglionic sympathetic nerves, sympathetic ganglia and the sympathetic trunk. Somewhat lower activity is present in the spinal cord and brain stem.

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*Values are expressed per g fresh weight unless otherwise stated.

Very low, but detectable, activity is present in the cerebral cortex. The authors noted at this time that nervous tissue can form noradrenaline. Kuntzman, Shore, Bogdanski and Brodie (1961) carried out a detailed study of decarboxylase in the central nervous system of the cat. Characteristically, the caudate nucleus (1.7 μmoles 5HT/g/h, 8.2 μmoles DA/g/h), and hypothalamus (0.9 μmoles 5HT/g/h, 4.9 μmoles DA/g/h) are high, and the cerebrum (0.11 μmoles DA/g/h) extremely low in activity.

McCaman and Aprison (1964) studied the enzyme in developing rabbit brain. At three days of age, the values are very low -- for cortex, 0.06 μmoles 5HT/g/h and for caudate 0.22. The values are doubled by age twelve days and they continue to increase until the adult levels are attained: 0.20 μmoles 5HT/g/h for cortex and 1.68 μmoles 5HT/g/h for caudate.

Aprison, Takahashi and Folkerth (1964) measured the enzyme in several areas of pigeon brain. Typically, cerebellum is low (0.03 μmoles 5HT/g/h) and the telencephalon and diencephalon higher (0.26). By comparison, the liver of this species has an activity of 0.57 μmoles 5HT/g/h. Pscheidt and Haber (1965) studied the regional distribution of dopa decarboxylase in the chicken central nervous system. Decarboxylase activity decreased in the following order: pons-medulla (2.5 μmoles Da/g/h), di- and mesencephalon (2.1), striatum (1.7), pallidum (1.2), spinal cord (0.5) and cerebellum (0.1).

Robins, Robins, Croninger, Moses, Spencer and Hudgens (1967) have reported that for human brain the enzyme is very low. For

example, human caudate has an activity of only 0.0028 μmoles 5HT/g/h. Constantidinis, de la Torre, Tissot and Geissbühler (1969) have reported dopa decarboxylase to be present in the parenchymal cells of the brain capillary walls, forming an effective barrier to the entry of DOPA into the brain, although if sufficient DOPA is given, some passes the barrier and is decarboxylated in neurones. This has clinical importance in conditions such as Parkinson's disease where brain DA is to be increased by treatment with DOPA.

According to Hakanson and Owman (1966) dopa decarboxylase is very high in the pineal gland of rabbit, rat and pig; in man and the cat the values are somewhat lower. The enzyme appears to exist in the parenchyma of the gland in the rabbit and rat since its activity is not diminished by sympathetic denervation. In the cat, however, the bulk of the activity seems to be in the sympathetic nerves. According to Anden, Magnusson and Rosengren (1964) denervation studies show that most of the dopa decarboxylase present in spleen is contained in its sympathetic nerves.

Several groups have studied the location of dopa decarboxylase within the cell. Dahlström and Jonason (1968) noted that in sympathetic nerves it is not located within the amine storage granules. DeRobertis (1964) has studied its subcellular distribution in rat brain. About half of the enzyme is soluble; the rest bound to particles. The particulate enzyme can be solubilized by osmotic shock. He concluded that the enzyme is loosely bound or occluded within the membranes of nerve endings. Ichiyama, Nakamura, Nishizuka and Hayaishi (1968) found similar subcellular distri-

bution of the enzyme in guinea pig brain stem.

d) Tumours

Langemann (1958) first reported that a tumour, namely argentaffinoma, contained dopa decarboxylase activity. Kizer and Chan (1961) investigated the relation between enzyme and tumour properties. They found the enzyme absent in five out of six transplanted hepatomas. In chemically-induced primary tumours, activity was absent in the tumour tissue, but present in the surrounding healthy tissue. They concluded that deletion of the enzymes from tumours was random. Hagen (1962) studied the high dopa decarboxylase activity present in both phaeochromocytomas and argentaffinomas.

e) Erythrocytes

There is one report of the enzyme being present in erythrocytes. Charezinski and Berbec (1968) found it present in duck erythrocytes with an activity of 34-122 μmoles 5HT/g protein/h.

f) Invertebrates

Dopa decarboxylase is found in various invertebrate orders including Gastropoda and Arthropoda. Welsh and Moorhead (1959) have measured the activity of the enzyme of the nervous tissues of the snails (Busycon (0.03-0.22 μmoles 5HT/g/h) and Venus (0.11-0.55 μmoles 5HT/g/h). Cardot (1964) has reported its presence in the nervous (0.4 μmoles DA/g/h) and cardiac tissues (0.3 μmoles DA/g/h) of the snail, Helix pomatia.

Several reports of its presence in arthropods have appeared, including that of Westerman (1957) for mealworms; Belzecka, Laskowska and Mochnacka (1962) for caterpillars of the moth Celerio euphorbiae (20 μmoles DA/g/h); Sekeris (1962) for the blowfly Calliphora erythrocephala (214 μmoles DA/g protein/h); and Colhoun (1963) for cockroach brain (1.7 μmoles 5HT/g/h). Whitehead (1970) has reported its presence in fly haemocytes. In the insect, according to Karlson, Sekeris and Sekeri (1962) the DA formed is N-acetylated and then serves as a substrate for the polyphenyloxidases that are important in tanning the insect cuticle. The enzyme is also present in the decapod crustacean, Upogebia littoralis (Marmaras and Fragoulis, 1970); activity is high in the eyestalks (16.9 μmoles 5HT/g protein/h with lower activities in the intestine (4.4), hepatopancreas (4.4) and eggs (1.1).

2. Substrate Specificity

Studies on the specificity of dopa decarboxylase have shifted in direction as knowledge of amino acid decarboxylations has accumulated. Early work was concentrated on the metabolic pathway of adrenaline biosynthesis. Some substrate specificity studies eliminated possible alternative routes. A second type of study has dealt with synthetic aromatic amino acids that do not occur naturally, to try to pinpoint the structural requirements for a compound to serve as substrate. A third type of study has tried to show the identity of dopa and 5HTP decarboxylases. Some attempt has also

been made to extend the list of natural substrates to such compounds as histidine.

Very soon after the discovery of dopa decarboxylase, Blaschko (1939) investigated the substrate specificity of the guinea pig enzyme and eliminated tyrosine, N-methyltyrosine and histidine as substrates. In the same year, Holtz, Credner and Walter concluded that three different enzymes were responsible for tyrosine, histidine and dopa decarboxylation. This was based on differences in activity found using different species, by competition experiments, and by removing tyrosine decarboxylase from a mixture by adsorption on kaolin.

In 1950 Blaschko, Burn and Langemann and Beyer, Blaschko, Burn and Langemann noted the formation of noradrenaline from 3,4-dihydroxyphenylserine (DOPS), by-passing DA, by both guinea pig kidney and brain preparations. The N-methyl compound was not decarboxylated. Various hydroxyphenylserines were studied by Werle and Sell (1954). They found that m-erythro-hydroxyphenylserine was decarboxylated by the kidneys of cattle, pig, guinea pig and dog, but the meta-threo, para-threo and para-erythro isomers were not. Both erythro- and threo- DOPS were decarboxylated by guinea pig, pig and beef kidney homogenates as well as beef liver and guinea pig brain. Hartman, Pogrund, Drell and Clark (1955) carried out an extensive study of the hydroxyphenylserines. Erythro-DOPS was decarboxylated by the hog kidney enzyme, but less actively than DOPA (approximately one-third as actively). However, threo-DOPS was decarboxylated only slowly (11% the rate of dopa) by kidney. The threo-isomer when

decarboxylated produces the natural isomer of noradrenaline (-) as was shown by its pressor activity in the cat. Rat liver was equally active on the erythro and threo isomers, but only 25% as active as dopa. Werle and Aures (1959) found that for their preparation of guinea pig kidney the activities towards DOPS remained 30% that of DOPA throughout the purification. Interest in DOPS has lagged since it has never been found in mammalian tissues and since neither Kirshner (1957) nor Pellerin and D'Iorio (1957) could detect its formation when ^{14}C-DOPA was incubated with bovine adrenal homogenates. However, DOPS can be a valuable tool in investigations which wish to pinpoint the effect of DA as distinct from noradrenaline. Blaschko and Chrusciel (1960) gave a hint of what sort of experiments are possible by demonstrating that DOPA will overcome reserpine-induced sedation in mice, whereas threo-DOPS will not.

Another set of investigations of substrate specificity has been that centred around ortho- and meta-tyrosine and other "unnatural"* amino acids. Blaschko and Sloane-Stanley (1948) reported that guinea pig kidney preparations were active on meta-tyrosine. Later Blaschko (1949) reported that rabbit liver and guinea pig extracts from carbon dioxide from ortho-tyrosine. He suggested that dopa decarboxylase was active toward DOPA and ortho- and meta-tyrosine, whereas a different enzyme (bacterial) was active towards para-tyrosine as well as DOPA, but not ortho-tyrosine. Sourkes (1955) reviewed the properties of a series of

*Fellman and Devlin (1958), and Fellman (1958) reported the occurrence and production of ortho-tyrosine in beef adrenals. Coulson, Henson and Jepson (1968) reported production of small amounts of meta-tyrosine from phenylalanine by rat liver homogenate.

aromatic amino acid decarboxylases including bacterial tyrosine decarboxylase and mammalian dopa decarboxylase. He summarized the activities of hog kidney and guinea pig kidney enzymes on various isomers of DOPA. With 3,4-DOPA as 100% activity, 2,3-DOPA gave 165%, 2,4-DOPA gave 42%, 2,5-DOPA 112%, 2,6-DOPA 53%, and 3,5-DOPA 23%. The hog kidney enzyme paralleled the activity of guinea pig, but was slightly more active (71% on 2,4-DOPA and less active (88%)) on 2,5-DOPA. More recently Bower and Lambooy (1969) have investigated other derivatives of hydroxyphenylalanines using guinea pig kidney enzyme. With 3,4-DOPA assigned 100% activity, ortho-tyrosine had 99%, 3-methyl-ortho-tyrosine 95%, 4-methyl-o-tyrosine 83%, and 6-methyl-o-tyrosine 31%. 5-Methyl-o-tyrosine was not a substrate. With a series of DOPAs, 2,4-DOPA was decarboxylated at 84% of the rate of 3,4-DOPA, 3-methyl-2,4-DOPA 44% and 6-methyl-2,4-DOPA 5%. Again the 5-methyl compounds, namely 5-methyl-2,4-DOPA was not a substrate. The authors speculate that the 5-methyl position prevents binding of the substrate to the enzyme since 5-methyl-o-tyrosine and 5-methyl-2,4-DOPA are neither substrates nor inhibitors of guinea pig kidney dopa decarboxylase. Both Blaschko (1950) and Sourkes see the meta-hydroxyl important in binding of substrate to enzyme. Sourkes (1955) showed that 3,5-DOPA has relatively low activity (there is a possibility of the two meta groups competing for a site). 5-Methyl corresponds to meta-methyl. It is possible that binding does not occur since the substitution is of a polar group (hydroxyl) by a nonpolar one (methyl). Ong, Creveling and Daly (1969) reported that the guinea pig enzyme is active on 6-hydroxydopa (2,4,5-trihydroxyphenylalanine), a noradrenaline-depleting agent, about as

efficiently as dopa. The use of a decarboxylase inhibitor showed that the amine, not the amino acid itself, is responsible for the depleting action.

One other group of unnatural amino acids is also decarboxylated by dopa decarboxylase, namely the α-methyl analogues. Carlsson and Lindquist (1962) reported on the in vivo decarboxylation of α-methyldopa and α-methyl-meta-tyrosine.

With the discovery by Clark, Weissbach and Udenfriend (1954) of an enzyme in guinea pig kidney capable of decarboxylating 5HTP, the question arose as to its possible relationship to dopa decarboxylase. The authors noted that tryptophan and 7-hydroxytryptophan were not substrates. Their partially purified preparation contained activity towards DOPA, but they thought at the time that two enzymes were involved since the pH optima were different and because DOPA decarboxylation required more pyridoxal phosphate (PLP) for activation whereas 5HTP decarboxylation showed little PLP activation. They also noted a difference in regard to inhibition with hydroxylamine and semicarbazide; using DOPA inhibition could be overcome by adding excess PLP, but not with 5HTP. Westermann, Balzer and Knell (1958) noted that in guinea pig and rabbit tissues activities towards 5HTP and DOPA were always found together and that both were inhibited by α-methyldopa. They concluded that one enzyme was involved. Yuwiler, Geller and Eiduson (1959) studied activity of the enzyme towards 5HTP, DOPA, erythro-DOPS and threo-DOPS by hog kidney homogenate. Mixtures of 5HTP and DOPA were decarboxylated at rates intermediate to those with the

single substrates. The same group (1960) extended the studies to inhibitors and suggested that one enzymic site was responsible for DOPA and 5HTP decarboxylation.

Fellman (1959) found that his purified preparation from bovine adrenal medulla retained its activity towards DOPA, ortho-tyrosine and 5HTP intact throughout purification. He also noted a mutual inhibition between substrates using the purified material. He did not commit himself as to whether there was one or two enzymes, but instead concluded that either adrenal dopa decarboxylase can sluggishly attack 5HTP or his preparation was contaminated by 5HTP decarboxylase. Rosengren (1960) posed the question as to whether DOPA and 5HTP were acted on by the same or different enzymes. He noted that rabbit kidney enzyme showed mutual competitive inhibition between DOPA and 5HTP. Decarboxylation of the latter two compounds was competitively inhibited by ortho- and meta-tyrosine and caffeic acid. When the enzyme preparation was passed through a DEAE-cellulose column, one peak with activity towards both 5HTP and DOPA was found. More interesting were the inhibition studies: ortho-tyrosine had a K_i of 7×10^{-4}M for both DOPA and 5HTP as substrates. Similarly meta-tyrosine had a K_i of 4×10^{-4}M for both DOPA and 5HTP as substrates. From this sort of data Rosengren concluded that one enzyme only was involved. Davis and Awapara (1960) investigated the activity of a variety of tissues of rats, guinea pigs and rabbits towards four substrates, namely DOPA, 5HTP and ortho- and meta-tyrosine. Ortho-tyrosine was the most active substrate followed by meta-tyrosine, DOPA and 5HTP respectively.

Awapara, Sandman and Hanly (1962) reported that their purified rat liver enzyme was active on ortho-tyrosine (160%), DOPA (100%), meta-tyrosine (86%) and 5HTP (22%).

Lovenberg, Weissbach and Udenfriend (1962) claimed that one enzyme in guinea pig kidney was responsible for the decarboxylation of DOPA and 5HTP. Further they extended the range of substrates to tryptophan, para-tyrosine, phenylalanine and histidine. This was based on a 50-100 fold purified preparation from guinea pig kidney. The authors renamed the enzyme "aromatic L-amino acid decarboxylase." This was because their purification did not dissociate activities, the lack of specificity even for unnatural amino acids, competitive substrate inhibition and because all activities were inhibited by α-methyldopa.

Hagen (1962) concluded that only one enzyme was involved from his study on dopa decarboxylase from ox adrenal medulla, human phaeochromocytoma and human argentaffinoma. He partially purified the enzyme from these sources using ammonium sulfate and found that DOPA was most active as substrate, followed respectively by meta-tyrosine, ortho-tyrosine and 5HTP. It is interesting to note that the relative activities with each substrate varied with the source of enzyme. Thus in ox adrenal medulla, if DOPA was assigned the activity of 100%, meta-tyrosine was 84%, ortho-tyrosine was 47% and 5HTP, 24%, whereas in human argentaffinoma the figures were 100, 33, 21 and 12, respectively. Hagen also found that the various amino acids acted as mutual competitive inhibitors. Using radio-active substrates he was unable to detect any decarboxylation of

para-tyrosine, tryptophan or histidine although he points out that he did not use kidney as an enzyme source.

The identity of 5HTP and DOPA decarboxylases appears to have been accepted in the literature although the wider claims for the enzyme as an aromatic L-amino acid decarboxylase have come under attack from a number of authors. Awapara, Perry, Hanly and Peck (1964) using radioactive carbon dioxide trapping, were unable to detect any activity of rat liver dopa decarboxylase for histidine, para-tyrosine or tryptophan. Urinary p-tyrosine was claimed to come from the gut flora. Coulson, Henson and Jepson (1968) have confirmed that rat liver enzyme is not active on phenylalanine or para-tyrosine.

Cardot's (1964) snail enzyme was active on both 5HTP and DOPA and inhibited by α-methyldopa for both substrates. Each amino acid acted as an inhibitor of the other. Tryptophan was also decarboxylated by this enzyme.

Reid and Shepherd (1964) found parallel reductions of decarboxylase activity to DOPA, 5HTP and histidine in tryptophan-deficient rats, and suggested a single, non-specific enzyme.

In 1967 Streffer, using mouse liver enzyme, and measuring each product independently of the other in competition experiments found evidence of one site being involved for both 5HTP and DOPA decarboxylation.

Sourkes (1966) reviewed the literature and concluded that DOPA, meta- and ortho-tyrosine, 5HTP and erythro-DOPS are substrates. In the same year Gonnard and Camier reopened the subject of substrate

specificity using inhibitor studies and concluded that dopa decarboxylase was active only upon DOPA and 5HTP among the natural amino acids and not on tyrosine, tryptophan and histidine.

On the other hand, DeQuattro and Sjoerdsma (1967) using antibiotics, monoamine oxidase inhibitors and glucose diet concluded that urinary tryptamine and tyramine are of tissue rather than bacterial origin, with the implication that they come from either dopa decarboxylase or some other enzyme.

3. Cofactor Requirement

Pyridoxal 5'-phosphate (PLP, codecarboxylase, 2-methyl-3-hydroxy-4-formyl-5-pyridylmethylphosphoric acid) was discovered by Gale and Epps (1944) and isolated from yeast. It was chemically synthesized by Umbreit, Bellamy and Gunsalus (1945).

Attempts to study its relationship to dopa decarboxylase have concentrated on resolution of the enzyme, purification of the enzyme, inhibition of the carbonyl group of the cofactor and the production of pyridoxine deficiency to influence tissue concentrations.

In 1945 Green, LeLoir and Nocito showed indirectly that pyridoxal phosphate was a cofactor for hog kidney dopa decarboxylase. They fractionated the enzyme with ammonium sulfate. The precipitate containing the enzyme was tnen resolved (the PLP was removed from the apoenzyme) by dialysis against 0.002M ammonia at 0° C for four days. Some activity could then be restored upon the addition of PLP and cysteine. Weissbach, Bogdanski, Redfield and Udenfriend (1957) could resolve crude rat kidney dopa decarboxylase by dialysis

after freezing its solutions, but this method did not serve for the guinea pig enzyme. Werle and Aures (1959, 1960) resolved their purified guinea pig enzyme by dialysis against EDTA. Awapara, Sandman and Hanly (1962) found that their purified rat liver enzyme was pale yellow and possessed an absorbance spectrum typical of pyridoxal phosphate enzymes. This preparation was still stimulated by additional PLP depending upon the substrate and upon the pH. Hakanson (1967) has suggested that, owing to the mechanism of the reaction with PLP, the pH optimum for dopa decarboxylase would shift with substrate concentration and the K_m would be pH-dependent.

Nutritional studies have provided some idea about the state of the enzyme in vivo. Blaschko (1950) noted that pyridoxine deficiency reduces the concentration of the liver enzyme. Dietrich and Shapiro (1953) showed that activity of dopa decarboxylase can be lowered in mouse liver both by pyridoxine-deficiency and by treatment with the antivitamin, desoxypyridoxine. The reduction is not particularly dramatic, however: the antivitamin treatment brought dopa decarboxylase to 82% of control, and aspartate-glutamate transaminase to 50% of control. Deficiency brought dopa decarboxylase to 84% of control and transaminase to 62%. From this Dietrich and Shapiro concluded that the PLP is very tightly bound by the decarboxylase. Buxton and Sinclair (1956) measured dopa decarboxylase in kidney of rats fed desoxypyridoxine plus a deficient diet and got a 25% decrease in activity over control in 8-10 weeks of treatment. Weissbach, Bogdanski, Redfield and Udenfriend (1957) used B_6-deficient chickens and found that the 5HT values were lowered, suggesting that PLP was

the cofactor. Buzard and Nytch (1957) studied the effects of pyridoxine-deficiency upon 5HTP decarboxylation by rat kidney. They considered that the ratio of the activity without additional PLP to the activity with PLP gave an index of the saturation of the apoenzyme with PLP, which they expressed as a percentage. Using this measure control rats showed values of 66-79% apoenzyme saturation, whereas deficient rats were in the range of 30-47%. Apoenzyme was also decreased by deficiency although not as dramatically; for example, it took two weeks on the deficient diet to halve the coenzyme content, but four weeks to halve the apoenzyme. Pogrund, Drell and Clark (1961) used indirect (the pressor effect of injected DOPA) and direct in vitro measurement of decarboxylase in liver and kidneys to show reduced decarboxylase, both due to cofactor and to apoenzyme in deficient rats. Eberle and Eiduson (1968) carried out an exhaustive study of the effect of pyridoxine deficiency on developing rat liver and brain. They measured per cent saturation of apoenzyme (cf. Buzard and Nytch). They found that they could reduce the saturation of the apoenzyme in both liver and various parts of the brain by pyridoxine deficiency. For example, in cerebellum controls increased in saturation from 9 to 65% with age, but the deficient rats remained in the range of 33-26% saturation. The authors noted that the foetus is protected from deficiency, but becomes deficient from post-partum suckling on a deficient mother.

The other main approach to the study of cofactor requirement has been the use of carbonyl reagents to bind the pyridoxal phosphate. Schales and Schales (1949) reported that hydroxylamine inhibited

guinea pig kidney preparations; inhibition was zero order. Clark, Weissbach and Udenfriend (1954) reported irreversible inhibition with hydroxylamine and semicarbazide by preparations of guinea pig kidney for decarboxylation of 5HTP. This did not occur with DOPA decarboxylation. Buzard and Nytch (1957) were able to overcome hydroxylamine inhibition of 5HTP decarboxylation in rat kidney by adding excess PLP. Schott and Clark (1952) made an extensive study of the reactions of PLP with various amino acids and amines. They postulated that DOPA reacts with PLP to form a tetrahydroisoquinoline condensation product, and prepared an analogous derivative by reacting pyridoxal phosphate with m-hydroxypropadrine 2-amino-1-(3'-hydroxy-phenyl-1-propanol) . The formation of this complex represents substrate inhibition of the enzyme by substrate. Para-tyrosine does not react appreciably with PLP. Sourkes (1954) using pig kidney cortex as enzyme source also studied the nonenzymic sequestering of PLP. Both 3,4-DOPA and DA form complexes to a considerable extent, but both α-methyldopa and α-methyl-m-tyrosine react much more slowly. Noradrenaline is also a noncompetitive inhibitor according to Buzard and Nytch (1959) which acts by binding the PLP. Tris and glycyl-glycine buffers form Schiff bases which tie up PLP according to these authors. Westermann, Balzer and Knell (1958) noted that DOPA and DA both inactivate PLP much faster than 5HTP and 5HT do.

Gonnard, Duhault, Camier, Nguyen-Philippon and Boigne (1964) reported that phospho-5'-pyridoximinotriazole is more active than PLP in stimulating dopa decarboxylase. Gonnard and Camier (1966) noted that various PLP hydrazones and semicarbazones would also

stimulate decarboxylation under conditions in which hydrolysis back to free PLP would not occur. This has led to some speculation that Schiff base formation is not involved in the mechanism of decarboxylation.

Vogel (1969) has reported that DOPA is decarboxylated non-enzymatically in buffer at pH 7.5 to the extent of 6% per hour. PLP decreased the decarboxylation, however.

Beiler and Martin (1954) reported inhibition of guinea pig kidney enzyme with rather high (10^{-2}M) concentrations of chelators such as EDTA, diphenylthiocarbazone and 3,5-dimethylpyrazole. They claimed a metal cofactor, either Zn^{++}, Mn^{++} or Mg^{++} was involved. No other workers have confirmed this. It is of interest, however, that the insect enzyme of Sekeris (1963) appears to require ferrous ion as well as PLP as a cofactor.

4. Inhibitors

Inhibition of dopa decarboxylase has been studied extensively since the discovery of the enzyme. Inhibitors can be classed into two main categories -- those which are aldehyde, i.e. pyridoxal, trapping agents, and those whose main action is upon the apoenzyme. The first category has already been dealt with in a previous section.

The whole field of dopa decarboxylase inhibitors has been extensively reviewed, notably by Clark and Pogrund (1961), Clark (1963), Sourkes (1966) and Sourkes and Rodriguez (1967). As these reviews are comprehensive and up-to-date they need not be duplicated here. However, it is noteworthy that the commonly used

inhibitors may have quite disparate effects in vivo. Bartholini and Pletscher (1969) have summed up the differences between four of the most popular, namely, N-(DL-seryl)-N'-(2,3,4-trihydroxybenzyl)-hydrazine known as Ro 4-4602, m-hydroxybenzylhydrazine (NSD 1015), β-(3,4-dihydroxyphenyl)-α-hydrazino-α-methylpropionic acid (MK 485) and α-methyldopa. NSD 1015 causes an inhibition of extracerebral dopa decarboxylase but, because it penetrates the brain, it inhibits the brain enzyme even in low doses. Ro 4-4602 readily inhibits the extracerebral dopa decarboxylase, but inhibits cerebral enzyme only in the large doses needed to get some of it across the blood-brain barrier. MK 485 inhibits extracerebrally, but even in high doses it does not appear to enter the brain. α-Methyldopa on the other hand has only a slight effect on the periphery and no effect on the cerebral enzyme.

5. Purification

Attempts at purification were made as early as 1945 by Green, LeLoir and Nocito. They used two fractionations with ammonium sulfate of their hog-kidney supernatant. Schales and Schales (1949) prepared a stable powder containing the enzyme from guinea pig and rabbit kidneys by drying in vacuo. The rabbit kidney extract had 75% of the activity of fresh homogenate; that of guinea pigs ranged from 45-60%.

Clark, Weissbach and Udenfriend (1954) went a little further in purification of guinea pig enzyme -- they prepared two "cuts" with ammonium sulfate, and then adsorbed the enzyme on alumina Cγ.

This gave a 37% yield of enzyme, with a specific activity of 1.3 μmole 5HT/mg protein/h. This purified material was extremely unstable, losing half of its activity overnight even if stored frozen.

Werle and Aures (1959, 1960) published a further purification of guinea pig kidney enzyme. It consisted of heat-shock at 50° for 5 min at pH 5.7 adsorption on kieselguhr, ammonium sulfate precipitation, followed by column chromatography on aluminum oxide. The most active fraction had a specific activity of 18.9 μmoles DA/mg protein/h; if one considers 5HTP to be about 20% as active as substrate, this would give 3.8 μmoles 5HT/mg protein/h. The authors considered this material to be 82-fold purified. After the final chromatography the enzyme was very unstable.

Lovenberg, Weissbach and Udenfriend (1962) also purified enzyme from guinea pig kidney. They followed the procedure of Clark et al. (1954), but then included a step of chromatography on DEAE-cellulose. This gave purified material with a specific activity of 253 μmoles DA/mg protein/h or 34.3 μmoles 5HT/mg protein/h. They claimed this material to be 56-fold purified. It is extremely difficult to compare their work to other workers since they measured enzymic activity at a very high pH (9.0). Further, their values for the crude and for the partially purified enzymes are very far out of line with those of Clark et al. Their material was relatively stable, losing 40% of its activity in 10 days when stored at 3° .

Fellman (1959) purified beef adrenal dopa decarboxylase. His method entails ammonium sulfate fractionation in small additive

steps followed by calcium phosphate adsorption of impurities. The remaining impurities are then precipitated at pH 4.9. The final purified material had a specific activity of 7.1 μmoles DA/mg protein/h or 0.5 μmoles 5HT/mg protein/h. This material yielded a single peak on paper electrophoresis and free boundary electrophoresis, as well as in the ultracentrifuge. The enzyme was quite unstable, but could be stored for a few days at 4° with little loss of activity. The molecular weight, calculated from its sedimentation coefficient, appeared to be 156,000.

A third source of purified dopa decarboxylase was that prepared from rat liver by Awapara, Sandman and Hanly (1962). It was purified by heating at 55° for 3 min, ammonium sulfate fractionation, and DEAE-Sephadex A50 column chromatography. Mercaptoethanol was present throughout the procedure. The most active material had a specific activity of 2.6 μmoles 5HT/mg protein/h or 11.9 μmoles DA/mg protein/h. Recently Coulson, Bender and Jepson (1969) have claimed the presence of four bands upon disc gel electrophoresis of rat liver dopa decarboxylase.

Sekeris (1963) has purified insect dopa decarboxylase from white prepupae of Calliphora erythrocephala (a type of blow fly). The method entails ammonium sulfate fractionation, and two fractionations on DEAE-cellulose. The final, very unstable material has a specific activity of 75 μmoles DA/mg protein/h or 22.9 μmoles 5HT/mg protein/h.

While this thesis was being prepared, a publication by Christenson, Dairman and Udenfriend (1970) has appeared which

deals with essentially the same material as the thesis, i.e. the purification of the hog kidney enzyme. The method entails in essence, fractionation with ammonium sulfate, heat treatment, alumina C_γ treatment, polyethylene glycol-6000 precipitation, DEAE-cellulose batchwise treatment, followed by chromatography on DEAE-Sephadex and hydroxylapatite. The purified material has a specific activity of 520 μmoles DA/mg/h, with a recovery of 5.9%. The assay of enzymic activity was done at pH 7.0. Mercaptoethanol was present throughout the purification procedure. This material was 332-fold purified. The purified enzyme has a half-life of 6 weeks stored at 0°. Purity as shown by disc gel electrophoresis was 97-100%. Immunological techniques were also employed to show purity.

6. Methods of Enzyme Assay

A recent thorough review of methods by Sourkes and Lancaster (in press) is available.

II. METHODS

1. Enzyme Assays*

a) 5-Hydroxytryptophan

1) Dietrich's method

The incubation mixture consisted of the following: 0.0015 M 5HTP, 0.001 M iproniazid (for crude material only), 7×10^{-5}M PLP, varying amounts of enzyme and 0.2 M phosphate buffer, pH 6.8, in a final volume of 3 ml. After a suitable incubation time, varying from 10 min to 1 hr, an aliquot was removed, boiled, diluted with glass-distilled water to 10 ml, and centrifuged if necessary to remove coagulated protein. A blank (boiled at zero time) was similarly prepared. The sample and blank were passed through activated Permutit columns as described by Dietrich (1953). The columns were washed with hot water, and the 5HT was eluted with 20% NaCl. The fluorescence was then determined at 350 nm (activation at 295 nm) and the 5HT determined from a standard curve prepared in 20% NaCl. This assay was used for all routine procedures such as monitoring purification steps.

2) Solvent extraction method

This was a modification of Kuntzmann's method (1961) for determining serotonin by butanol extraction, using 5HTP-^{14}C-3 as substrate. This method was developed to allow competitive inhibition studies by fluorescent substrates on purified enzyme. In essence, different concentrations of 5HTP, PLP, inhibitor and 0.2 M phosphate buffer, pH 6.8, in a final volume of 2 ml were incubated an appropriate

*Time kinetics were determined for each method.

length of time (10 to 60 min) at 37°. Appropriate blanks were used for each condition. The reaction was stopped by boiling. Two grams of NaCl were then added, as well as 5 ml of 0.5 M borate buffer saturated in NaCl, pH 10, and 10 ml of 1-butanol. The mixture was extracted for 1 min and centrifuged to destroy the emulsion. Two ml of the butanol extract were mixed with 15 ml of scintillation cocktail (toluene: ethylene glycol monomethyl ether 2:1 with 5.5 g/l of PPO and 100 mg/l of dimethyl POPOP) and counted. The blank values were then subtracted.

3) Amberlite CG50

The resin was washed according to Sharman's instructions (personal communication) as follows: 10 g of resin was washed with 200 ml of 2N HCl and the fine particles discarded. The resin was next washed 3 times with water. The resin was then washed with 200 ml of 2N NaOH and again the fine particles were discarded. This was followed by three more washes with water. The NaOH-water washes were repeated 3 times in succession. Finally, a wash with 2N HCl was followed by 5 washes of water. The resin was stored in water until use. For 5HT determinations, columns were packed with 4.5 cm x 0.5 cm of resin. The sample to be determined was then applied at neutral pH. Six ml of water were then passed through the column to remove non-adsorbed compounds. Finally the 5HT was eluted with 3 ml of 1.2N HCl and determined fluorometrically at 530 nm (activation at 290 nm). Absolute amounts of 5HT were determined by treating standards in a similar manner.

b) DOPA

1) Method of Laduron and Belpaire (1968)

This method was used when DOPA was substrate, except for crude preparations of enzyme. For crude preparations of kidney, artifacts are produced owing to the conversion of some of the D-isomer of the radioactive compound to the L-isomer which gives misleadingly high values. The method used the following incubation mixture: 0.6 μmoles L-DOPA, in phosphate buffer, pH 6.8, 0.1 ml DL-^{14}C-2 DOPA (0.2 μc/ml) and enzyme to make a total volume of 1 ml. Other reagents such as PLP or inhibitors were added depending upon the experiment. The mixture was then incubated at 37^{o} for a convenient period (10-60 min), boiled, 1 g of NaCl was added along with 10 ml of 1-butanol which had previously been washed with phosphate buffer, pH 6.8 to remove acid. After 1 min of extraction and centrifugation to break the emulsion, 1-2 ml aliquots of the organic phase were mixed with 15 ml of scintillation cocktail described earlier and counted. The blanks were subtracted and the counts per min converted into μmoles of DA produced -- based on only L-DOPA being utilized and a 50% extraction.

2) Amberlite CG50

This procedure was used in a similar manner to that described in detail for 5HT measurement with the differences that elution from the column was in 2 N HCl and that the fluorescence was read at 330 nm (excitation at 285 nm).

c) DL-ortho-Tyrosine and DL-meta-tyrosine

These two compounds were assayed as in the Dietrich method for 5HTP, but the concentration of substrate used was 10^{-3}M. After elution from Permutit the corresponding amines were determined using Folin reagent as follows: the amine or blank in a volume of 4.2 ml was mixed with 1.8 ml of 0.67 N Folin-Ciocalteu reagent and 6 ml of 10% Na_2CO_3. They were then left to incubate at 37° for 15 min and centrifuged, if necessary, to remove turbidity. The absorbance at 500 nm was determined. A standard curve based on the parent amino acid (since no standard amines were available) converted optical density to μmoles of amine.

d) DL-erythro-DOPS and DL-threo-DOPS

These two compounds were assayed using the Amberlite CG50 method outlined for DOPA. Fluorescence was read at 325 nm (excitation at 285 nm). Standard noradrenaline treated in the same manner was used to quantitate the results.

e) L-para-Tyrosine

This compound was passed through Permutit as described for ortho- and meta-tyrosine, but was determined fluorimetrically instead of colorimetrically. Acidified samples were read at 310 nm (excitation at 275 nm). p-Tyramine was used as standard.

2. Determination of Pyridoxal Phosphate

This was carried out by the method of Adams (1969) in which the fluorescent cyanide derivative of PLP is prepared. A sample

of 0.2 ml of enzyme was mixed with 0.2 ml of 11% TCA and incubated at 50° for 15 min. After this time, 0.14 ml of 3.3 M K_2HPO_4 and 0.05 ml of 0.02 M NaCN were added and the sample was incubated at 50° for a further 25 min. The pH was then lowered by adding 0.07 ml of 28% H_3PO_4 and 1.0 ml of 2 M acetate buffer, pH 3.8. The sample and a similarly treated blank were read at 420 nm (activation at 325 nm) in the Aminco-Bowman fluorimeter.

3. Preparation of DEAE-Sephadex A50

Fresh ion-exchange resin was equilibrated in 0.05 M Tris-HCl, pH 7.2, containing 0.1 M NaCl and 10^{-4}M PLP. To regenerate, the ion-exchanger was washed with 2 M NaCl to strip off most of the residual material, washed with glass-distilled water and then re-equilibrated with Tris-HCl, NaCl, PLP buffer.

4. Preparation of Hydroxylapatite

Biogel HTP was suspended in 0.002 M phosphate buffer, pH 6.8, left several hours to equilibrate, and then used without further preparation.

5. Acrylamide Disc-Gel Electrophoresis

a) pH 8.9

A liter of "running buffer", pH 8.9, was made by neutralizing 46 g of Tris with 4 ml of concentrated HCl and adjusting the pH to 8.9. "Spacer buffer" was made by adding 7.5 g of Tris to 4 ml of concentrated HCl in a liter final volume and adjusted the pH. to 6.7. "Electrode buffer", pH 8.3 was prepared by mixing 1.2 g

of Tris to 5.8 g of glycine to make a final volume of 2 liters. "Running gel solution" was prepared by mixing Cyanogum-41 (1.4 g) with running buffer (20 ml), TMED (0.02 ml) and ammonium persulfate catalyst (20 mg). These proportions give a 7% gel, which is the one routinely used. On various other occasions 5% (1.0 g Cyanogum) and 10% (2 g Cyanogum) gels were prepared. The "spacer gel solution" (4.0%) was prepared by dissolving 0.4 g of Cyanogum in 10 ml of spacer buffer, adding 0.01 ml of TMED and 10 mg of ammonium persulfate just before using.

Gels were prepared in the upper chamber of the Buchler Co. Polyanalyst (Cat. No. 3-1750). First, running gel solution was pipetted into the corked tubes to a height of 5 cm. After all tubes had been filled, a few drops of water were carefully layered over the top of the gel to insure a smooth surface. After polymerization had occurred (30 min) the water was pipetted off, and spacer gel solution to a height of 0.3 cm was added. A water layer was pipetted on in this case, and was removed just before using. The corks were removed and the bottom chamber of the apparatus was filled with running buffer. Samples were mixed with a few grains of sucrose to increase their density and then approximately 0.075 ml of sample was carefully pipetted on top of the spacer gel. Electrode buffer was layered on top of the samples and the upper chamber of the Polyanalyst filled. A drop of 0.05% bromophenol blue (aq) was then added to the electrode buffer in the upper chamber, to act as tracking dye. This was omitted in the cases where enzymic activity

was looked for. A current of 1.25 mA was applied until the discs entered the gel; the current was then increased to 2.5 mA for the rest of the run.

Gels were removed from the tubes by chilling them in icewater, and forcing water into them with a hypodermic needle. The gels were stained in 0.1% Buffalo Black NBR in 7% acetic acid for about an hour, and were then electrophoretically destained. To do this, the gels were placed in the destaining tubes, 7% acetic acid was placed in both the upper and lower chambers of the Polyanalyst and a current of 5 mA per tube applied until the background stain had migrated out of the gel.

b) pH 2.7

Upper buffer contained 28.1 g of glycine and 3.05 ml of acetic acid per liter to give a pH of 4.0. The lower buffer contained 43 ml of acetic acid and 120 ml of 1 N KOH per liter to give a final pH of 4.3. The running gel solution consisted of a mixture in the ratio of 1:2:1 of the following three stock solutions: acrylamide 30 g, and bis-acrylamide 0.8 g in 100 ml, 12 ml of 1 N KOH, 53.2 ml of acetic acid and 0.24 ml of TMED, all in 100 ml in the second stock solution; 120 mg of potassium persulfate and 2 mg of riboflavin per 100 ml were in the third. This gave a final gel concentration of 7.5%. The spacer gel solution, pH 6.7, consisted of 0.4 g of Cyanogum and 10 mg of ammonium persulfate dissolved in 10 ml of the following buffer: 3 ml of 1 N KOH, 0.185 ml acetic acid, 0.01 ml TMED made up to 50 ml. Fluorescent light was necessary

in order to promote polymerization of the running gel. The polymerization took several hours. The rest of the procedure was similar to that for pH 8.9 except that (a) the electrodes were reversed so that the proteins migrate towards the cathode, and (b) methyl green, instead of bromophenol blue, was used as a dye front indicator.

6. Continuous Gel Electrophoresis

This was carried out in a similar manner to the disc-gel procedure already outlined. Gel was made up in 0.05 M Tris-HCl buffer, pH 7.4, or 7.2 to 5, 7 or 10% gel concentrations. The sample was added to the top of the gel with bromophenol blue mixed with it, as well as the sucrose, and the run was made using the same buffer in both upper and lower chambers.

7. Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

This was carried out according to a method outlined by Weber and Osborn (1969). Protein samples were prepared by denaturing for 2 hr at 37° in 0.01 M potassium phosphate buffer, pH 7.0, 1% in sodium dodecyl sulfate and 1% in β-mercaptoethanol. The buffer contained 7.8 g $NaH_2PO_4.H_2O$, 38.6 g of $Na_2HPO_4.7H_2O$ and 2 g of sodium dodecyl sulfate per liter. A stock acrylamide solution contained 22.2 g of acrylamide and 0.6 g of methylenebisacrylamide in 100 ml of water. A 10% final concentration of acrylamide was achieved by mixing 15 ml of gel buffer, 13.5 ml of acrylamide stock, 1.5 ml of ammonium persulfate solution (15 mg/ml) and 0.045 ml of TMED. Sample application solution was made up as follows:

one part 0.01 M phosphate buffer, pH 7.0, containing 0.1% sodium dodecyl sulfate and 0.1% β-mercaptoethanol, one part glycerol, one-tenth part β-mercaptoethanol, and 0.06 parts 0.05% bromophenol blue. Samples were mixed half and half with this sample-application solution. The electrophoresis was performed with gel buffer diluted 1:1 with water. The same buffer was used in both chambers. The lower chamber contained the positive electrode. A current of 8 mA per gel was employed. After the run, the gels were removed from the tubes. The length before staining and the distance of dye migration were then measured before fixing and staining in 7% acetic acid Buffalo Black. After electrophoretic destaining the length of the gel and the distance of the protein migration were determined. Mobility (μ) was calculated as equal to:

$$\frac{\text{distance of protein migration}}{\text{length after destaining}} \times \frac{\text{length before destaining}}{\text{distance of dye migration}}$$

III. RESULTS

1. Purification of DOPA decarboxylase from Hog Kidney

a) Preparation of the homogenate

Frozen pig kidney cortex (250-300 g) was homogenized in two volumes of chilled 0.8% saline in a Waring blender. The homogenate was then centrifuged for 30 min at 35,000 g in a Sorval refrigerated centrifuge. The supernatant was considered the starting material.

b) Heat treatment

The starting material was adjusted to pH 6.8 with 0.2 M phosphate buffer. PLP was added to give a final concentration of 4×10^{-4}M. The homogenate was heated in a water bath to 50^{o}, held at that temperature for 5 min, and then chilled in ice. The supernatant obtained was saved after centrifugation for 15 min at 35,000 g.

c) Ammonium sulfate fractionation

Solid ammonium sulfate was added slowly to the supernatant with constant stirring to give a concentration of 18% w/v. After 30 min, the solution was centrifuged for 15 min at 35,000 g and the supernatant retained. To the supernatant 12 g of solid ammonium sulfate per 100 ml of solution was added with stirring. After 30 min the solution was centrifuged and the precipitate collected.

d) Desalting

The brownish precipitate was dissolved in a minimal amount of glass-distilled water. This was then desalted by passing through a column of Sephadex G25 (4 x 25 cm) equilibrated with glass-distilled water.

e) DEAE-Sephadex A50 Column Chromatography

The desalted preparation was introduced into a column of DEAE-Sephadex A50 (4 x 58 cm) equilibrated with 0.05 M Tris-HCl, pH 7.2, containing 0.1 M NaCl and 10^{-4}M PLP. After washing out the protein which did not adsorb to the column, the column was eluted with 0.05 M Tris-HCl, pH 7.2, containing 0.18 M NaCl and 10^{-4}M PLP. The enzyme-containing fractions were pooled and precipitated with 30% w/v ammonium sulfate. The precipitate was dissolved in water and desalted by passage through a column (1.5 x 30 cm) of Sephadex G25 equilibrated with 0.002 M phosphate buffer, pH 6.8. Figure 1 shows the elution profile for the DEAE-Sephadex column.

f) Hydroxylapatite Column Chromatography

The desalted material from the previous step was applied to a column (1.5 x 9 cm) of hydroxylapatite. The column was equilibrated with 0.002 M phosphate buffer, pH 6.8. The column was then washed with the equilibrating buffer. Elution was performed stepwise using 0.008 M, 0.020 M and 0.200 M phosphate buffers, pH 6.8, consecutively. The most active fractions, those eluted

at 0.02 M phosphate, were then pooled. Figure 2 shows a typical column profile.

g) Sephadex G150 column chromatography

The active material from the previous step was precipitated with 30% w/v ammonium sulfate, taken up in a minimal amount of water (less than 1 ml) and applied to a column of Sephadex G150 (1.5 x 90cm) equilibrated with 0.02 M phosphate buffer, pH 6.8. Fractions of 1.6 ml were collected, and those with highest enzyme activity were pooled as the purified enzyme. Figure 3 shows a typical elution profile.

Table I shows a typical protocol of the purification scheme.

2. Notes on Purification

a) Heat treatment

Several compounds were added to see if they would stabilize the enzyme during heat treatment. Some data obtained using the postmitochondrial supernatant fraction, which had been treated at pH 5, are shown in Table II. From these data it can be seen that PLP protects the enzyme against heat denaturation at 50°. Higher temperatures are not suitable, as can be seen by the data shown in Table III. In this case all samples contained 10^{-4}M PLP.

b) Ammonium sulfate precipitation

The conditions for this procedure were obtained from Green et al. (1945). An experiment was performed in which solid ammonium sulfate was added to make a concentration of 18% w/v, the solution

Figure 1

Chromatography was carried out in 0.05 M Tris HCl, pH 7.2 containing the amounts of NaCl indicated at the top of the figure and 10^{-5} M PLP. Dopa decarboxylase activity was present in the protein peak indicated by the arrow.

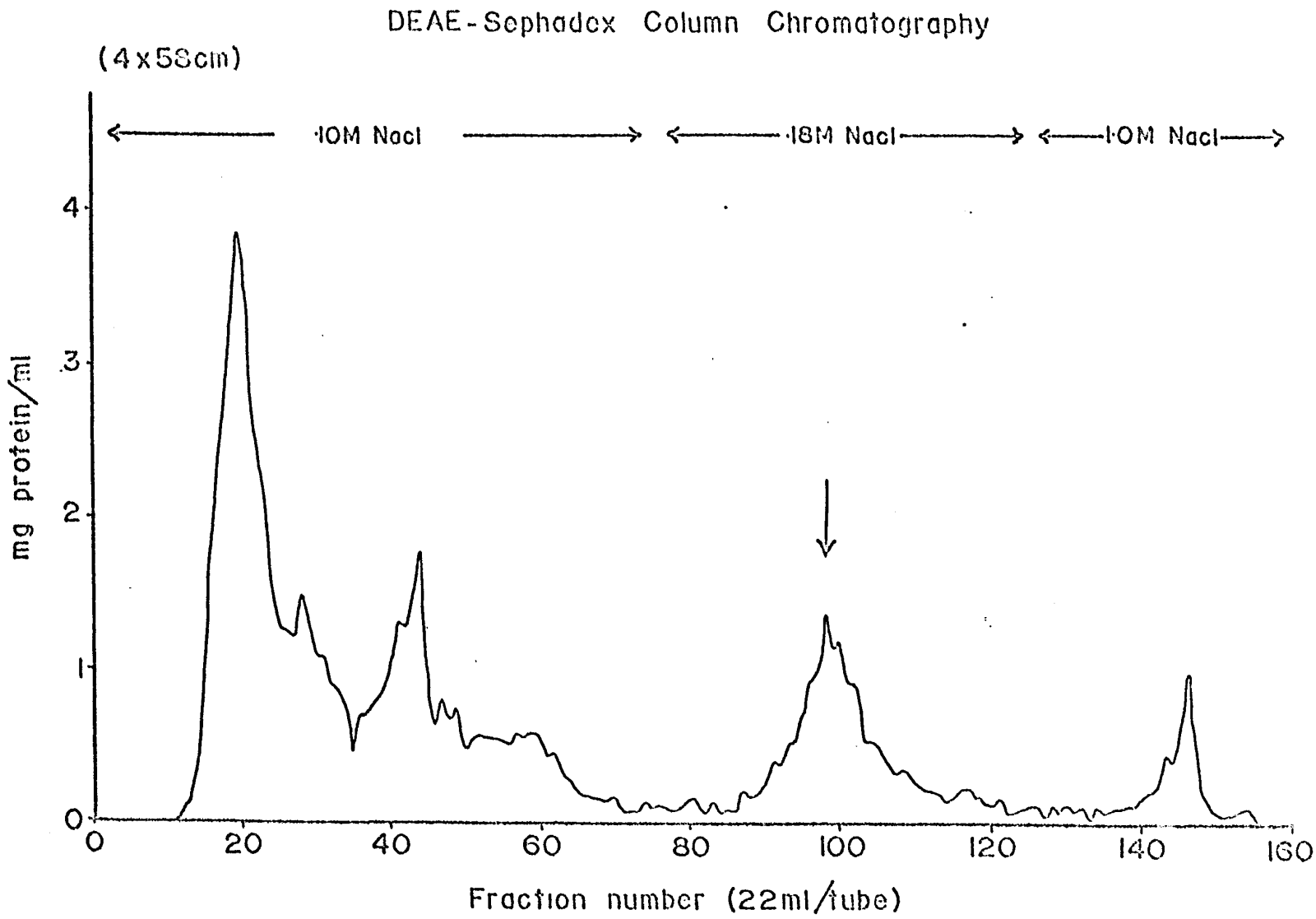

DEAE-Sephadex Column Chromatography
(4x58cm)
·1OM Nacl
·18M Nacl
·1·0M Nacl
mg protein/ml
0
1
2
3
4
0
20
40
60
80
100
120
140
160
Fraction number (22ml/tube)

Figure 2

Initially the column was equilibrated with 0.002 M phosphate buffer, pH 6.86. Elution was carried out step-wise using 0.008 M, 0.02 M and 0.200 M phosphate buffers, pH 6.86, respectively. Dopa decarboxylase activity was present in the protein peak indicated by the arrow.

Hydroxylapatite Column Chromatography

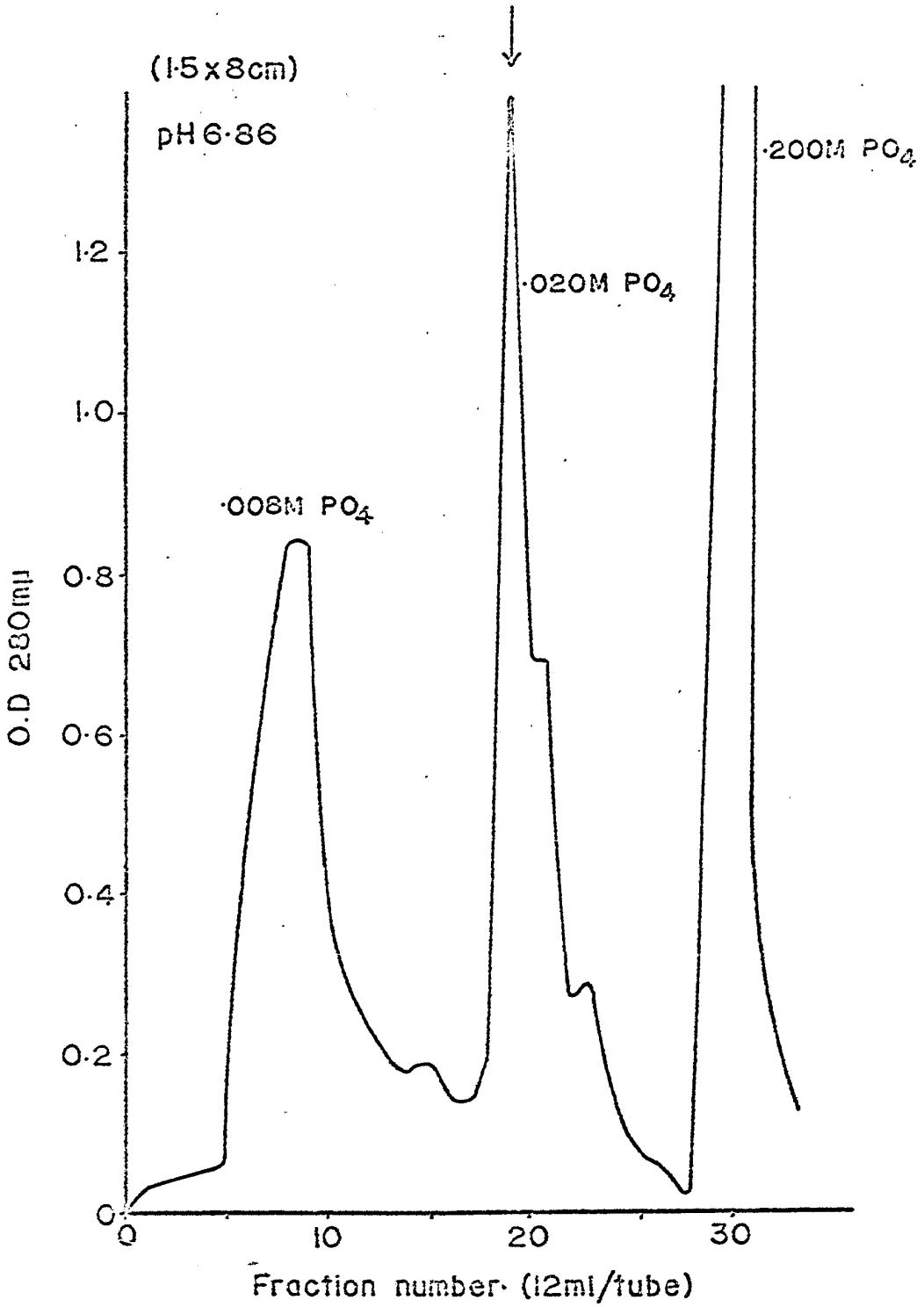


Figure 3

Chromatography was carried out in 0.05 M phosphate buffer, pH 6.86. Dopa decarboxylase activity was present in the protein peak indicated by the arrow.

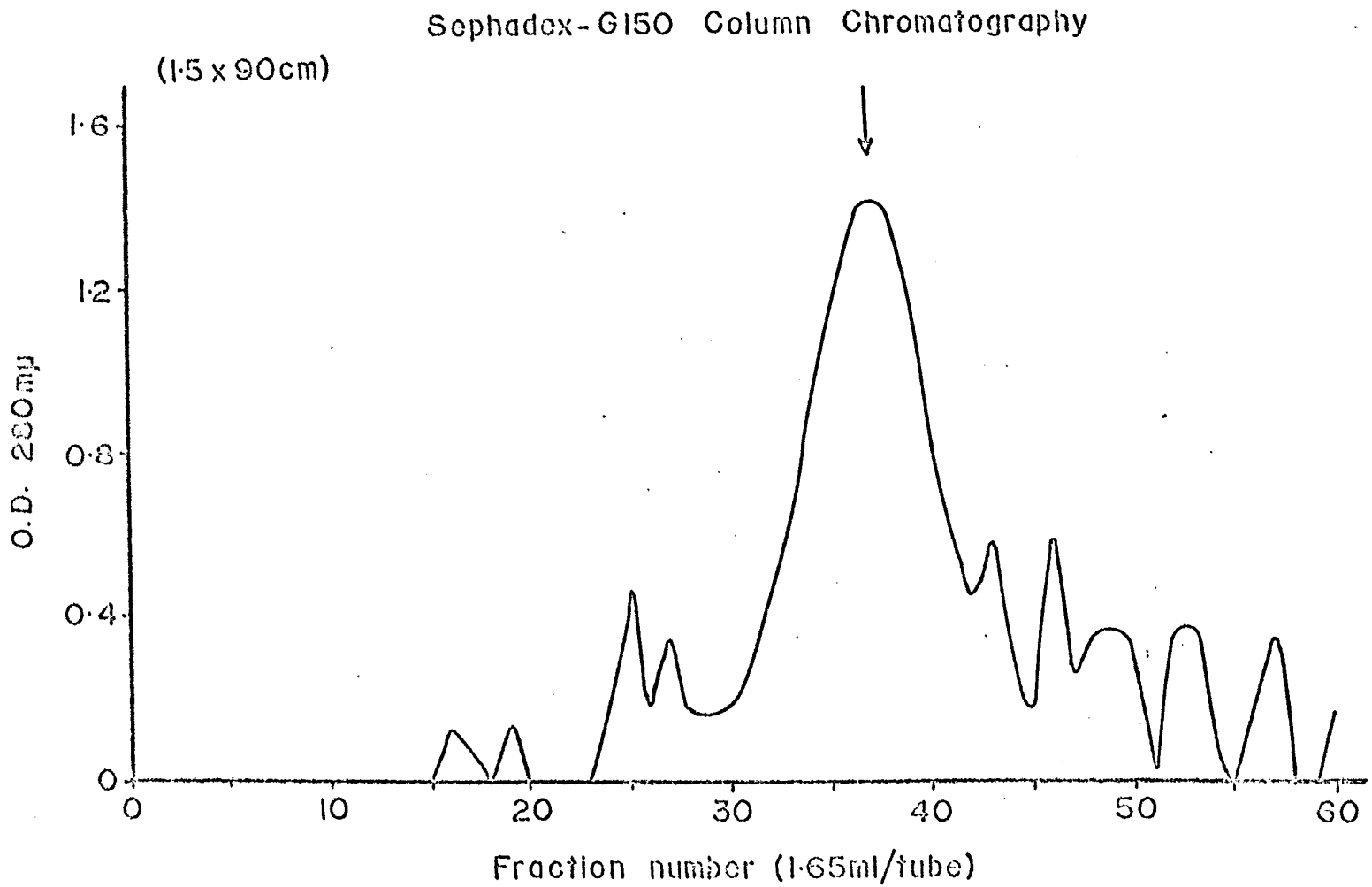

Sephadex-G150 Column Chromatography
(1·5 x 90cm)
1·6
1·2
0·8
0·4
0
O.D. 280mμ
0
10
20
30
40
50
60
Fraction number (1·65ml/tube)

Table I

Purification of Dopa Decarboxylase

Step	Units of enzymic activity*	Protein (mg)	Specific Activity**	% Recovery
Crude	2282	20520	0.11	100
Heat treatment	1891	10416	0.182	82.9
Ammonium sulfate	1188	4560	0.261	52.1
DEAE-Sephadex	1137	425	0.675	49.8
Hydroxylapatite	405	33	12.3	17.7
Sephadex G150	401***	19	21.1	

* One unit = the production (from DL-5HTP) of one μmole of 5HT per hr.

** Units of activity per mg protein.

***The specific activity of 21.1 is the highest ever obtained, and is found only in peak tubes. The units shown are those from the column.

Table II

Stability of Dopa Decarboxylase to Heat Shock at 50°*

Addition	Units recovered	Protein recovered	S.A.
None	720	3720	0.19
10^{-4}M PLP	1051	3880	0.27
0.01% β-mercapto-ethanol	634	3840	0.17
0.01% β-mercapto-ethanol + 10^{-4} PLP	950	3920	0.24
Unheated	1066	4120	0.26

*Enzyme used was post-mitochondrial supernatant which had been treated at pH 5. Units = μmoles 5HT/h, assayed in presence of PLP. Protein was determined by biuret method after precipitating the protein with 10% trichloroacetic acid. S.A. = specific activity = units/mg protein. See text for details of the procedure.

Table III

Effect of Temperature on Heat Stability of Dopa Decarboxylase*

Treatment	Units recovered	Protein recovered	S.A.
50° (5 min)	626	2730	0.23
60° (2 min)	132	2100	0.06
65° (2 min)	51	1440	0.04
Unheated	529	3090	0.17

*S.A. = specific activity = μmoles 5HT/mg protein/h. Enzyme used was post-mitochondrial supernatant after precipitation of impurities at pH 5.

was centrifuged and then more solid ammonium sulfate was added to give a final concentration of 30%, followed again by centrifugation. The two pellets were resuspended in water and all three fractions (0-18%, 18-30% and greater than 30%) were desalted using Sephadex G25. Eighty-nine per cent of the units were recovered in the 18-30% w/v ammonium sulfate fraction.

c) DEAE-Sephadex A50

In working out the purification procedure this step, i.e. recovering a reasonable percentage of the enzyme units from the column, proved especially troublesome. If the column is equilibrated with a buffer of low ionic strength recovery of enzyme is very low, although only one peak of activity is found. Increase in specific activity is also very poor despite the removal of large amounts of protein. The addition of PLP or reducing agents does not aid in the recovery of enzyme under these conditions.

The problem was solved when it was observed that PLP binds to the DEAE-column under conditions of low ionic strength, but not so tightly as dopa decarboxylase. Using an ionic strength high enough to prevent binding of PLP, but low enough to permit adsorption of the enzyme it became possible to have high recovery of units and large increase in specific activity. PLP was added to the buffer to promote stability further. DEAE-Sephadex was preferred over DEAE-cellulose because of its faster flow rate and higher binding capacity. Table IV shows the effects of ionic strength on DEAE-Sephadex chromatography of pig kidney dopa decarboxylase.

Table IV

Effect of Initial Ionic Strength on DEAE-Column Chromatography

Preparation Number	Equilibration Conditions	Recovery of Units* %
20	DEAE-cellulose equilibrated with 0.005 M phosphate, pH 6.8	14
57	DEAE-Sephadex equilibrated with 0.05 M Tris HCl, pH 7.2	8
59	DEAE-Sephadex equilibrated with 0.05 M Tris-HCl, pH 7.2 containing 0.10 M NaCl	65
69	as above	77

*Units expressed as μmoles 5HT/h. 10^{-5} M PLP was present in all buffers.

d) Alumina Cγ

The conditions for adsorption and elution in a batch-wise procedure were worked out as follows: a partially purified preparation in 0.05 M Tris-HCl, pH 7.2, was adjusted to pH 6.5 with 0.1 HCl. A slurry of alumina Cγ was then added to aliquots of enzyme; the tubes were centrifuged and the supernatants assayed. The results are shown in Table V. From these data the ratio of gel (dry solids of alumina Cγ) to protein was determined as 0.75.

Gel with adsorbed enzyme was then eluted with increasing concentrations of phosphate buffer, pH 7.0. The results of this experiment are set out in Table VI. As can be seen from the table, 0.02 M phosphate buffer (or lower) was sufficient to elute the enzyme. In enzyme purification work the gel with adsorbed proteins was eluted twice to regain the enzyme. Adsorption on alumina Cγ was inserted between the DEAE-Sephadex chromatography and hydroxylapatite step. It was later eliminated from the purification scheme, since the recovery of units was not consistently high and since it duplicated the hydroxylapatite step.

e) Precipitation at pH 5

This was used for a time in the purification procedure as an initial step, but was later eliminated as redundant. Recovery of activity was good (940 units out of 1052) in the supernatant, but the heat treatment removed the same amount of protein as adjustment of pH, and was quicker to perform.

Table V

Conditions for Adsorption of Dopa Decarboxylase on Alumina Cγ

Ratio* of dry solids of Alumina Cγ to protein	Supernatant Activity (Units**)
1.0	44
0.75	130
0.50	440
0.25	2800
0.10	2500

*Ratio of weights.

**Units expressed as μmoles 5HT/h.

Supernatant activity gives an indirect measure of adsorption (disappearance of activity from the supernatant indicates adsorption to the gel). Enzyme used for this experiment was post-mitochondrial supernatant. Adsorption was performed from Tris-HCl buffer adjusted to pH 6.5 with 0.1 N HCl.

Table VI

Conditions for the Elution of Dopa Decarboxylase from Alumina C_7*

Concentration of Eluting Buffer (molarity)	Activity in Eluting Buffer (units)	S.A. (units/mg protein)
0.02	0.3140	0.224
0.04	0.0588	0.04
0.06	0.0276	0.07
0.08	0.002	0.005
0.10	0	0

*All eluting buffers were phosphate, pH 6.8. Activity is expressed as μmoles 5HT/h. Enzyme used was post-mitochondrial supernatant.

f) Hydroxylapatite

Columns were run under low air pressure (from the building compressor) to speed elution. No advantage was apparent in making the elution with smaller differences between the ionic strength of successive steps since under those conditions, instead of getting higher resolution of enzyme, the enzyme merely comes out at more than one ionic strength with little gain in specific activity.

3. Subcellular Distribution

The subcellular fractionation was carried out according to an abbreviated version of the method of DeRobertis (1964). The kidney was homogenized in 0.32 M sucrose using a glass, hand-homogenizer with a Teflon pestle. The "nuclei" were prepared by spinning at 800 g for 10 min and then washed twice with sucrose. The "mitochondria" were prepared by centrifuging at 12,000 g for 30 min and were washed once in sucrose. Nuclei and mitochondria were assayed together. The microsomes were sedimented by centrifuging at 111,000 g for 60 min and were washed once in sucrose. The supernatant fraction was the soluble material left over. As can be seen in Table VII most of the enzymic activity is present in the supernatant fluid. A portion of the enzyme is loosely bound to particular matter, but this can be released by mild sonication. Table VIII shows the effect of sonication (5 min using a Branwell sonicator at maximum energy) on a particulate fraction obtained by centrifuging the post-mitochondrial supernatant at 100,000 g for 1 hour. Further demonstration that some enzyme was indeed bound to a particulate fraction was

Table VII

Crude Subcellular Fractionation of Dopa Decarboxylase*

Fraction	Total Units	Specific Activity
Crude homogenate (before fractionation)	3110	0.114
Nuclei and mitochondria	683	0.069
Microsomes	198	0.083
Supernatant	2819	0.390

*Homogenization was performed in 0.32 M sucrose. See text for details of preparing each fraction. Units are μmoles 5HT/h; specific activity is μmoles 5HT/mg/h.

Table VIII

Presence of Particulate Dopa Decarboxylase *

Fraction assayed	Specific Activity	Total Units
"Microsomal"	0.055	115
Supernatant of above after sonication**	0.126	45

*The "microsomal" fraction was the pellet obtained by centrifuging the post-mitochondrial supernatant at 100,000 g for 1 hr. It was washed once with 0.32 M sucrose to remove any contaminating supernatant enzyme and resuspended in 0.32 M sucrose. Specific activity is defined as μmoles 5HT/mg/h. Units are defined as μmoles 5HT/h.

**Sonication was performed for 5 min using a Branwell sonicator at maximum energy.

obtained by density gradient centrifugation of the post-mitochondrial supernatant. Gradients were prepared by layering successively 4.5 ml of 40%, 35%, 30%, 25%, 20%, 15%, 10% sucrose carefully on top of each other. On top of this was layered 0.5 ml of sample. The tube was then centrifuged for 5 hr at 45,000 g in a SW25-1 rotor. Fractions of 28 drops were then collected from the bottom of the tube by puncturing the bottom. A pellet was then collected last by adding 2 ml of phosphate buffer, pH 6.8 and pipetting off this with a Pasteur pipette. Table IX shows the presence of decarboxylase in two discrete parts of the gradient, namely the least dense sucrose near the top of the tube, and in the pellet.

4. Criteria of Purity

a) Acrylamide gel electrophoresis

1) pH 8.9

The purified enzyme was run at both 5% and 7% gel concentrations. If a heavy sample was applied (100γ) a faint band consisting of an impurity appears above the major band. This is not detectable with small samples. Figure 4 shows the effect of gel concentration.

2) pH 2.7

This was run at a gel concentration of 7.5%. Figure 5 shows that one major band only was present.

3) Continuous electrophoresis in 0.05 M Tris-HCl, pH 7.4

Table IX

Density Gradient Centrifugation of Dopa Decarboxylase*

Fraction	Units	Protein (mg)
Pellet	0.245	10.20
Tubes 1-7	0	8.82
Tube 8	0.029	1.50
Tube 9	0.049	1.68
Tube 10	0.101	3.78
Tube 11	1.350	1.14
Tube 12	0.480	5.72

*Tubes are arranged in order of decreasing density. Units are μmoles 5HT/h. Post-mitochondrial supernatant was applied to the top of a discontinuous sucrose gradient. Full details are in the text.

Figure 4

Two concentrations of gel were used, 7% and 5%. The dye front was marked by Bromcresol Blue. The protein bands were stained with Amido Black and were indicated by the cross-hatched areas. Other details ot electrophoresis are outlined in the text.

Acrylamide Gel Electrophoresis (discontinuous)

pH8·9

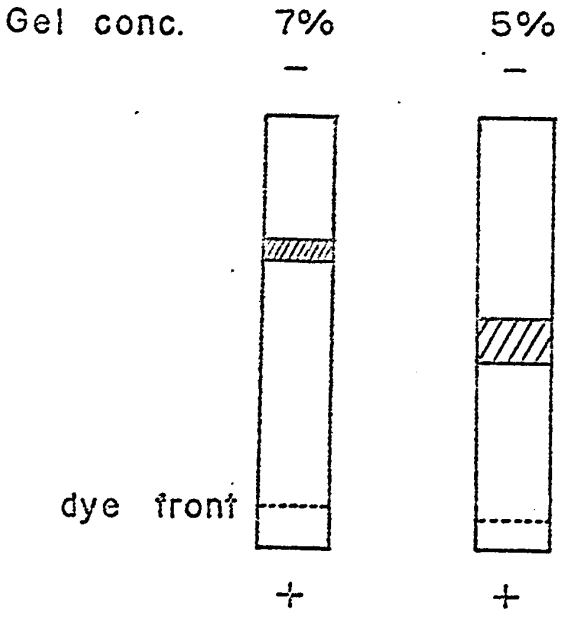


Figure 5

A gel concentration of 7.5% was used for this procedure. The dye front was marked by methyl green. Protein was stained with Amido Black and is represented by the cross-hatched area. Further details of the electrophoresis procedure are outlined in the text.

Acrylamide Gel Electrophoresis (discontinuous)

pH 2·7

Gel conc. 7·5%

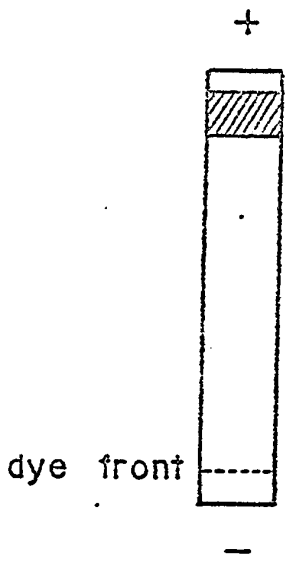
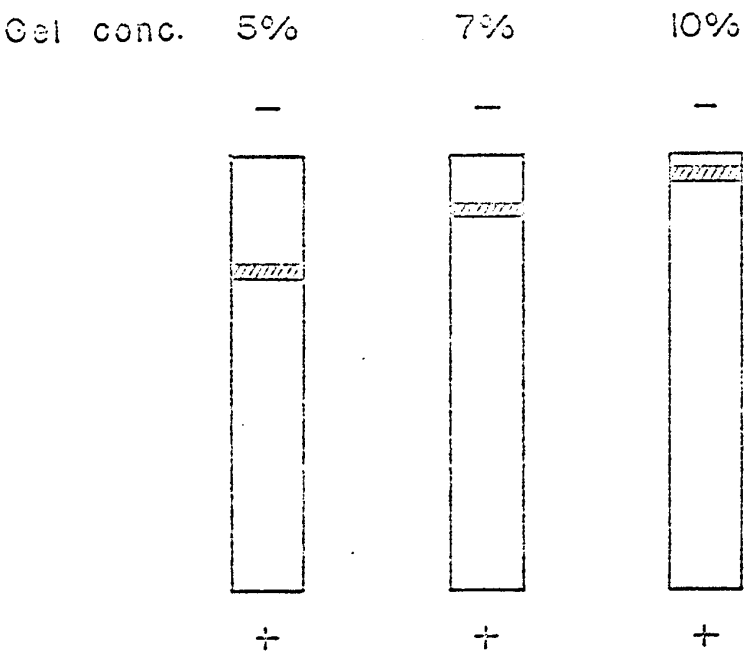


Figure 6

Three gel concentrations are shown, namely 5, 7 and 10%. Protein was stained with Amido Black and is represented by the cross-hatched area. Further details of the electrophoresis procedure are outlined in the text.

Acrylamide Gel Electrophoresis (continuous)

0·05M Tris-HCl pH 7·4



This was run in gel concentrations of 5, 7, and 10%. One major band only was present. Figure 6 shows the effect of gel concentration.

4) Attempts to elute activity from the gel

Numerous attempts have been made to release activity from the gel after electrophoresis. They have all been unsuccessful. Enzyme at stages of purity ranging from crude to purified have been used. Gels include those run at pH 7.2 and 8.9. Methods tried without success include:

i) incubation of unstained gel on filter paper impregnated with ^{14}COOH-DOPA, cutting the paper into 0.2 cm strips, eluting each strip with toluene and 95% ethanol (10:4) containing 50 mg/l dimethyl POPOP and 4 g/l of PPO and then counting and looking for a loss of radioactivity.

ii) Cutting unstained gels into pieces, eluting each piece with 0.5 ml of 0.2 M phosphate buffer, pH 6.8 overnight, incubating the eluting solution with ^{14}C-2-DOPA, adding 0.5 µmoles of cold L-DOPA, extracting the DA with n-butanol and counting.

iii) As in (ii), but adding PLP to the incubation mixture.

iv) As above, but homogenizing each gel piece to aid elution.

Coulson et al. managed to elute rat liver dopa decarboxylase run preparatively at pH 8.7 using electrophoretic elution. This might be the method of choice.

5. Determination of Molecular Weight

a) Gel filtration

The method described by Andrews (1965) for determining the molecular weight of proteins was used. The molecular weight of hog kidney dopa decarboxylase was estimated using Sephadex G150 in a column 1.5 x 90cm, equilibrated with 0.05 M Tris-HCl, pH 7.4. Purified enzyme was applied and this gave a single peak on elution. The eluate was checked enzymically for dopa decarboxylase activity which was found to be present. The column was then calibrated with Blue Dextran (Pharmacia), rabbit muscle glyceraldehyde 3-phosphate dehydrogenase (Calbiochem), beef serum albumin (Calbiochem), ovalbumin (Nutritional Biochemicals Co.) and horse heart cytochrome c (Sigma). When log (molecular weight) was plotted against elution volume, the value of 8.5-9 x 10^4 was obtained as the molecular weight of hog kidney dopa decarboxylase. The calibration curve is shown in Figure 7. As this method does not require purified material, an estimate of the molecular weight of dopa decarboxylase from rat liver, guinea pig liver, and guinea pig kidney was performed in a similar manner. Table X shows molecular weight data for dopa decarboxylases from a number of sources.

b) Dodecyl-sulfate polyacrylamide gel electrophoresis

The method of Weber and Osborne (1969), which has been outlined in the section "Methods" was followed. Standards included catalase (Nutritional Biochemicals Co.), ovalbumin, bovine gamma-globulin (Nutritional Biochemicals Co.) and cytochrome c. Purified enzyme

Figure 7

A Pharmacia (1.5 x 90 cm) column was used for these determinations. Fractions of 1.6 ml were collected. The figure shows a plot of log molecular weight vs elution volume. a represents Blue Dextran, b = glucose 3-phosphate dehydrogenase, c = beef serum albumin, d = ovalbumin, e = cytochrome c. The arrow indicates where dopa decarboxylase is eluted; this corresponds to a m.w. of 90,000.

Determination of Molecular Weight

Gel Filtration

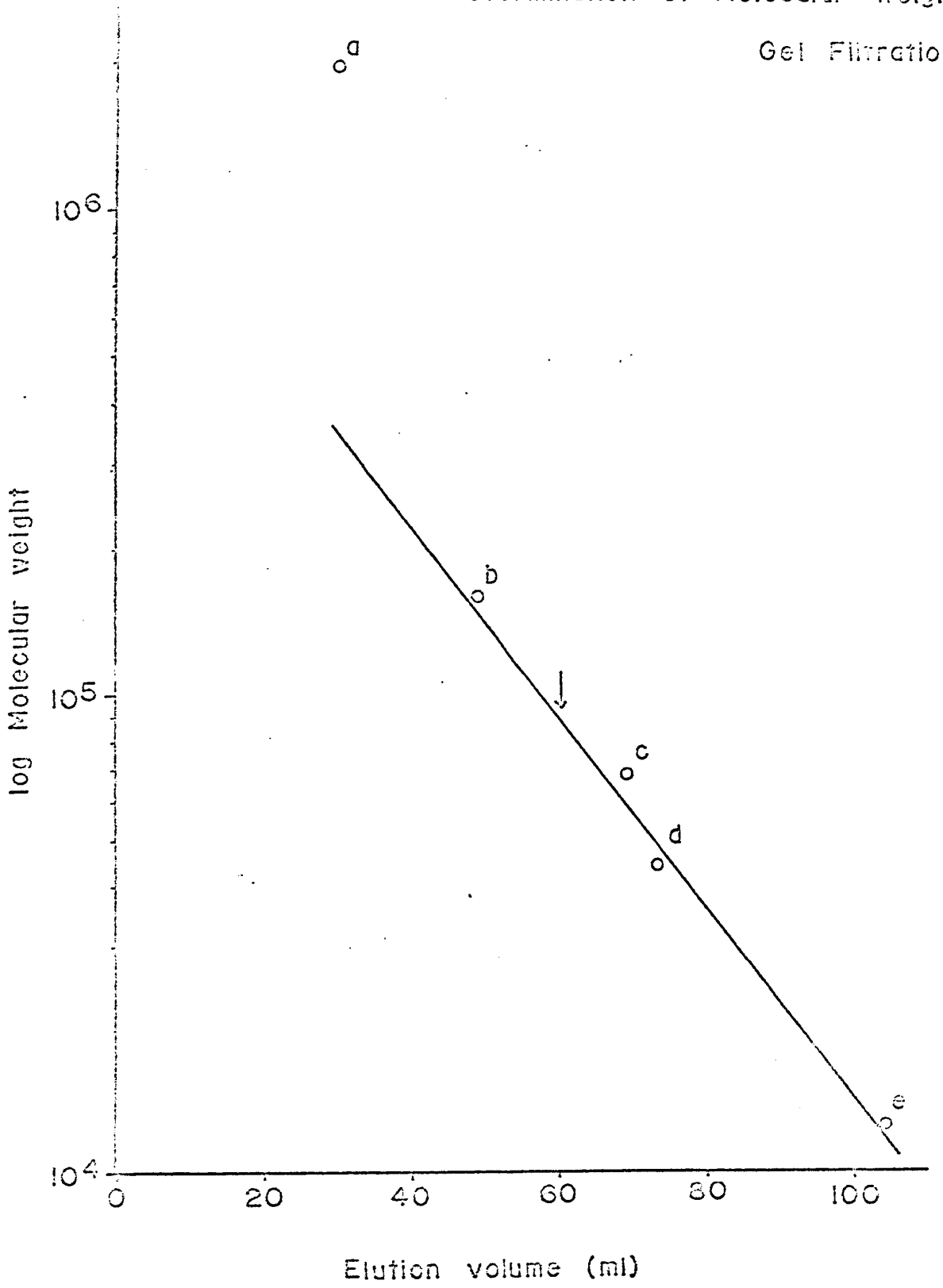


Figure 8

Details of the preparation of samples and standards are described in the text. The figure shows the calibration curve obtained with the following standards: a = catalase, b = ovalbumin, c = γ-globulin, d = cytochrome c.

Determination of Molecular Weight

SDS Gel Electrophoresis

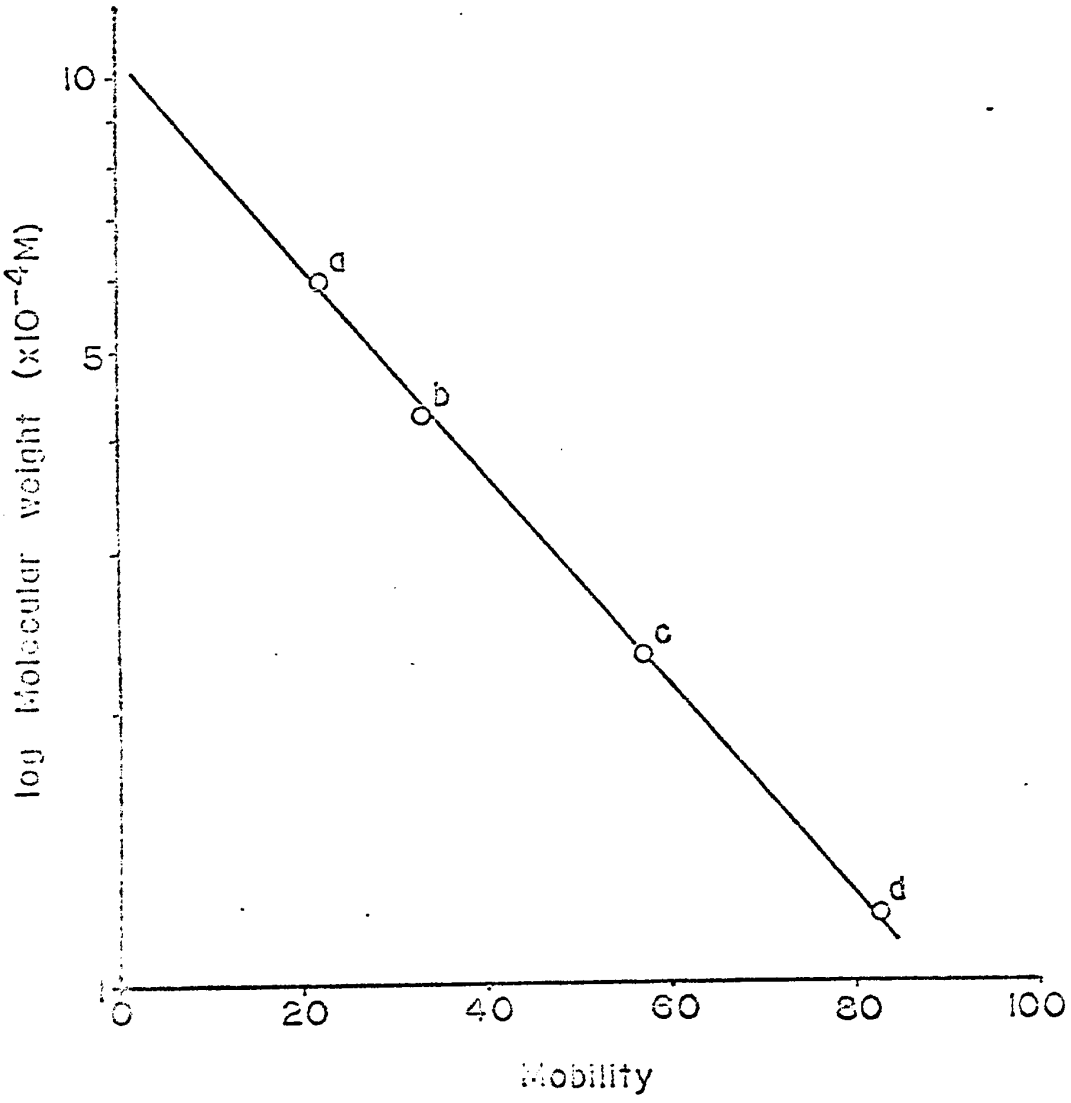


Table X

Molecular Weights of Dopa Decarboxylases

Source of Enzyme	MW x 10^{-4}	Method
Pig kidney	8.5 - 9.0	Gel filtration
Pig kidney	8.8 - 9.4	Dodecyl-sulfate polyacrylamide gel electrophoresis
Guinea pig kidney	9.0 - 10	Gel filtration
Guinea pig kidney	8.5 - 9.1	Gel filtration
Rat liver	8.3 - 9.1	Gel filtration
Bovine adrenal	15.6	Calculated from sedimentation data of Fellman (1969)

was used as sample. After denaturation, two bands were found, one corresponding to a weight of 48-50 x 10^3 and the other 40-44 x 10^3. When these are totalled a molecular weight of 8.8-9.4 x 10^4 is found which is consistent with that obtained by gel filtration. This method of determining the molecular weight was performed in duplicate on two different batches of enzyme. Figure 8 shows the calibration curve used in this method.

6. Stability

Several chemicals were added to the enzyme in the attempt to promote stability; these experiments were unsuccessful. The chemicals included thiols, such as cysteine and mercaptoethanol, as well as glycerol and EDTA.

The crude enzyme loses about half of its activity in a month if stored at -20° C. With purified material stored frozen for 3 months at the same temperature the activity was approximately one-tenth that of the starting material. The addition of excess PLP did not prevent storage loss in the purified or crude material. However, PLP stabilizes the enzyme to heat shock (see Table II).

7. Substrate Specificity

During purification the ratio of activities of DOPA to 5HTP as substrates remains constant. Table XI shows the behaviour towards DOPA and 5HTP during purification. Table XII shows the activity of purified dopa decarboxylase on a variety of substrates both in the presence and absence of PLP. Under the assay conditions, exogenous PLP stimulated activity markedly for o- and

Table XI

Activity towards DOPA and 5HTP during Purification of Dopa Decarboxylase*

Step	μmoles 5HT/h	μmoles DA/h	DA/5HT
pH 5	0.1854	1.2855	6.9
DEAE-Sephadex	0.2360	0.8185	6.5
Alumina C_γ	0.0702	0.5150	7.3
Hydroxylapatite	0.0882	0.6090	6.9

*All these determinations were done on a single preparation. 5HT was determined by the method of Dietrich. DA was determined by the method of Laduron and Belpaire.

Table XII

Substrate Specificity of Purified Hog Kidney Dopa Decarboxylase

Substrate	Activity (μmoles amine/h)	
	with PLP added	without additional PLP
5HTP	0.30	0.30
DOPA	1.69	1.52
threo-DOPS	n.d.*	n.d.
erythro-DOPS	0.040	0.039
ortho-tyrosine	2.1	0.70
meta-tyrosine	2.5	1.0
para-tyrosine	n.d.	n.d.

*No activity detectable. Values are the average of three determintations.

m-tyrosine, but not for any of the others tested. Activity towards α-methyl-5HTP was detected fluorimetrically. After incubating α-methyl-5HTP with enzyme purified up to the hydroxylapatite stage for 2 hr at 37° under nitrogen and then separating the amine from the parent amino acid with paper chromatography using Wh 3MM (butanol: acetic acid: water), the paper was cut into one-half cm strips, eluted and read at 530 nm (activation at 290 nm). Two spots were recovered by this procedure. One corresponded to authentic α-methyl-5HTP and the other to authentic α-methyl-5HT (K. Missala, unpublished).

8. Competitive Inhibition Studies

A few inhibition studies were performed to get some insight into whether one or two sites on the enzyme were responsible for the binding of DOPA and 5HTP. Since these two compounds have been reported to be mutual inhibitors (see Introduction), K_I's for each in the presence of the other as well as K_M's under different conditions were determined for several preparations. Since PLP was not always added, apparent K_M's were obtained which were higher than the real K_M as the cofactor was occasionally limiting (depending upon the enzyme preparation). Table XIII shows some data which suggest that one site on the enzyme is involved.

9. Effect of pH

The enzyme was assayed at various pH's in phosphate buffer using 5HTP as substrate. pH 6.8 was the pH with the highest activity. Stability was also tested. Enzyme in phosphate buffer,

Table XIII

Mutual Inhibition of DOPA and 5HTP of Dopa Decarboxylase

Preparation	Substrate	Inhibitor	Assay Condition	K_I	K_M
88	DOPA	5HTP	PLP added	1.3-2.0*	
88	5HTP	---	PLP added		1.3*
90	DOPA	5HTP	no PLP added	5.0, 5.1	
90	5HTP	---	no PLP added		4.8
91	5HTP	DOPA	no PLP added	25, 50	
91	DOPA	---	no PLP added		99

*Values in these columns represent K x 10^4M. Assays for 5HTP were by the Dietrich method for K_M's and by solvent extraction for K_I of DOPA; assays for DOPA were by the Laduron and Belpaire method.

pH 6.8, was adjusted to a variety of pH's between 3 and 10 using carefully measured amounts of standard 0.1 N NaOH using a pH meter and constant stirring. The samples were then incubated at 50° for 5 min, to stress the enzyme, and then they were chilled. They were then readjusted to pH 6.8 by the addition of complementary acid or base. Finally the samples were diluted in more 0.2 M phosphate buffer to give a final constant volume for each sample. Each of the samples was then assayed. As can be seen in Figure 9, pH 6.8 is the pH of maximum stability.

The K_M using 5HTP as substrate also varies with pH, as can be seen in Figure 10.

10. Cofactor Requirement

Adams' (1969) microtechnique was used to detect and measure PLP. The method was applied to the purified enzyme and the results are shown in Figure 11. Figure 11 shows the activation spectrum and the emission spectrum of purified enzyme as determined by this technique. Authentic PLP was used for comparison. PLP content is variable in different enzyme preparations, indicating that different degrees of resolution are obtained. Table XIV shows the PLP content of two different batches of enzyme. As can be seen from the table, PLP is present in less than stoichiometric amounts, to give one mole of PLP per mole of enzyme if the molecular weight of dopa decarboxylase is considered to be 90,000. Since no enhancement of activity to most substrates (see Table XII) was obtained by additional PLP a certain portion of the protein present is

Figure 9

Samples of enzyme were adjusted to various pH's, heated to 50° C, adjusted back to pH 6.8 and assayed, using 5HTP. pH 6.8 is the most stable pH.

pH for Maximum Stability

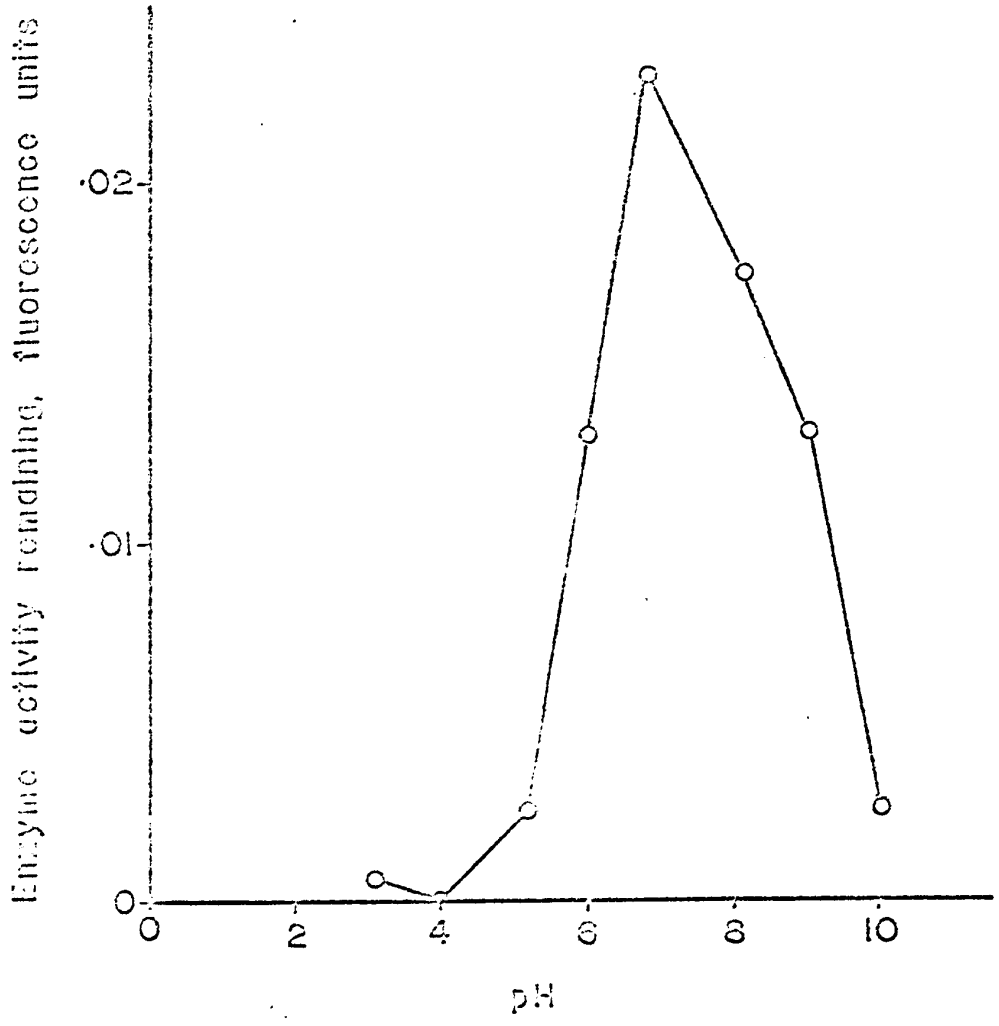


Figure 10

Apparent K_M's were obtained at various pH's for purified enzyme in the presence of additional PLP. The affinity of enzyme for substrate appears highest in the range pH 6-7, but falls off rapidly on either side of this range.

Effect of pH on the K_M for 5HTP Decarboxylation

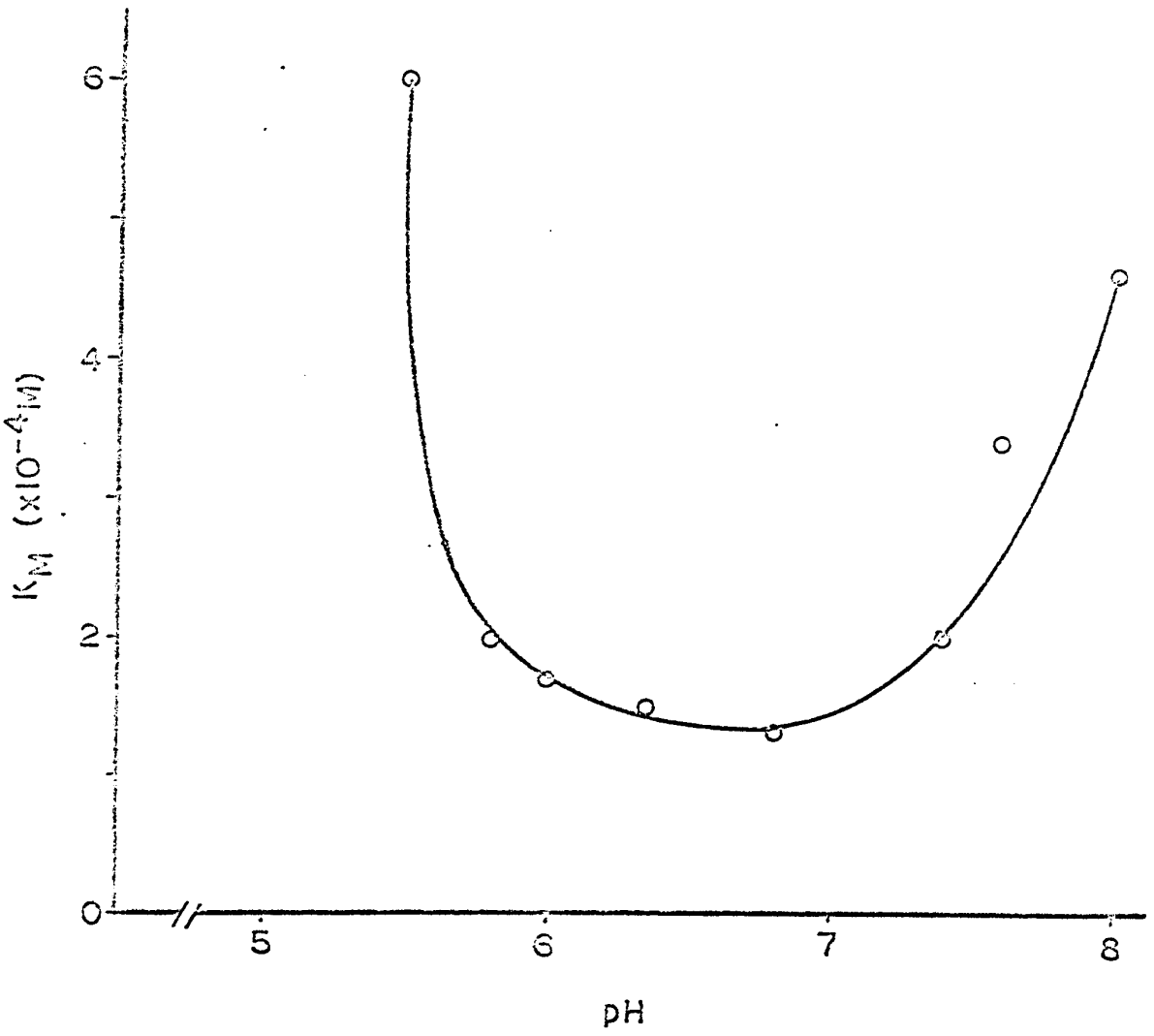


Table XIV

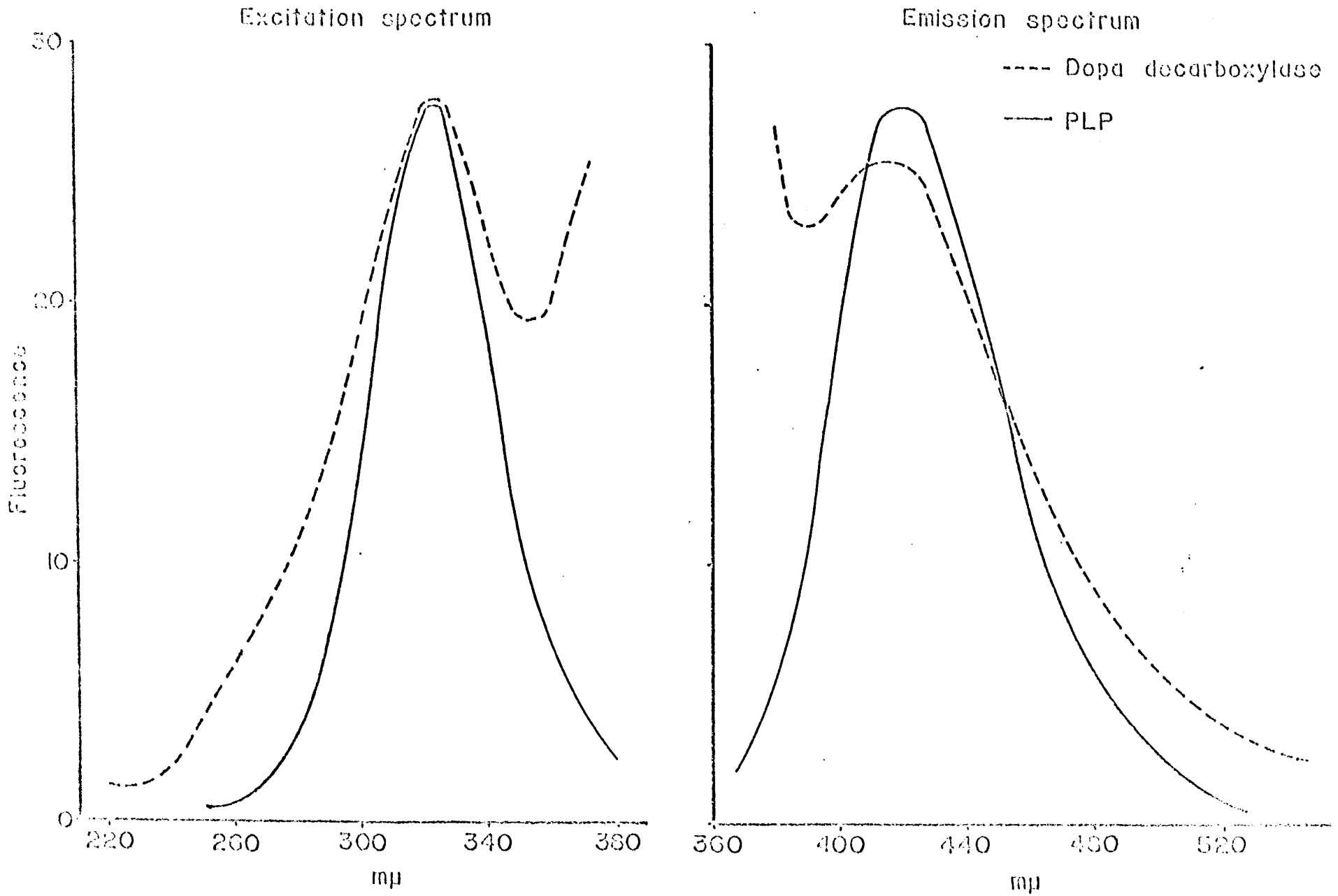
PLP Content of Purified Dopa Decarboxylase

Preparation	nmoles enzyme	nmoles PLP	PLP/mole of enzyme*
96	0.71	0.14	0.2
97	1.1	0.44	0.4

*Molecular weight of dopa decarboxylase was taken to be 9.0×10^{-4}. PLP was determined by the method of Adams (1969). Protein was measured by the method of Lowry et al. (1951).

Figure 11

This figure indicates the presence of PLP in purified dopa decarboxylase as is evidenced by their similar emission and excitation spectra after preparing the cyanide derivative by the method of Adams (1969).


Excitation spectrum
Emission spectrum
---- Dopa decarboxylase
—— PLP
Fluorescence
30
20
10
0
220
260
300
340
380
mμ
360
400
440
480
520
mμ

probably inactivated enzyme or impurity. From the wide variety of conditions used to check purity the former seems likely. On the basis of these considerations, it seems probable that one mole of PLP is bound per mole of active enzyme.

IV. DISCUSSION

1. Enzyme Purification Method

The work outlined in this thesis gives one more source of purified dopa decarboxylase. The specific activity found is higher than that obtained by Awapara et al. (rat liver), Werle and Aures (guinea pig kidney) or Fellman (beef adrenal). The guinea pig kidney preparation of Lovenberg et al. was slightly more active, which may represent a species difference. They assayed the enzyme at a different pH (pH 9 instead of 6.8) which might also explain the discrepancy. The preparation of Christenson et al. from hog kidney is slightly more active.

In regard to purity, only two authors (Fellman and Christenson) gave any indication of the purity of their preparations. Fellman used paper electrophoresis at pH 8.6 and 7.0 and free boundary electrophoresis at pH 8.6 as well as ultracentrifugal analysis to show homogeneity. Christenson et al. obtained 97-100% homogeneity as estimated by polyacrylamide gel disc electrophoresis at pH 8.3 and 7.5 and Ouchterlony double diffusion technique. Coulson et al. (1969) have claimed four dopa decarboxylases in rat liver as shown by mobility on polyacrylamide gel electrophoresis. The purification method outlined in this thesis gives material which is essentially homogeneous, although a faint contaminant does show up upon electrophoresis with concentrated samples. The variety of pH's employed, i.e. 8.9, 7.0 and 2.7 would tend to separate proteins with different charged groups; the different gel concentrations (5%, 7%, and 10%)

would separate the proteins by molecular weight. Upon rechromatography on Sephadex G100 the enzyme gives a single symmetrical peak, which further suggests homogeneity. The cofactor content of the purified enzyme is consistent with the view that the major band and not the minor band (the contaminant) is dopa decarboxylase since calculated on the basis of 100% purity, one-half mole PLP is present per mole of dopa decarboxylase, i.e. the stoichiometry is of the same order of magnitude. Some of the protein isolated in this manner is inactive enzyme as is apparent by the lack of stimulation by additional PLP, although only one-half PLP was found per mole of protein.

The yield of enzyme is quite high for such a cheap, easily available source of pig kidney. Fellman obtained 36 mg of protein from 95-105 g of beef adrenal medullas. Lovenberg et al. do not state their yield, but by calculation, considering their maximum load to the DEAE-cellulose column of 200 mg and a purification of 7-fold using the column, a 60% yield off the column would give them approximately 14 mg. Awapara et al. obtained approximately 40 mg with various specific activities from 5-10 rat livers. Werle and Aures also do not state their yield, but by a similar calculation, it must have been about 12 mg. The yield obtained from pig kidney was 19 mg, which was mostly dopa decarboxylase and not contaminating proteins. The yield and purity obtained would make it suitable material for immunological studies. Christenson et al. obtained recoveries of 5-14%, i.e. 4 mg from 300-400 g of pig kidney cortex.

The purification method used by Christenson et al. is strikingly similar to the one outlined in this thesis, with several marked differences noted in the ensuing text. Approximately the same amount of starting material is used. Ammonium sulfate, heat treatment, chromatography on DEAE-Sephadex and hydroxylapatite are all similar. Alumina Cγ was deleted from my procedure when it was noticed that it removed the same proteins as hydroxylapatite (noticed on polyacrylamide assays). My procedure is notably faster since it has fewer steps and employs desalting on Sephadex G25 columns in place of dialysis over night. Mercaptoethanol is omitted from my procedure since acid-washed (heavy metal-free) glassware was used throughout.

2. Subcellular Distribution

The results obtained here were similar to those obtained in nervous tissue by DeRobertis (1964) and Ichiyama et al. (1968), i.e. the presence of most of the dopa decarboxylase activity in the supernatant, but also the presence of a small amount in the washed particulate fraction which could be released by mild sonication and hypotonic shock. Further investigation is necessary to determine whether the "bound" and soluble enzyme are identical or different. It is of some interest that in brain two forms of glutamic acid decarboxylase have been reported by Haber et al. (1970), one existing in the mitochondria and one in the nerve endings. These two forms are claimed to have different behaviour towards pyridoxal trapping agents. Another possibility is that

one enzyme might be present in the capillary walls and another in the parenchymal cells of the organ in question.

As can be seen from Table X, most of the dopa decarboxylases that have been purified are rather similar as to their molecular weight, namely in the range of 8.5-9.0 x 10^4, the notable exception being the one isolated from beef adrenals which is about twice the weight (a dimer?). My estimate of the molecular weight of 8.5-9.0 x 10^4 by gel filtration and the Weber and Osborn method differs from the value of 112,000 given by Christenson et al. obtained by sedimentation. The Weber and Osborn technique suggests that hog kidney dopa decarboxylase is formed of two peptide chains of differing molecular weight (40-44 x 10^3 and 48-50 x 10^3) joined by disulfide bridges.

3. Stability

Dopa decarboxylase isolated from pig kidney is stable compared to the preparations described from other sources which ranged from a few days (beef adrenal enzyme) to a half-life of about two weeks (guinea pig kidney). The hog kidney enzyme has a half-life of about a month when stored frozen. Christenson et al. give a half-life of six weeks for their preparation which is in good agreement.

Awapara et al. and Christenson et al. used mercaptoethanol in their buffers to protect the enzyme against oxidation and heavy metals. Mercaptoethanol did not promote stability towards heat denaturation (see Table II). As glass-distilled water was used for all reagents, and all glassware was washed in 3N HCl, heavy metals did not pose a problem.

The extremesusceptibility of dopa decarboxylase to denaturation on ion exchangers unless the conditions are carefully controlled has been indicated in Table IV.

4. Substrate Specificity

The constant ratio of activity towards DOPA and 5HTP throughout purification (see Table XI) is expected since as has been shown, one enzyme is involved for both substrates. A change in the ratio of two substrates during purification is a classical method of demonstrating the presence of two enzymes, not one. These results agree with similar findings in other tissues, for example, Lovenberg et al. with guinea pig kidney enzyme and Rosengren with rabbit kidney.

The mutual inhibition studies shown in Table XIII give independent support for the existence of one enzyme, with one site used for both substrates, since the K_I and K_M for 5HTP are the same suggesting the compound is being bound with the same affinity as a substrate and as an inhibitor. The results with DOPA are ambiguous, but this is probably due to DOPA acting as a carbonyl-trapping agent as well as a competitive inhibitor. The ability of DOPA to tie up PLP is well documented (Schott and Clark, 1952; Sourkes, 1954).

The response of the purified enzyme to a variety of substrates (Table XII) is unusual in that only ortho- and meta-tyrosine were markedly stimulated by additional PLP. This may be an artifact produced by the relatively long incubation time (30 min) since

both substrates sequester PLP - the additional PLP may protect the PLP bound to the enzyme. In the case of DOPA, this effect was minimized due to a shorter incubation period (10 min or 5 min). No activity was found for para-tyrosine which is in agreement with the work of Hagen (1962) using ox adrenal enzyme, Awapara et al. (1964) and Coulson et al. (1968) using rat liver enzyme and Gonnard et al. (1966) using pig kidney and rabbit kidney. This is in disagreement with Lovenberg et al. (1960) who used guinea pig enzyme. Christenson et al. reported that a very low activity towards para-tyrosine by pig kidney enzyme can be detected (about 0.3% of the activity of DOPA) if the enzyme is assayed at pH 8.5.

The decarboxylation of erythro-DOPS by hog kidney homogenates was reported by Hartman et al. (1955) with about one-third the activity with DOPA. My figures of 1.69 μmoles DA/h vs 0.04 μmoles NA/h are more on the order of two per cent. It is interesting that activity towards this compound could be detected even with highly purified preparations. The failure to detect decarboxylation of the threo-isomer of DOPS is in line with the relatively weak activity reported for kidney by the same authors.

5. Effect of pH

There has been some dispute in the literature about the pH optimum for dopa decarboxylase. A pH for optimum activity in the region of 6.86 is in agreement with most authors - for example, Werle et al. (1959, 1960), Fellman (1959), Awapara et al. (1962)

and Sekeris (1963). Lovenberg et al. (1960) used pH 9.0 which, as already mentioned makes comparison with other work very questionable. Christenson et al. assayed DOPA at 7.0 and other substrates at pH 8.5. No authors mention carrying out pH stability studies. Hakanson (1967) suggests that pH optimum for dopa decarboxylase would vary with the concentration of substrate.

The effect of pH on K_M gives an indication that ionic groups are involved in the substrate-enzyme complex.

6. Cofactor Requirement

The emission and activation spectra of purified enzyme after treatment by Adam's technique indicate that PLP is indeed present. The low yield of PLP per mole of enzyme may be explained by the presence of inactive protein. Christenson et al. also postulate one mole PLP per mole of enzyme. The inactive protein present in my preparation may be oxidized enzyme since Christenson et al. (1970) reported some activation by pretreatment with thiols even though these were present throughout the purification procedure. As mentioned earlier, they were not included in my procedure.

V. SUMMARY

This thesis outlines a purification procedure for dopa decarboxylase starting with 250-300 g of hog kidney cortex. The method consists of heat-treatment of a homogenate and two fractionations with ammonium sulfate. After desalting on Sephadex G25, the material is chromatographed on a column of DEAE-Sephadex, using a step-wise procedure. This material after concentration with ammonium sulfate and desalting, is chromatographed on columns of hydroxylapatite and Sephadex G150, respectively. Various modifications of this basic procedure, such as precipitation at pH 5 and Alumina C_γ adsorption, are discussed.

A crude subcellular distribution showed that most of the enzyme was present in the soluble supernatant, but a small amount of particulate enzyme was present

The purified enzyme was homogeneous by means of acrylamide gel electrophoresis using a discontinuous system at pH 8.9 and 2.7 and various concentrations ot gel. A continuous system at pH 7.4 at three gel concentrations failed to show additional components. Attempts to elute enzymic activity from the gel were unsuccessful.

Molecular weight determinations were made on purified enzyme using both gel filtration and dodecyl-sulfate polyacrylamide gel electrophoresis. Gel filtration gave a molecular weight of $8.5\text{-}9.0 \times 10^4$. The dodecyl-sulfate method gave two bands - $4.8\text{-}5.0 \times 10^4$ and $4.0\text{-}4.4 \times 10^4$. The sum of these two bands is $8.8\text{-}9.4 \times 10^4$ in close agreement with the other method.

Since this method breaks disulfide bands it is probable that dopa decarboxylase from hog kidney consists of two unequal peptide chains.

The stability of purified enzyme was found to be similar to that of crude enzyme, with a half-life of approximately one month, when stored frozen.

The substrate specificity of purified enzyme has been determined for a number of possible substrates. Highest activity was found for DOPA. Activity was measured directly for 5HTP, o-tyrosine, m-tyrosine and erythro-DOPS. Activity was detected chromatographically for α-methyltryptophan. No activity could be detected for p-tyrosine or threo-DOPS under the assay conditions used. The rates of activity towards DOPA and 5HTP remained unchanged throughout purification

Competitive inhibition studies on purified enzyme showed that DOPA and 5HTP act as mutual competitive inhibitors and probably bind at the same site on the enzyme.

A pH activity for 5HTP was determined giving an optimum at pH 6.8. This pH was also the pH of maximum stability to heat shock. The K_M of the enzyme was pH-dependent.

Using a method for direct measurement of purified enzyme, it was found that one PLP is bound per mole of enzyme. Because no stimulation was obtained for the majority of substrates by addition of exogenous PLP it was concluded that the enzyme had been isolated as holoenzyme.

VI. SOMMAIRE

Une methode de purification de la DOPA décarboxylase à partir de 250 a 300 g de cortex renal de porc est décrite. L'homogénat subit tout d'abord un traitement thermique, suivit de deux fractionnements par le sulfate d'ammonium. Après désalage sur une colonne de Séphadex G-25, la préparation enzymatique est chromatographiée sur une colonne de DEAE-Séphadex A-50 utilisant un gradient discontinu. La fraction active, concentrée par précipitation au sulfate d'ammonium et desalée subséquemment, est rechromatographiée sur une colonne d'hydroxylapatite et ensuite sur une colonne de Séphadex G-150. Plusieurs variantes de cette technique initiale sont critiquées. Lors d'une étude préliminaire de la distribution intracellulaire de l'enzyme, la majorité de l'activité a été retrouvée dans la fraction cytoplasmique.

L'homogénéité de l'enzyme purifié est démontrée par électrophorèse discontinue sur gel de polyacrylamide de concentrations variables et aux pH 8.9 et 2.7. Les electrophorèses continues à trois concentrations differentes de gel de polyacrylamide a pH 7.4 n'ont pu démontrer la présence de proteines contaminantes. Les essais d'extraction de l'enzyme du gel ont été infructueux.

Le poids moléculaire de l'enzyme purifié estimé par tamis moleculaire varie entre 85,000 et 90,000. Par electrophorèse sur gel de polyacrylamide en presence de dodécyl-sulfate de sodium et de mercaptoéthanol, nous obtenons deux bandes dont la somme des poids moléculaires fluctue entre 88,000 et 94,000. Ces derniers

resultats corroborent les premiers. Étant donne que les liens disulfurés sont scindés par la deuxième méthode, nous pensons que la DOPA décarboxylase de rein de porc consiste de deux chaînes polypeptidiques inégales.

La stabilité de l'enzyme purifié se compare à celle de l'enzyme non purifié dont la demi-vie est d'un mois lorsque congelé.

La spécificité de l'enzyme a été verifiée en utilisant plusieurs substrats dont la DOPA, 5HTP, l'ortho et la meta-tyrosine, l'erythro-DOPS et l'α-méthyltryptophane. Une activité maximale a été obtenue avec la DOPA. D'autre part, aucune activité n'a pu être décélée avec la para-tyrosine ou la thréo-DOPS comme substrats. Le rapport entre l'affinite de l'enzyme pour la DOPA et pour la 5HTP est demeuré constant durant toute la purification.

Nous avons demontré par cinétique enzymatique l'inhibition compétitive reciproque de la DOPA et de la 5HTP suggérant que les deux substrats utilisent le même centre actif.

L'activité enzymatique optimale est a pH 6.8 en utilisant la 5HTP comme substrat; et est aussi celui auquel l'enzyme est le plus resistant aux chocs thermiques.

En utilisant une méthode de mesure directe, nous avons pu mettre en évidence qu'une mole de PLP est fixée par mole d'enzyme. Avec la plupart des substrats aucune stimulation de l'activité enzymatique n'a pu être obtenue avec l'addition de PLP exogène. Nous concluons donc que l'enzyme isolé est une holoprotéine.

VII. Claims of Contribution to Knowledge

1. A method is given to purify dopa decarboxylase from hog kidney to homogeneity.

2. The purified enzyme has a molecular weight of 85,000-94,000.

3. The enzyme consists of two unequal peptides - one with a molecular weight of 48-50 x 10^3 and the other 40-44 x 10^3.

4. Purified dopa decarboxylase contains 1 mole PLP per mole of enzyme.

5. Purified enzyme is active towards the following substrates: DOPA, 5HTP, erythro-DOPS, ortho-tyrosine, meta-tyrosine. Activity towards para-tyrosine and threo-DOPS could not be detected.

6. The ratio of activity towards DOPA and 5HTP does not change during purification.

VIII. Abbreviations

DA	3,4-dihydroxyphenylethylamine = dopamine
DEAE	diethylaminoethyl
Dimethyl POPOP	1,4,bis-2-(4-methyl-5-phenyloxazolyl)-benzene
DOPA	3,4-dihydroxyphenylalanine, except where otherwise stated as in 2,5-DOPA, etc.
DOPS	3,4-dihydroxyphenylserine
EDTA	ethylenediaminetetracetic acid
5HT	5-hydroxytryptamine = serotonin
5HTP	5-hydroxytryptophan
Iproniazid	isonicotinyl-2-isopropylhydrazine = marsilid
PLP	2-methyl-3-hydroxy-4-formyl-5-pyridylmethyl-phosphoric acid = pyridoxal 5-phosphate = codecarboxylase
PPO	2,5-diphenyloxazole
TMED	N,N,N',N'-tetramethylethylenediamine
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
K_I	is the dissociation constant of the enzyme inhibitor (EI) complex
K_M	Michaelis constant. $K_M = (k_{-S} + k_P)/k_S$ where k_S is the rate constant for the reaction E + S → ES k_{-S} is the rate constant for ES → E + S and k_P is the rate constant for ES → E + P. E = enzyme, S = substrate P = product. ES = enzyme substrate complex.

IX. BIBLIOGRAPHY

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