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STUDIES ON

AROMATIC L-AMINO ACID DECARBOXYLASE

by

JAMES G. CHRISTENSON

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1972

This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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What is this thing called life? --- But I believe

That the earth and stars too, and the whole glittering
universe, and rocks on the mountain have life,

Only we do not call it so -- I speak of the life

That oxydizes fats and proteins and carbo-

Hydrates to live on, and from that chemical energy

Makes pleasure and pain, wonder, love, adoration, hatred
and terror: how do these things grow

From a chemical reaction?

I think they were here al-
ready. I think the rocks

And the earth and the other planets, and the stars and
galaxies

Have their various consciousness, all things are conscious;

But the nerves of an animal, the nerves and brain

Bring it to focus; the nerves and brain are like a burning-
glass

To concentrate the heat and make it catch fire . . .

From "The Beginning and the End"
by Robinson Jeffers.

DEDICATION

This thesis is dedicated to my wife, Karin.

ACKNOWLEDGEMENTS

I wish to express my gratitude to my adviser, Dr. Sidney Udenfriend, whose knowledge and scientific insight were of inestimable value in this work.

I also wish to acknowledge my indebtedness to my colleague, Dr. Wallace Dairman, who is mainly responsible for whatever knowledge of the field of catecholamine research I have acquired.

Thanks are also due to Drs. Aaron Lukton, James Hogg, and Thomas Haines, who provided indispensable help in dealing with the rules, regulations, and bureaucracy of The City University of New York.

I am grateful to my employer, Hoffmann-La Roche Inc., for the tuition reimbursement program.

Expert advice and technical assistance has been received from the following individuals: Barbara Segen, James Stone, Daniel Luk, Paul Rubinfeld, Gwendolyn Reed, and Drs. Paul Bartl, Barry Berkowitz, Jack Coffey, and Sydney Spector. The work of Dr. B. Hecht and his staff at the Roche animal farm was especially important.

Many of my colleagues have given of their time and understanding in valuable discussions. Among them, I especially wish to thank Drs. Robert Niederman, Frank Margolis, Hiroyoshi Hidaka, James McGee, Robert Rhoads, and Kenneth Gibson.

Finally, I wish to thank Miss Dianna Trout for her skillful typing and preparation of the manuscript.

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INTRODUCTION

Aromatic L-amino acid decarboxylase* is a member of a large class of enzymes known as "carboxy-lyases" (EC 4.1.1.-). These enzymes may generally be divided into those which decarboxylate α-amino acids and those which act on substrates other than amino acids. Most amino acid decarboxylases have been found to utilize pyridoxal 5'-phosphate as a cofactor, although this has not been firmly established in all cases. Only two exceptions are known: a histidine decarboxylase from Lactobacillus 30a (Rosenthaler et al., 1965) and an S-adenosylmethionine decarboxylase from E. coli W (Tabor, 1962). Neither enzyme was activated by pyridoxal phosphate, suggesting either that the cofactor was tightly bound or not required at all. No evidence was found that the highly purified and crystalline histidine decarboxylase contained pyridoxal phosphate. Nevertheless, both enzymes were found to be inhibited by carbonyl reagents and this observation led to the demonstration that they contain covalently bound pyruvate (Riley and Snell, 1968; Wickner, Tabor, and Tabor, 1970). Riley and Snell suggested a mechanism in which the pyruvate functions catalytically in much the same way as that proposed for pyridoxal phosphate.

Mechanism of decarboxylation catalyzed by pyridoxal phosphate. Most of the biochemical reactions catalyzed by pyridoxal phosphate enzymes have been fruitfully studied in nonenzymatic model systems (for reviews, see

* Because of the evidence that this enzyme decarboxylates many aromatic amino acids of the L-configuration, Lovenberg, Weissbach, and Udenfriend (1962) suggested that it be named "aromatic L-amino acid decarboxylase" and this name is now in common usage. However, in many papers, the enzyme has been called "DOPA decarboxylase" (EC 4.1.1.26) or "5-hydroxytryptophan decarboxylase (EC 4.1.1.28). In this Introduction, the author has generally used the term used by the original authors in order to specify the actual substrate(s) studied.

Metzler, Ikawa, and Snell, 1954; Snell, 1958; Braunstein, 1960; Bruice and Benkovic, 1966). The first step in all these reactions is thought to be formation of a Schiff base by condensation of the α-amino group of the amino acid with the aldehyde function of pyridoxal. Since pyridoxal phosphate is thought to be already bound to the enzyme as a Schiff base, this step is presumably a transaldimination in the case of enzymatic reactions. The resulting highly planar structure facilitates delocalization of electrons from the α-carbon of the amino acid, the pyridine nucleus serving as an "electron sink", thus labilizing the bonds about the α- and β-carbons of the amino acid. Bruice and Benkovic (1966) point out that pyridoxal (and thiamine) may be formally "considered to play their roles by stabilizing or obviating the necessity to stabilize carbanions" at these positions.

Pyridoxal-catalyzed decarboxylation has been studied in only a few nonenzymatic systems. Part of the reason for this may be that, while metal ions greatly stimulate most nonenzymatic pyridoxal reactions, they inhibit decarboxylation (Kalyankar and Snell, 1962). Metal ions are thought to form chelates with the imine nitrogen of the Schiff base, the phenolate group at the 3-position, and the carboxylate group. While this chelate formation would be expected to further labilize the bonds to the α-hydrogen and the side chain by increasing the planarity of the intermediate and providing an additional electron sink, it should be expected to stabilize the bond to the carboxyl group by the energy conferred through charge neutralization and chelate ring formation.

Werle and Koch (1949) first reported the decarboxylation of histidine in a nonenzymatic system containing either pyridoxal or pyridoxal phosphate. They proposed a mechanism based on the tautomerization of the initial aldimine

to an α-imino acid followed by cleavage of CO_2. This mechanism was consistent with earlier observations that α-imino acids are readily decarboxylated and that amino acids, such as α-aminoisobutyric acid, which have no imino form are relatively resistant to decarboxylation (Wieland and Bergel, 1924).

An alternative mechanism in which the imino acid does not participate was proposed by Westheimer. This mechanism was based on the studies of Herbst and Rittenberg (1943) on the decarboxylative transamination of α-phenylglycine and pyruvic acid. These workers observed that when the reaction was carried out in D_2O-H_2O mixtures, the product alanine contained deuterium in the α- and β-positions, while the aldehyde hydrogen of the product benzaldehyde had not exchanged. Likewise when α-deutero-α-phenylglycine reacted with pyruvic acid in water, the deuterium was retained in benzaldehyde. These results are clearly inconsistent with the participation of the imino derivative of the α-phenylglycine and imply that loss of CO_2 occurs before, or concomitant with, the prototropic shift.

Mandeles, Koppelman and Hanke (1952) tested and confirmed Westheimer's mechanism for three bacterial, pyridoxal phosphate-dependent decarboxylases. When tyrosine, lysine, and glutamic acid were decarboxylated by their respective enzymes in 99% D_2O, one, and not more than one, atom of deuterium was incorporated per molecule of product amine. The contrary results of Osipenko (1950) in Braunstein's laboratory were subsequently retracted (Braunstein, 1960). These results clearly indicate that the α-hydrogen of the amino acid is not labilized during decarboxylation.

Amino acids which lack an α-hydrogen cannot undergo decarboxylation by a mechanism, such as that of Werle and Koch, which requires the participation of an imino acid form. However, Weissbach, Lovenberg, and Udenfriend (1960) have shown that aromatic α-methyl amino acids were decarboxylated by a

partially purified preparation of aromatic L-amino acid decarboxylase. Furthermore, nonenzymatic decarboxylation, both with and without transamination, of α-amino-isobutyric acid and α-methylserine in the presence of pyridoxal was demonstrated by Kalyankar and Snell (1962). Nonenzymatic decarboxylation of α-methylaminomalonic acid in the presence of pyridoxal or pyridoxal phosphate was observed by Thanassi and Fruton (1962).

In addition to showing that one and only one atom of deuterium is introduced into the product amine by enzymatic decarboxylation in D_2O, Mandeles, Koppelman, and Hanke (1952) showed that decarboxylases catalyze two other reactions: hydrogen exchange of amines in D_2O with the introduction of not more than one atom of deuterium and carboxylation of amines to produce L-α-amino acids. This is strong evidence that the mechanism of decarboxylase action is stereospecific.

The product of decarboxylation in D_2O must be either the R or the S enantiomer, the former corresponding to retention of configuration and the latter to inversion. Retention of configuration might be expected since the side chain probably remains bound to the enzyme in the same orientation throughout the reaction (cf. Ogston, 1948; Palekar, Tate and Meister, 1971). Thus, deuterium should enter at the same stereospecific site from which CO_2 is removed. That this is the case was elegantly demonstrated by Belleau and Burba (1960). These workers prepared both enantiomers of α-deuterotyramine by stereospecific synthesis. The deuterium isotope effects on oxidation of the amines by monoamine oxidase were then determined (2.00 for the R and 1.25 for the S enantiomers) and compared with the isotope effect for the enantiomers prepared enzymatically by decarboxylation of tyrosine in D_2O or of α-deuterotyrosine in water (2.3 and 1.00, respectively). Thus, the

product of decarboxylation in D_2O must correspond to the R-form, proving that configuration is retained.

O'Leary (1969) has studied the ^{13}C isotope effect in the enzymatic decarboxylation of glutamic acid. Using a highly purified preparation of the E. coli enzyme, an isotope effect of $k^{12}/k^{13} = 1.0172$ was observed. Although this isotope effect is somewhat smaller than those usually obtained in nonenzymatic decarboxylations (Yankwich and Buddenbaum, 1967), O'Leary argued that the greater stabilization of the transition state afforded by enzyme catalysis would necessitate less carbon-carbon bond breaking in the transition state and he concluded that decarboxylation was the rate-limiting step. This is in contrast to the results of Seltzer, Hamilton, and Westheimer (1959) who studied oxalacetate decarboxylase (a non-pyridoxal phosphate enzyme which requires metal ions) from Micrococcus lysodeikticus. These authors found a ^{13}C isotope effect of 1.06 in the nonenzymatic, metal ion-catalyzed decarboxylation, but no measurable effect for the enzyme catalyzed reaction.

Almost nothing is known about the role of the protein portion of enzyme in the mechanism of decarboxylation. Strausbauch and Fischer (1970b) determined the sequence of a nonapeptide presumed to be near the active site of glutamate decarboxylase from E. coli. Similar but less complete results had been reported by Mekhanik and Torchinskii (1970). The enzyme was reduced with $NaBH_4$ and then digested with trypsin. The peptide containing the 5'-phosphopyridoxal residue was purified and its sequence determined as:

Ser-Ile-Ser-Ala-Ser-Gly-His-Lys(P-Pxy)-Phe (Strausbauch and Fischer, 1970b)

Ser-(Gly,Ala,Ile,His,Ser_2)-Lys(P-Pxy)-Phe (Mekhanik and Torchinskii, 1970).

Strausbauch and Fischer (1970b) specifically note three features of this structure. First, hydrophilic and hydrophobic residues alternate for six

residues preceding the lysyl residue, suggesting the possibility that the peptide lies in a β-conformation with hydrophilic side chains on one side and hydrophobic residues on the other. Second, the lysyl residue is followed by phenylalanine, which is also found after the pyridoxal phosphate-binding site of rabbit muscle glycogen phosphorylase. Third, the lysyl residue is preceded by a histidyl residue which may participate in the reaction. There are also several seryl residues present, which could also be reactive. It is interesting to note that isoleucine precedes the active serine in rabbit phosphorylase and that alanine precedes the active serine in phosphoglucomutase from several species (Dayhoff and Eck, 1968).

The amino acid sequence near the pyridoxal phosphate-binding site arginine decarboxylase from E. coli has recently been reported as:

Ala-Thr-His-Ser-Thr-His-Lys(P-Pxy)-Leu-Leu-Asn-Ala-Leu-Ser-Gln-Ala-Ser-Tyr

(Boeker, Fischer and Snell, 1971).

As the authors note, "of the eight corresponding residues known for each enzyme, three are identical (Ser,His,Lys) and two represent conservative substitutions (Thr-Ser and Leu-Phe) in terms of both function and the genetic code". A histidyl residue precedes the pyridoxal phosphate-binding residue in both decarboxylases and in the β chain of the tryptophan synthetases from E. coli and Pseudomonas putida (Fluri et al., 1971; Maurer and Crawford, 1971). Since both α-decarboxylation and β-replacement reactions are thought to require specific protonation of the α-carbon of the substrate, Boeker, Fischer, and Snell (1971) suggest this as a possible role for the conserved histidyl residue.

Both arginine and glutamate decarboxylases from E. coli contain cysteinyl residues (Boeker, Fischer, and Snell, 1969; Strausbauch and Fischer, 1970a)

although most of them are not available for titration in the native enzymes. In arginine decarboxylase, no sulfhydryl groups are titrated in the native, associated (23S) enzyme, only one in the 8S subunit, and seven in the denatured enzyme. In glutamate decarboxylase, one sulfhydryl group per subunit molecular weight was titrated, while ten were titrated in the presence of sodium dodecylsulfate. Neither enzyme contains cystine. Tate and Meister (1968) studied the sulfhydryl groups of aspartate β-decarboxylase from Alcaligenes faecalis and found that p-mercuribenzoate titration of the holoenzyme increased its activity, while similar treatment of the apoenzyme resulted in a partial loss of activity which could not be restored by pyridoxal phosphate. When p-mercuribenzoate was removed from either apo- or holoenzyme by dialysis against cysteine, the original activity was restored. These authors concluded that free sulfhydryl groups were not directly required for catalytic activity, or for the binding of coenzyme, but that the introduction of p-mercuribenzoate could affect the conformation so as to increase or decrease the activity.

Physiological and pharmacological properties of the product amines. Aromatic L-amino acid decarboxylase is widely distributed in animal tissues and is thought to catalyze the decarboxylation of a number of aromatic amino acids to the corresponding amines. These amines and many of their derivatives -- among which are dopamine, norepinephrine, epinephrine, serotonin, phenylethylamine, tryptamine, the tyramines, and histamine -- have pronounced physiological effects both peripherally and in the central nervous system, hence the terms "biogenic" or "sympathomimetic" amines. All these amines are found in normal human urine (Sjoerdsma et al., 1959 a,b; Jepson et al., 1960; Rodnight, 1956; von Euler and Hellner, 1951; Roberts and Adam, 1950). They are apparently produced by mammalian decarboxylase(s), since

neither controlled diets nor sterilization of the gut changes significantly the pattern of amine excretion (Jepson et al., 1970).

Dopamine. The pathway of norepinephrine biosynthesis was established in the late 1950's although originally proposed by Blaschko as early as 1939 (see Udenfriend, 1966). Blaschko (1957) was apparently the first to suggest that dopamine was not simply an intermediate in this pathway, but might have regulatory significance itself. Shortly thereafter, Carlsson (1959) proposed that it might have a role as a neurotransmitter in the central nervous system.

Carlsson noted that administration of reserpine depleted dopamine in the corpus striatum and produced extrapyramidal effects not unlike those seen in Parkinson's syndrome. Sano et al (1959) determined the distribution of dopamine in various regions of human brain. High concentrations were found in the caudate nucleus, putamen and hypothalamus. With the development of sensitive histochemical fluorescence techniques, it became possible to identify and map dopaminergic neurons in the brain (Hillarp, Fuxe, and Dahlström, 1966). These workers have found cell bodies which apparently contain dopamine in the lower portion of the mesencephalon, particularly the substantia nigra and nucleus interpeduncularis. The corpus striatum, caudate nucleus, putamen, nucleus accumbens, and tuberculum olfactorium were shown to be rich in very fine nerve terminals containing dopamine. These results must be interpreted with caution, since the histofluorescent method does not directly distinguish dopamine from norepinephrine. Dopamine and norepinephrine neurons are identified by introducing perturbations, e.g., lesions or drug treatment, and correlating the fluorescence results with specific biochemical assays for each amine in particular regions of the brain.

Hornykiewicz and his coworkers assayed the dopamine content of the brains of parkinsonian patients after autopsy and found strikingly reduced levels in the striatum (see Hornykiewicz, 1966). The finding that reduced striatal dopamine was correlated with parkinsonism suggested a possible mode of therapy; if dopamine levels could be replenished, the symptoms might be alleviated. Since dopamine does not pass the blood-brain barrier, DOPA was investigated as a therapeutic agent. Carlsson (1959) had already noted that DOPA could reverse the effects of reserpine. The success of L-DOPA therapy has been the subject of much recent publicity and a number of scholarly reviews (for example, Cotzias et al., 1971; Calne and Sandler, 1970). Briefly, its history is as follows. Birkmayer and Hornykiewicz (1961) were the first to administer L-DOPA in 50-150 mg intravenous doses to parkinsonian patients. Although there was noticeable improvement, especially in the akinesia, the results were not especially exciting. These results were widely confirmed, but Hornykiewicz (1966) noted as late as 1966 that "the therapeutic value of dopa . . . [had] not yet been definitely established . . . due to the fact that the unpleasant side-effects of dopa . . . make it practically impossible to inject doses which are high enough to increase the dopamine level sufficiently in the striatum". Cotzias, van Woert, and Schiffer (1967), however, were able to give very large oral doses of DOPA by gradually increasing the dose over a long period of time and titrating the rate of increase and the maximum dose against the adverse effects. In eight of sixteen cases, substantial, even dramatic, improvement was observed. These results have been generally confirmed and L-DOPA treatment is now the method of choice in the management of parkinsonism.

The requirement of large doses of DOPA for therapeutic results is probably at least partly due to the high level of the decarboxylating enzyme in the periphery. For example, Wurtman, Chou, and Rose (1970) found that, in mice, less than 0.1% of injected ^{14}C-DOPA entered the brain. Decarboxylase inhibitors which do not penetrate the brain, such as N-(DL-seryl)-N'-(2,3,4-trihydroxybenzyl)-hydrazine (HCl, Ro 4-4602), might be of great value in this regard (see Bartholini et al., 1967; Bartholini and Pletscher, 1968).

A possible explanation for the increasing therapeutic efficacy of the drug has been suggested by Dairman, Christenson, and Udenfriend (1971) who observed that the chronic administration of L-DOPA to rats led to a decrease of as much as 50% in the content of the decarboxylase in the liver. Since the liver accounts for most of the total decarboxylase activity in the rat, a substantial decrease in this activity could alleviate the peripheral side effects while making more DOPA available for entry into the brain.

Although the development of L-DOPA therapy is an example of a relatively rational, deductive approach to drug therapy, the specific lesion which results in parkinsonism is still not known. The detailed etiology may be complex, as is suggested by the degeneration of entire groups of neurons, or it may be a relatively simple defect in one or two enzymes. In the latter case, the two obvious possibilities are tyrosine hydroxylase and DOPA decarboxylase. Of these, the former appears the more likely at first, since there is substantial evidence that tyrosine hydroxylase is the rate-determining step (Udenfriend, 1966) and since parkinsonian patients clearly retain at least some decarboxylase activity. In fact, Martin (1971) has proposed, on the basis of epidemiological and biochemical evidence (which is indirect at best), that a genetic deficiency of tyrosine hydroxy-

lase is responsible for spontaneously occurring parkinsonism. Unfortunately, it has not been possible to establish whether tyrosine hydroxylase levels are reduced in Parkinson's disease, due to the difficulty in obtaining fresh autopsy material and to the problem of defining proper control levels, which show great variations. The few data which have been obtained have not been published (Udenfriend, personal communication; McGeer, 1971). On the other hand, it should be remembered that the kinetic studies were done with guinea pig heart; there is no evidence that tyrosine hydroxylase is the rate-limiting enzyme in human brain. Lloyd and Hornykiewicz (1970b) have published data showing that the mean levels of DOPA decarboxylase in the putamen, caudate nucleus, and hypothalamus of patients with Parkinson's disease were much lower than the mean levels in the same regions of patients with no known neurological disorder. Again, enormous individual deviations were observed. For example, the mean level of decarboxylase in the putamens of nine control patients was 864 $\pm$ 271 (S.E.). This would imply a standard deviation almost as large as the mean itself. It is clearly impossible to define a meaningful "normal" level in the face of such variation. Still, the differences in the regions listed do appear to be statistically significant. Thus, if decarboxylase levels in Parkinson's disease are lower than those of tyrosine hydroxylase, this may explain the reduced content of dopamine. As Lloyd and Hornykiewicz point out, however, the data do not distinguish cause and effect; that is, whether the lower decarboxylase levels are a primary event or a consequence of some other prior lesion.

It should be mentioned that the mode of action of L-DOPA in parkinsonism outlined above has been questioned (Sourkes, 1971; Sandler *et al.*, 1971). While these questions must be investigated seriously, it is this author's

view that there is not sufficient experimental evidence at this time to justify modification of the simplest hypothesis; i.e., that L-DOPA replenishes depleted dopamine stores in the central nervous system through the action of the decarboxylase.

Norepinephrine. Dopamine β-hydroxylase (EC 1.14.2.1), a copper-containing mixed-function oxidase, catalyzes the conversion of dopamine to norepinephrine (Neri et al., 1956; Levin, Levenberg, and Kaufman, 1960; Friedman and Kaufman, 1965). Von Euler (1948) was able to establish that norepinephrine is the neurotransmitter of the sympathetic nervous system. In the periphery, norepinephrine is localized in the adrenal medulla, where it comprises about 10% of the total catecholamines (von Euler, Franksson, and Hellström, 1954) and in sympathetic nerves (von Euler and Purkhold, 1951). It is also found in the central nervous system and neurons containing norepinephrine have been mapped by the histofluorescence method (Hillarp, Fuxe, and Dahlström, 1966). Most of the cell bodies containing norepinephrine were found in the pons and medulla oblongata. Nerve terminals containing norepinephrine were found in the prosencephalon, especially the hypothalamus and limbic lobe, and in the neocortex. Axons from the norepinephrine-containing neurons in the medulla oblongata were found to extend down the spinal cord as far as the lumbosacral region. Pharmacologically, norepinephrine has many and pronounced actions, especially vasoconstrictor and pressor effects (Franzen and Eysell, 1969).

Epinephrine. In the adrenal medulla, there is a specific enzyme, phenylethanolamine N-methyl transferase, which catalyzes the transfer of a methyl group from S-adenosylmethionine to norepinephrine (Kirshner and Goodall, 1957). Epinephrine, the product of this reaction, is the major adrenal

medullary catecholamine in man (von Euler, Franksson, and Hellström, 1954). Epinephrine has many pronounced physiological effects, a number of which are mediated through adenosine-3',5'-monophosphate via activation of the enzyme adenyl cyclase (Robison, Butcher, and Sutherland, 1968). Among its pharmacological effects are vasoconstriction or vasodilation (depending on dose); it stimulates respiration, and dilates the bronchi; it increases the pulse frequency, the basal metabolic rate and blood glucose levels (Franzen and Eysell, 1969).

Serotonin. Serotonin, or 5-hydroxytryptamine, was first isolated by Rapport, Green and Page (1948) and identified by Rapport (1949) in bovine serum. It is present in many peripheral tissues, especially the platelets. Twarog and Page (1953) demonstrated the presence of serotonin in the brain. Its presence in the brain and Gaddum's finding (1953) that lysergic acid diethylamide, a powerful hallucinogen, antagonized the contractile effect of serotonin on rat uterus, suggested that it might have a role in brain function. Hillarp, Fuxe and Dahlström (1966) have reviewed the histofluorescent mapping of neurons containing serotonin in the brain. The nerve cells are found mainly in the mesencephalon and are almost exclusively localized in the raphe complex, as opposed to the lateral position of the catecholamine-containing nerve cells. After unilateral lesions in the medial forebrain bundle, the subthalamus, and the ventral part of the crus cerebri, marked decreases in the number of serotonin-containing terminals in the hypothalamus, globus pallidus, amygdaloid cortex, and septal area were observed. Serotonin-containing terminals were observed to make contacts only with cells which did not contain monoamines.

Serotonin has been implicated in a variety of physiological and mental processes and disorders. The following were selected as being among the more interesting to the author. Armstrong and Robinson (1954) showed the urinary excretion of serotonin, 5-hydroxyindoleacetic acid, and tryptamine was markedly reduced in phenylketonuric patients. Lance, Anthony and Gonski (1967) have found that serotonin has a strong constrictor effect on the arteries of the scalp and that migraine attacks were correlated with reduced plasma levels of the amine. Bazelon et al. (1967) have found reduced blood levels of serotonin in infants with Down's syndrome. When 5-hydroxytryptophan was fed, the hypotonia was significantly improved. No inferences have been drawn as to the effect of the amino acid on intelligence in mongolism. Jouvet (1969) placed experimental lesions in the raphe system of cats, and observed a decrease in sleeping time which was correlated with the degree of depletion of cerebral serotonin. Moreover, p-chlorophenylalanine, a specific inhibitor of tryptophan hydroxylase, also caused insomnia which could be reversed by administration of 5-hydroxytryptophan. Coleman (1971) has noted unusually low serotonin levels in the platelets of hyperactive children. In two cases, in which variations in the serotonin levels were observed, the severity of hyperactivity was quantitatively correlated with decreased serontonin levels. Carcinoid tumors of the argentaffin cells produce a variety of clinical symptoms, at least some of which are thought to be due to the excess production of serotonin by the tumor (Page, 1968; Franzen and Eysell, 1969). Certainly the metabolism of tryptophan and other indole derivatives is profoundly disturbed. For example, Sjoerdsma, Weissbach, and Udenfriend (1956) found that as much as 60% of the dietary intake of tryptophan may be accounted for by urinary 5-hydroxyindoles, compared with only 1% in normal persons. These workers also observed moderately to greatly increased blood levels of serotonin.

Phenylethylamine. Phenylethylamine, the decarboxylation product of phenylalanine, is normally present in human urine and its level is increased in the urine of phenylketonuric patients (Jepson et al., 1960). It has potent pharmacological effects, including hypertension and stimulation of the central nervous system sometimes leading to convulsions. These effects apparently result mainly from the release of norepinephrine from tissue stores (Franzen and Eysell, 1969).

Tryptamine. Tryptophan is also a substrate of aromatic L-amino acid decarboxylase and its decarboxylation product, tryptamine, is a constituent of normal human urine (Sjoerdsma et al., 1959). Tryptamine causes vasoconstriction and bronchoconstriction and increases the pulse rate. It is a stimulant of the central nervous system and may cause convulsions and tremor (Franzen and Eysell, 1969).

Tyramine. The ortho- and meta- isomers of tyrosine are thought to be relatively good substrates for aromatic L-amino acid decarboxylase, while ordinary p-tyrosine is decarboxylated at a slow, but readily measurable, rate (Lovenberg, Weissbach, and Udenfriend, 1962). The products of this reaction, the o-, m- and p-tyrmaines, are all found in normal human urine (Jepson et al., 1969), but their physiological role, if any, is not understood. Pharmacologically, tyramine causes vasoconstriction and increases in the rate and force of the heartbeat, among other effects (Franzen and Eysell, 1969).

Histamine. Lovenberg, Weissbach, and Udenfriend (1962) reported that histidine is also decarboxylated by aromatic L-amino acid decarboxylase, although the kinetics of this reaction are such that it is probably not of great physiological significance. There exists another decarboxylase (EC 4.1.1.22) which is specific for histidine (Weissbach, Lovenberg, and

Udenfriend, 1961; Lorenz et al., 1969; Schwartz, Lampart and Rose, 1970), and which is probably responsible for the physiological production of histamine. It has been speculated that histamine may have a role as a neurotransmitter in the central nervous system (see Schwartz, Lampart, and Rose, 1970). Pharmacologically, histamine is an extremely potent dilator of the capillaries, but with increasing size of the vessels, its action becomes vasoconstrictor, especially in animals. It is excitatory toward smooth muscle and is liberated in the course of certain antigen-antibody reactions (Franzen and Eysell, 1969).

Previous studies of aromatic L-amino acid decarboxylase. It will be assumed that DOPA and 5-hydroxytryptophan decarboxylase are the same enzyme. The justification of this assumption will become apparent in the course of this dissertation.

Distribution. Many studies of the distribution of aromatic L-amino acid decarboxylase have appeared. DOPA decarboxylase activity was first demonstrated in guinea pig kidney (Holtz, Heise, and Ludtke, 1938), which is the richest known source of the enzyme. Indeed, the kidneys of many species have high levels of the enzyme (see references below and Table I of this dissertation). Holtz and coworkers showed that the activity was also present in liver, intestine, and pancreas (Holtz, Credner, and Reinhold, 1959; Holtz, Credner, and Strübing, 1943; Holtz, Reinhold and Credner, 1939). Langemann (1951) was apparently the first to demonstrate the presence of the enzyme in the adrenal gland. Clark, Weissbach, and Udenfriend (1954) found 5-hydroxytryptophan decarboxylase activity in the kidneys, stomach, liver, intestine, and lungs of guinea pig, the kidneys and liver of hog,

and the kidneys of rat, rabbit and dog. Holtz and Westermann (1956) found DOPA decarboxylase activity in the peripheral sympathetic ganglia, in the postganglionic adrenergic neurons, and in the sympathetic trunk. Lower levels were also found in certain regions of the brain and in the spinal cord. Davis and Awapara (1960) found aromatic L-amino acid decarboxylase activity in the kidneys, liver, small intestine, brain, lungs, and spleen of rats. In rabbits and guinea pigs, they also found activity in the heart and adrenals.

Bogdanski, Weissbach, and Udenfriend (1957) reported the distribution of serotonin, 5-hydroxytryptophan decarboxylase, and monoamine oxidase in dog brain. High decarboxylase levels were found in the caudate nucleus, hypothalamus, septal region, and midbrain. There was a qualitative correlation between serotonin content and decarboxylase levels, the notable exceptions being the amygdala (high serotonin, low enzyme) and caudate nucleus (high enzyme, low serotonin).

In an important study, Kuntzman et al. (1961) reported the detailed mapping of DOPA and 5-hydroxytryptophan decarboxylase activities in cat brain. The ratio of activities for the two substrates was approximately constant in all areas. This, of course, suggested that the two activities are properties of a single protein. Activity was high in brain stem, the caudate nucleus, the reticular formation, the hypothalamus, and certain portions of the thalamus and rhinencephalon. It was particularly low in the white matter, cerebellum, and cortex. Although levels of enzyme and the levels of norepinephrine were not strictly correlated in the various regions of the brain, those areas which contained high levels of the amine also had relatively high enzyme levels. The highest enzyme level found

was in the caudate nucleus, which does not have a high level of norepinephrine or serotonin, but which does have a high level of dopamine (Sano et al., 1959). A similar, though less detailed, distribution was reported by de Ropp and Furst (1966) in rat brain.

In view of the demonstrable presence and physiological importance of catecholamines and serotonin in human brain, it is surprising that many workers have failed to detect significant levels of aromatic L-amino acid decarboxylase in this tissue (Robins et al., 1967; Vogel, Orfei, and Century, 1969; Vogel, McFarland, and Prince, 1970). Lloyd and Hornykiewicz (1970a) finally succeeded in demonstrating the presence of a low but significant level of decarboxylase activity in human brain. The activity showed considerable variation among individuals and even between the right and left sides of the same brain. These workers (1970b) also reported that the level of decarboxylase was significantly reduced in the caudate nucleus and putamen of patients with Parkinson's disease.

The subcellular distribution of aromatic L-amino acid decarboxylase has been considered in many studies. In most studies the enzyme was found almost exclusively in the soluble fraction, suggesting that it exists, in vivo, free in the cytoplasm (Clark, Weissbach, and Udenfriend, 1954; Blaschko, Hagen, and Welch, 1955; Laduron and Belpaire, 1968). However, Udenfriend (1966) questioned this hypothesis, suggesting that the decarboxylase may, in fact, be particle-bound in vivo and "leak out" during homogenization. He pointed out that the enzymes catalyzing the preceding and following steps in norepinephrine synthesis have been shown to be associated with particulate fractions (Nagatsu, Levitt, and Udenfriend, 1964; Levin, Levenberg, and Kaufman, 1960). Thus, if the decarboxylase

is cytoplasmic, two further steps become necessary: transport of DOPA into the cytoplasm and transport of dopamine back into the particles. It might be added that, in the adrenal medulla, phenylethanolamine N-methyl transferse is also thought to be cytoplasmic (Kirshner and Goodall, 1957; Axelrod, 1962) necessitating still two more steps in epinephrine synthesis: transport of norepinephrine into the cytoplasm and transport of epinephrine into the storage granules. The interjection of such seemingly unecessary transport processes does not appear to be efficient and one would expect accumulation of DOPA or dopamine. This is particularly so when one notes that the K_m value of tyrosine hydroxylase is about one-tenth that of the decarboxylase, which in turn is about one-tenth that of dopamine β-hydroxylase. Yet, these intermediates "seldom accumulate in sympathetic nerves" (except in certain tissues where dopamine has a role other than as an intermediate). Udenfriend presented evidence that in brain stem some 60% of the decarboxylase activity was associated with particles when tissues were carefully homogenized and that it could be released by sonication. Similar results were presented by Rodriguez deLores Arnaiz and deRobertis (1964). It is likely, however, that this "bound" enzyme is contained in "pinched off" nerve endings -- i.e., "synaptosomes" (Whittaker, Michaelson, and Kirkland (1964) -- which contain a considerable amount of trapped cytoplasm. The problem of the subcellular localization of aromatic L-amino acid decarboxylase must, therefore, be regarded as unsolved. It is hoped that the development of electron-dense immunological staining procedures (see, for example, McLean and Singer, 1970) will soon provide an answer.

Substrate specificity. At the time of the discovery of 5-hydroxytryptophan decarboxylase activity (Udenfriend, Clark, and Titus, 1953), evidence from

the laboratories of Blaschko and of Sourkes was accumulating that "DOPA decarboxylase" was not strictly specific for DOPA, but could act on a variety of phenylalanine derivatives (Blaschko et al., 1949; Blaschko, 1949; Blaschko, 1950; Blaschko and Langemann, 1951; Sourkes, Heneage, and Trano, 1952; Sourkes, 1954a; Sourkes, 1954b; see also Ferrini and Glässer, 1964). It was not until some years later that investigators began to suspect that DOPA decarboxylase and 5-hydroxytryptophan decarboxylase might be the same enzyme. In their most highly purified preparations of 5-hydroxytryptophan decarboxylase from guinea pig kidney, Clark, Weissbach and Udenfriend (1954) found a significant level of DOPA decarboxylase activity. They ascribed this to contamination by another enzyme for the following reasons:

(1) the ratio of DOPA to 5-hydroxytryptophan decarboxylase activity could be varied by a factor of more than twenty by inactivating and purifying procedures;

(2) the pH optima were widely different;

(3) pyridoxal phosphate requirements were different.

More recent studies, however, have generally favored the hypothesis that a single enzyme acts on both substrates. The evidence may be classified as follows:

(1) Inhibitors of DOPA decarboxylation also inhibit 5-hydroxytryptophan decarboxylation (Westermann, Balzer, and Knell, 1958; Yuwiler, Geller and Eiduson, 1959, 1960; Smith, 1960; Lovenberg, Weissbach, Udenfriend, 1962). Moreover, α-methyl-5-hydroxytryptophan is a good inhibitor of DOPA decarboxylation in vivo (Murphy and Sourkes, 1961).

(2) DOPA and 5-hydroxytryptophan are mutually inhibitory. This was first shown by Yuwiler, Geller and Eiduson (1959), who found that the rate of CO_2 formation in the presence of both substrates was not the sum of the rates in the presence of either substrate alone, but was intermediate between them. This was soon confirmed by Werle and Aures (1959). Likewise, Fellman (1959) reported that 5-hydroxytryptophan inhibited the decarboxylation of DOPA. However, his results do not support his assertion that the inhibition is competitive. On the basis of similar experiments which also included o- and m-tyrosine, Hagen (1962) concluded that all four amino acids were substrates for the same enzyme. Rosengren (1960) and Streffer (1967) showed that the mutual inhibition of DOPA and 5-hydroxytryptophan was competitive. Lovenberg (1962) has shown that 5-hydroxytryptophan inhibits the decarboxylation of phenylalanine and that phenylalanine competitively inhibits the decarboxylation of tryptophan, suggesting the existence of a single decarboxylase for all these amino acids.

(3) DOPA and 5-hydroxytryptophan decarboxylase activities are not separated by purification procedures (Werle and Aures, 1959; Fellman, 1959; Rosengren, 1960; Hagen, 1962; Awapara, Sandman, and Hanly, 1962; Coulson, Bender and Jepson, 1969). Lovenberg, Weissbach, and Udenfriend (1962) have provided the strongest evidence of this type. These investigators demonstrated that decarboxylase activities for all the naturally occurring aromatic L-amino acids were purified some 60-fold in parallel. Furthermore, the activities for 5-hydroxytryptophan, tryptophan, and

histidine in a partially purified preparation cochromatographed on DEAE-cellulose. Their most purified preparations also were active toward a variety of aromatic L-amino acids which do not occur naturally. In view of this broad substrate specificity, these authors proposed the name, "aromatic L-amino acid decarboxylase", which is now in general use.

Although it is generally agreed that the substrate specificity of aromatic L-amino acid decarboxylase is rather broad, not all authors are in agreement about every putative substrate (see, for example, Awapara, Sandman, and Hanly, 1962; Hagen, 1962; Coulson, Henson and Jepson, 1968). It is possible that species differences are involved in at least some cases. Furthermore, many different assay procedures have been used in different laboratories. Some of these may not be sufficiently sensitive to detect decarboxylation of the poorer substrates, such as p-tyrosine and histidine. Most of the studies discussed above made use of enzyme preparations from peripheral tissues. Some evidence has recently been reported (Sims and Bloom, 1971) that DOPA and 5-hydroxytryptophan are decarboxylated by two distinct enzymes in rat brain. These authors reported that, after intracisternal administration of 6-hydroxydopamine to rats, DOPA decarboxylase activites were substantially reduced in certain areas of the brain, while 5-hydroxytryptophan decarboxylase activities were no different from controls.

By contrast, all investigators apparently are agreed that the enzyme is specific for the L-form of all substrates. In every case in which D-isomers have been tested, they have been found inactive (Lovenberg, 1962). Furthermore, only the L-isomer of the competitive inhibitor, α-methylDOPA, is active (Lovenberg et al., 1963).

Interaction with coenzyme. Green, Leloir and Nocito (1945) showed that DOPA decarboxylase could be inactivated by dialysis against dilute ammonia, and that the activity could be partially restored by the addition of pyridoxal phosphate and cysteine. Schales and Schales (1949) obtained approximately a two-fold stimulation of their preparation by pyridoxal phosphate. Furthermore, the enzyme was inhibited by hydroxylamine. Schott and Clark (1952) demonstrated that DOPA decarboxylase was inhibited at high concentrations of substrate. This effect could be prevented by incubating the enzyme with pyridoxal phosphate either before or during assay. However, when the pyridoxal phosphate was preincubated with DOPA, its ability to activate the enzyme and prevent inhibition was lost. The inactive product had an absorption peak at 327 nm and it was shown that a variety of aromatic amino acids and amines could react with pyridoxal phosphate to give similar spectra. Synthesis of a model compound suggested that the product of the reaction is a tetrahydroisoquinoline compound (see also Buzard and Nytch, 1959).

Clark, Weissbach, and Udenfriend (1954) found that their partially purified 5-hydroxytryptophan decarboxylase from guinea pig kidney was almost completely inhibited by 10^{-3} M hydroxylamine or semicarbazide. However, the addition of pyridoxal phosphate afforded only a slight stimulation of enzyme activity and did not reverse the inhibition due to the carbonyl reagents. On the other hand, pyridoxal phosphate did reverse the inhibition of DOPA decarboxylation by semicarbazide in the same preparations.

Buzard and Nytch (1957a) observed a two-fold stimulation of 5-hydroxytryptophan decarboxylase from rat kidney in the presence of 7×10^{-4} M pyridoxal phosphate. Partial inhibition of the enzyme by 5×10^{-4} M hydroxylamine was

reversed by the addition of 10^{-3} M pyridoxal phosphate. Furthermore, pyridoxine deficiency resulted in a lower enzyme level which could be restored to normal by the addition of pyridoxal phosphate *in vitro* (see also Buzard and Nytch, 1957b). Almost simultaneously, Weissbach *et al.* (1957) reported a partial reversal of semicarbazide inhibition by pyridoxal phosphate. It was also shown that the enzyme from rat kidney was partially resolved by freezing and thawing of the tissue and that the *in vivo* enzyme activity in chicks was evidently reduced by vitamin B_6 deficiency. Eberle and Eiduson (1968) examined the effects of maternal pyridoxine deficiency on aromatic L-amino acid decarboxylase activity in rats during development. Fetuses were apparently protected from the deficiency during gestation, but during nursing of the vitamin-deficient mothers, the enzyme levels failed to increase normally. The activity could be substantially increased by the addition of pyridoxal phosphate to the *in vitro* assay mixture.

Werle and Aures (1959) were able to resolve their enzyme preparation by extended dialysis against EDTA and found a K_m of 9×10^{-8}M for the apoenzyme-coenzyme complex. The DOPA decarboxylase preparation of Fellman (1959) from bovine adrenal medulla showed an obligate requirement for the cofactor.

Awapara, Sandman, and Hanly (1962) purified DOPA decarboxylase activity from rat liver about 60-fold. This preparation had a weak absorption band at 415 nm which was not increased by incubation of the enzyme with pyridoxal phosphate, although the enzyme activity was stimulated by the addition of pyridoxal phosphate. The extent of stimulation depended upon a number of factors, notably the pH and the substrate. The authors concluded that a portion of the coenzyme was tightly bound, while another portion was easily removed.

All of the above studies strongly indicate that pyridoxal phosphate is the coenzyme of the decarboxylase and there appears to be no reason to doubt that this is the case. However, there is much contradictory evidence concerning the nature of the binding of the cofactor, with reports ranging from very loose binding to very tight, if not irreversible, binding. As pointed out by Werle and Aures (1959) and by Awapara, Sandman, and Hanly (1962), many of these contradictions may be due to the effects of such variables as pH, prior treatment, substrate, and cofactor concentration. Some may be due to species differences as well.

Effect of pH. Although results vary from laboratory to laboratory, it may be generally stated that the pH optimum for enzymatic decarboxylation of DOPA (approximately 7) is apparently somewhat lower than that for the other substrates (approximately 8) (Schales and Schales, 1949; Clark, Weissbach, and Udenfriend, 1954; Werle and Aures, 1959; Yuwiler, Geller and Eiduson, 1960; Awapara, Sandman, and Hanly, 1962; Lovenberg, Weissbach, and Udenfriend, 1962; de Ropp and Furst, 1966; Håkanson, 1967; Coulson, Bender, and Jepson, 1969). Lovenberg, Weissbach, and Udenfriend (1962) attributed the lower pH optimum for DOPA to the instability of catechols at alkaline pH. Werle and Aures (1959) showed that the pH optima for DOPA, 3-hydroxyphenylserine, and 3,4-dihydroxyphenylserine depended upon the chemical nature of the buffer and Håkanson (1967) has argued on theoretical grounds that, for pyridoxal phosphate enzymes generally, the pH optimum should depend upon the concentration of substrate. Thus, the diversity of values reported in the literature may not be merely artifactual. Of course, species differences may also be involved.

Sulfhydryl requirement. A few workers have investigated the effect of sulfhydryl reagents (i.e., chemicals which react with thiols) and sulfhydryl

compounds on the enzyme. Buzard and Nytch (1957a) showed that their preparation of 5-hydroxytryptophan decarboxylase from rat kidney was partially inhibited by 10^{-3} M p-chloromercuribenzoate, o-iodosobenzoate, and γ-(p-arsenosophenyl) butyrate. The inhibition could not be prevented or reversed by $5x10^{-3}$ M cysteine or 2,3-dimercaptopropanol (BAL). The thiols alone had no effect. Fellman (1959) reported that DOPA decarboxylase from bovine adrenal medulla was completely inhibited by $5x10^{-4}$ M N-ethylmaleimide. Lovenberg, Weissbach, and Udenfriend (1962) found that aromatic L-amino acid decarboxylase from guinea pig kidney was inhibited by 10^{-5} M p-chloromercuribenzoate, but, again, the addition of sulfhydryl compounds to enzyme assay mixtures had no effect. Vogel, Snyder and Hare (1970) reported 50% inhibition of the enzyme from human liver by $3x10^{-5}$ M p-chloromercuribenzoate. It should be noted that all these investigators simply added the sulfhydryl reagents to the assay mixtures. To rule out possible side reactions with cofactor or substrate, unreacted reagents should have been removed by the addition of a thiol compound in excess and/or dialysis before assay.

Effect of metal ions. With the exception of two unconfirmed reports (Beiler and Martin, 1954; Steensholt, Flikke, and Joner, 1955, cited by Fellman, 1959) no evidence has been obtained that any metal ion is required by the enzyme. Beiler and Martin (1954) found that EDTA inhibited the decarboxylation of 5-hydroxytryptophan and that the inhibition could be overcome by any of several divalent cations. However, these workers assayed at pH 6.7, far below the pH optimum for 5-hydroxytryptophan. Lovenberg (1962, pp. 44-45) found that 10^{-3} M EDTA inhibited by about 40% at pH 6.7, but had no effect at his optimal pH of 9.0. Furthermore, Mg^{++} had no effect at either pH. In general, chelating agents and metal ions have been found to

be without effect, except at high concentrations (>10^{-3} M) (Buzard and Nytch, 1957; Fellman, 1959; Perry, Schwartz, and Sahagian, 1969; Vogel, Snyder and Hare, 1970). However, a few metal ions have been found to be strongly inhibitory. These include Cu^{++}, Cd^{++}, Hg^{++}, and Zn^{++}.

Existence of multiple forms of the enzyme. Two reports have appeared which suggest that rat liver aromatic L-amino acid decarboxylase may exist in multiple forms, possibly with different substrate specificities. Coulson, Bender, and Jepson (1969) found four electrophoretically separable activities, all four showing decarboxylase activity toward both DOPA and 5-hydroxytryptophan. Antonas, Coulson, and Jepson (1971) ran rat liver homogenates on polyacrylamide gels. The gels were then incubated with the various amino acids, monoamine oxidase, and a tetrazolium dye. The reduced and precipitated dye was assumed to stain the site of decarboxylation within the gel. A single band stained for 5-hydroxytryptophan, o-tyrosine, and m-tyrosine. Four additional bands were observed with o-tyrosine and 5-hydroxytryptophan, but only two with m-tyrosine. Unfortunately, a full-length report of this work has not yet appeared so a complete evaluation of these data is not possible.

MATERIALS AND METHODS

Ammonium sulfate, urea, guanidine hydrochloride (all "ultra pure"), streptomycin sulfate and amino acids (all L-isomers, unless otherwise specified) were obtained from Mann Research Labs. Omnifluor and radioactive compounds were products of New England Nuclear, unless otherwise specified. L-[Carboxy-^{14}C]tyrosine was freed of possible contamination with DOPA by treatment with alumina, according to the method of Crout (1961); other compounds were used without further purification. Pyridoxal phosphate was supplied by Hoffmann-La Roche, Basle, Switzerland. NCS* was obtained from Amersham-Searle. Polyethylene glycol-6000 was purchased from Matheson, Coleman and Bell. Alumina Cγ gel and hydroxylapatite (Bio-Gel HTP) were from Bio-Rad Labs. The ultrafiltration apparatus was the product of Amicon Corp. and the variable gradient maker was from Phoenix Precision Instrument Co.

Dialysis tubing was treated by boiling for at least 30 minutes, or by stirring at 80° for at least two hours, in a solution containing 0.03M 2-mercaptoethanol, 0.01M sodium phosphate, pH 7.2, and 10^{-3}M sodium EDTA. It was then thoroughly rinsed and stored in distilled water at 5°.

Pyridoxal phosphate was assayed by the fluorometric method of Adams (1969). Protein was assayed by the method of Lowry et al (1951), except in monitoring column effluents, when the absorbance at 280 nm was taken as a measure of protein concentration.

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* All abbreviations are standard IUPAC-IUB abbreviations. "NCS" is a trademark of the Amersham-Searle Corp. for 0.6N solution of a quaternary ammonium base in toluene.

Polyacrylamide gel electrophoresis. Analytical polyacrylamide gel disc electrophoresis was performed by a modification of the method of Davis (1964) or of Williams and Reisfeld (1964). Both stacking and resolving gels were photopolymerized in the presence of 5 or 2.5 μg/ml, respectively, of riboflavin. Electrophoresis was at a constant current of 2mA per gel column and was ended when the bromothymol blue tracking dye reached the anode end of the gel. The gels were stained for at least 3 hours in a solution of Coomassie brilliant blue R250 prepared by diluting a 1% solution 1:20 with 12.5% trichloroacetic acid. They were destained overnight in 50-75 ml/gel of 12.5% trichloroacetic acid and stored permanently in 7.5% acetic acid. Relative amounts of protein in the bands were estimated by scanning the destained gels at 580 nm in a Gilford Model 240 recording spectrophotometer equipped with a Model 2410 linear transport device.

When it was desired to assay gels for enzyme activity, the gels were cut by hand into slices 1 to 5 mm thick. The slices were extruded through an 18 ga. needle in the presence of 0.75 ml of 0.5M sodium phosphate buffer, pH 7.0, directly into assay tubes. Cofactor and substrate were added and the assay carried out as described below.

Assay for DOPA decarboxylase activity. This assay is based on $^{14}CO_2$ evolution from [carboxy-^{14}C]DOPA and makes use of apparatus and procedures previously described by Rhoads and Udenfriend (1968) and by Ellenbogen, Markley and Taylor (1969) for other enzymatic decarboxylations. Assays were performed in a 21 x 145-mm tube having a closed side arm, about 50 mm from the bottom, to which was added 0.3 ml of 35% trichloroacetic acid. The preincubation mixture contained, in a total volume of 1.25 ml: 120 μmoles

of sodium phosphate, pH 7.0; 0.105 μmole of pyridoxal phosphate; 15 μmole of 2-mercaptoethanol; and enzyme. This was preincubated at 37° for 15 minutes, then cooled in an ice bath. Strips of Whatman 3 MM paper (about 15 x 23 mm) were suspended from rubber stoppers by a stainless steel hook, wetted with 0.05 ml of NCS, and allowed to dry for 10-20 minutes. Then 0.25 ml of 0.018M DOPA containing 0.1 μCi of DL-[carboxy-^{14}C]DOPA in 0.02N HCl was added to each tube. The blank was the same, except that water was added instead of enzyme. The tubes were stoppered with the paper strip centered in the tube and were incubated at 37° for 15 minutes. After incubation, the tubes were placed in an ice bath and trichloroacetic acid in the side arm was immediately tipped in and mixed. The tubes were then incubated at 37° for at least 30 minutes, after which the strips were removed and counted in 10 ml of Omnifluor-dioxane scintillation mixture. The production of labeled CO_2 was linear within experimental error as a function of time and enzyme concentration for at least 20 minutes and the first 800 nmoles of CO_2. Although Vogel (1969) reported significant nonenzymatic decarboxylation of DOPA under similar conditions, blank values were consistently low in this work. One unit of activity is defined as that amount of enzyme which produces 1 nmole of CO_2 per minute under the specified conditions.

The other amino acids were assayed in a similar manner substituting Tris-HCl buffer, pH 8.5, for the sodium phosphate. Histidine decarboxylation could be assayed by the above method, but was at the limit of its sensitivity. In this case, the fluorometric procedure of Lovenberg, Weissbach, and Udenfriend (1962) was found to be satisfactory and was therefore used.

Assay for 5-hydroxytryptophan decarboxylase activity. Serotonin extraction method. In the early stages of this work, carboxyl-labeled 5-hydroxytryptophan was not available, so another type of assay was developed. This assay is based on the standard extraction assay for serotonin (Udenfriend, Weissbach, and Clark, 1955), modified to permit extraction of serotonin in a single step with relatively little contamination by 5-hydroxytryptophan. The preincubation mixture contained, in a total volume of 1.25 ml: 125 μmoles of Tris-HCl, pH 8.5; 0.105 μmole of pyridoxal phosphate; 15 μmoles of 2-mercaptoethanol; and enzyme. This was preincubated at 37° for 15 minutes and cooled in an ice bath. Then 0.25 ml of 0.018 M L-5-hydroxytryptophan (Calbiochem), containing 0.5 μCi of 5-hydroxy-DL-[methylene-^{14}C]tryptophan (Amersham-Searle), in 0.02N HCl was added to each tube. The blank was the same, but without enzyme. Incubations were carried out at 37° for 25 minutes after which the reaction was stopped by placing the tubes in a boiling water bath for 2 minutes. They were then cooled on ice, 0.16 g of solid sodium carbonate was added, and the mixtures were extracted with 1.5 ml of a mixture of benzene and butanol. This solvent mixture was prepared by mixing equal volumes of 1-butanol and benzene, then washing the solution with equal volumes of 0.5N sodium hydroxide, 0.5N hydrochloric acid, three times with distilled water, and finally 0.08M Tris-HCl buffer, pH 8.5. Control tubes containing 0.05 μCi of [3'-^{14}C]serotonin (creatinine sulfate complex, Amersham-Searle) and 3 x 10^{-3}M 5-hydroxytryptophan, but no radioactive 5-hydroxytryptophan or enzyme, were also included in order to determine the efficiency of the serotonin extraction, which was about 60%, compared with 0.5% for 5-hydroxytryptophan. A portion (0.5-1.0 ml) of the organic

phase was counted in 10 ml of Omnifluor-dioxane scintillation mixture. The production of [^{14}C]serotonin was linear within experimental error as a function of time and enzyme concentration for at least 25 minutes and the first 300 nmoles formed.

$^{14}CO_2$ Method. About the time the immunological studies of the identity of DOPA decarboxylase and 5-hydroxytryptophan decarboxylase were begun, a source of carboxyl-labeled 5-hydroxytryptophan was located (Dhom Products, Ltd., No. Hollywood, Calif.). This made it possible to assay for 5-hydroxytryptophan decarboxylation by a method similar to that used for the other substrates. The buffer used was Tris-HCl, pH 8.5, the incubation time was 30 min, and the final concentration of L-5-hydroxytryptophan was 4×10^{-4}M. The blank was the same except that the enzyme was preincubated in the presence of 1.25×10^{-4}M NSD-1055 (Brocresine; 3-hydroxy-4-bromobenzyloxyamine) and the absence of pyridoxal phosphate. Pyridoxal phosphate and substrate were then added before the incubation period. The production of labeled CO_2 was linear with time and with enzyme concentration for at least 30 min and for the first 100 mnoles of CO_2.

Preparation of hydroxylapatite. The commercial hydroxylapatite gel used in the enzyme purification was found to give excellent results when carefully prepared. As supplied, the material contains a large proportion of very fine particles which must be removed to obtain satisfactory flow rates. About twenty grams of Bio-Gel HTP were suspended in about 400 ml of distilled water at room temperature with very gentle stirring by hand. The hydroxylapatite crystals were apparently very fragile so mechanical and magnetic stirring were avoided. After settling for a short time, the milky supernatant was decanted. This was repeated at least five times,

until the supernatant was relatively clear. The material was then resuspended and decanted in about 400 ml of cold 0.01M sodium phosphate buffer, pH 7.2, containing 0.01M 2-mercaptoethanol a total of three times. It was then poured into a 1.5 cm diameter column to obtain a packed bed height of 25-30 cm and allowed to settle. The column was washed with at least 500 ml of 0.01M sodium phosphate buffer, pH 7.2, containing 0.01M 2-mercaptoethanol over a period of at least 24 hours.

Although Levin (1962) has reported that hydroxylapatite columns may be regenerated, it was found that the enzyme chromatographed differently on columns which had been regenerated. Therefore, a fresh column was prepared for each run and the used material was discarded.

Analytical ultracentrifugation. Samples of enzyme were dialyzed overnight at 5° against 1000 vol of 0.005M sodium phosphate, pH 7.2., containing 0.2M sodium chloride and 0.01M 2-mercaptoethanol. Centrifugation was performed in a Spinco Model E analytical ultracentrifuge equipped with ultraviolet absorption optics and a photoelectric scanner. A sample of the dialysate was used as blank in all cases.

For determination of the molecular weight by the Archibald (1947) method, the optical densities at 280 nm from the meniscus or the bottom of the cell to the plateau region were fitted to third-order polynomials as a function of radial distance, using a computer program based on the method of least squares.* The protein concentrations at the meniscus and bottom were determined by substitution of the radial distances of the meniscus and bottom into these equations and the partial derivatives of concentration with respect to radial distance at the meniscus and bottom were estimated by differentiation and substitution.

* The computer program used for curve fitting, known as POLFIT, was available through the General Electric Time Sharing Service.

Amino acid analysis. A solution of enzyme containing 0.60 mg protein/ml was dialyzed exhaustively against distilled water in the cold room. Two or three ml of twice-distilled, constant boiling hydrochloric acid which had been flushed with nitrogen was added to vials containing 0.2 or 0.3 ml of the dialyzed enzyme solution. The vials were then repeatedly evacuated and backfilled with oxygen-free nitrogen and sealed under vacuum. Hydrolysis was in a forced-draft oven at 108-111° for 23, 48, or 92 hours, after which the vials were opened and dried *in vacuo* over sodium hydroxide pellets. Cystine and cysteine were determined by the performic acid oxidation method of Moore (1963). Tryptophan was determined essentially by the method of Duggan and Udenfriend (1956) on a sample hydrolyzed in 5N sodium hydroxide in a polypropylene tube. Acid hydrolysates were analyzed with a Beckman Model 120C instrument.

Immunization. About 1 mg of 99+% pure enzyme was subjected to electrophoresis by the method of Davis (1964), loading about 100 µg of protein on each of 10 gels. The gels were stained with 8-anilinonapthalene sulfonate according to the method of Hartman and Udenfriend (1969). Only one fluorescent band per gel was visible under ultraviolet light. The fluorescent bands (3-4 mm wide) were cut out of the gels, extruded through an 18-gauge needle, and allowed to stand in an equal volume of physiological saline overnight in the cold room. An equal volume of complete Freund's adjuvant was added and thoroughly mixed. The entire mixture was injected subcutaneously into a young, female goat at four sites in the neck region. After approximately six months, the immunization was repeated. Small samples of blood were taken two, five, and seven days after injection and the sera roughly titrated by Ouchterlony double diffusion. The titer of the serum taken on the seventh

day was approximately the same as that taken on the fifth and was about four-fold higher than serum taken three weeks after the first injection. It was assumed that the titer was maximal and 400 ml of blood was drawn.

Quantitative precipitin assay. Antiserum was centrifuged at 100,000xg for 60 min and the homogeneous enzyme preparation (0.20 mg/ml) at 35,000xg for 30 min. Incubation was carried out in 5 ml conical centrifuge tubes with glass stoppers. Each tube contained bovine serum albumin (1 mg/ml) in buffered saline*, 10 µl of antiserum, and 10 to 80 µl of enzyme solution (2.0 to 16 µg of enzyme) in a final volume of 1 ml. Blanks contained 10 µl of antiserum but no enzyme. The tubes were incubated at room temperature for 30 min and at 4° for 180 hours. The tubes were rotated between the palms of the hands at least once daily to resuspend any precipitate. A visible precipitate had formed in the tube nearest the equivalence point after 48 hours. After the incubation, the tubes were centrifuged at about 800xg for 60 min. The supernatants were decanted and saved and the pellets were thoroughly drained. The pellets were washed twice by resuspending them in 0.2 ml of buffered saline, then adding 0.8 ml of buffered saline and repeating the centrifugation. The washed precipitates were assayed for protein by redissolving them in 0.1 ml of Reagent C (Lowry et al., 1951); then adding 0.9 ml of Reagent C and completing the assay as usual. The supernatants were assayed for enzyme and antibody activity.

Preparation of a solid-phase immunoadsorbent. Ten ml of antiserum was brought to 35% saturation by the dropwise addition of 5.4 ml of neutralized saturated ammonium sulfate solution. This was stirred for 20 min, then centrifuged at 20,000xg for 20 min. The precipitate was redissolved in

* Buffered saline refers to 0.9% NaCl in 0.005M sodium phosphate buffer, pH 7.2.

12.5 ml of 0.2M sodium citrate buffer, pH 6.5. A γ-globulin fraction was similarly prepared from 19 ml of pooled normal goat serum (the γ-globulin level of the normal serum was found to be about half that of the antiserum). Both redissolved fractions were dialyzed overnight at 4° against 0.2M sodium citrate, pH 6.5. The following morning, the dialysis was continued for four hr against fresh buffer. The volume of the fraction prepared from antiserum was made equal to the volume of that prepared from control serum (25 ml) by the addition of dialysate. The total absorbances at 280 nm were 345 and 316, respectively.

The activation and coupling procedures were essentially those of Cuatrecasas and Anfinsen (1971). Cyanogen bromide (7.5g) was added all at once to 50 ml of 50% (v/v) Sepharose 4B in water with vigorous magnetic stirring. The pH was immediately brought to and maintained at 11.0 ± 0.3 by the addition of 5N NaOH. The temperature was maintained at 25° ± 2° by the addition of crushed ice, either to a pan of water in which the reaction vessel was placed or directly to the reaction vessel itself. After 30 min, all the cyanogen bromide had disappeared and the rate of proton release had slowed by a factor of about 20. The reaction was assumed to be essentially complete. The mixture was poured over crushed ice in a 150 ml coarse fritted funnel under vacuum and immediately washed with 500 ml of 0.2M sodium citrate, pH 6.5. The funnel was removed from vacuum and all 25 ml of γ-globulin solution immediately added. The Sepharose was suspended in this solution and the suspension was then transferred to a beaker and stirred overnight at 4°.

The materials were then placed on coarse fritted funnels and washed under gravity with 50 ml aliquots of water (about 1000 ml, total), 50 ml of

2M NaCl, and five 30 ml aliquots of 6M guanidine HCl. The total recoveries of A_{280} in the washes were 44 and 32 units for the antibody- and γ-globulin-Sepharoses, respectively, corresponding to coupling yields of about 87% and 90%.

The materials were thoroughly washed with buffered saline and stored at 2° in buffered saline containing 0.1% NaN_3.

Preparation of ^{125}I-enzyme. This procedure was modified from that of Hunter and Greenwood (1962). Two ml of homogenous enzyme (0.62 mg/ml) was dialyzed overnight at 4° against 1 l of 0.05M sodium phosphate, pH 7.2. The iodination was carried out at room temperature and the gel filtration at 4°. Fifty µl of dialyzed enzyme was added to 5 mCi of $Na^{125}I$(14 Ci/mg I) in 47 µl of dilute NaOH, pH 7-9, as supplied by Amersham/Searle. To this was added 20 µl of 0.25% chloramine-T in 0.05M sodium borate, pH 8.4, by means of a Hamilton syringe. The reaction was stopped after 2 sec by injecting 20 µl of 1% sodium metabisulfite in the same borate buffer. The mixture was allowed to stand a few minutes, and then was diluted by adding 0.5 ml of 1% bovine serum albumin and 0.1 ml of 1M potassium iodide. The solution was then placed on a 1 x 45 cm column of Sephadex G-25 in buffered saline. To minimize nonspecific adsorption of enzyme by the column, 0.5 ml of 1% bovine serum albumin had previously been passed through it. The column was eluted with buffered saline and fractions of about 1 ml were collected. Aliquots (25 µl) of each fraction were counted to locate the ^{125}I-enzyme. Fractions 16-20 contained over 90% of the ^{125}I found in the void volume (approximately $1x10^9$cpm, total). After the appearance of the ^{125}I-enzyme peak, the column was closed at both ends and discarded to avoid any unnecessary risk of contamination.

RESULTS

I. PURIFICATION OF THE ENZYME

Selection of enzyme source. Material obtained from a local abattoir is a convenient source of mammlian enzymes for purification, because it is available fresh, cheaply, and in bulk. A survey of several slaughterhouse materials was undertaken in order to choose the best source of aromatic L-amino acid decarboxylase. Specific activities were compared with that of guinea pig kidney, which is known to have a high level of decarboxylase (Lovenberg, Weissbach, and Udenfriend 1962). The results are shown in Table 1, along with data for other sources which were obtained subsequently. Hog kidney was found to have a decarboxylase level about half as high as guinea pig kidney and far higher than any of the other slaughterhouse materials tested. Accordingly, hog kidney was chosen as the enzyme source.

General notes. Most of the procedures described below are those finally arrived at after much experimentation. Many details of the methods were not originally part of the scheme, but were added as knowledge of the properties of the enzyme grew. For example, 2-mercaptoethanol was added to the buffers after it was found that the enzyme was sensitive to sulfhydryl reagents. In some cases, the evolution of a particular procedure will be described in some detail.

All procedures in the purification were carried out at 0-5°, unless otherwise stated. Usually, fresh kidneys were obtained for each preparation, but the activity appeared to be quite stable when intact or coarsely chopped kidneys were stored at -20° for as long as several months.

The enzyme was relatively unstable in the crude homogenate and the crude supernant, so the ammonium sulfate fractionation was completed on

TABLE 1

AROMATIC L-AMINO ACID DECARBOXYLASE LEVELS IN VARIOUS TISSUES

Species	Tissue	Specific Activity (units/g wet tissue)
Beef	Adrenal medulla	470
	Kidney	110
	Liver	380
Hog	Kidney	1700
	Liver	490
	Adrenal medulla	180
	Brain	12
Sheep	Kidney	570
	Liver	470
Guinea Pig	Kidney	3200
	Brain	30
	Adrenal	30
Tetrahymena pyriformis	Sonicated cells	50
Rabbit	Kidney	2400
	Brain (less cerebrum)	30
	Adrenal	20
	Pineal Gland	2200
Rat	Kidney	630
	Liver	520
	Brain	40
Frog	Heart	2
	Kidney and Adrenal Body	80
	Brain	35
Human	Kidney	3
	Liver	27

This Table is provided only as an approximate guide and substantial deviation from the values presented should be expected. This is because the data were obtained on several different occasions over the course of about two years, during which time modifications were made in the assay and other experimental procedures. Also, in some cases, only single tissue samples were tested, so normal variation among individuals may be a source of error.

the first day. The enzyme was not stable to freezing at any stage tested, so enzyme solutions were always stored at 0°.

Preliminary treatment. Three kidneys (300-400 g) were defatted, minced, and homogenized in a Waring blender for one minute with approximately three volumes of 0.005M sodium phosphate buffer, pH 7.2, containing 0.01M 2-mercaptoethanol. The crude homogenate was centrifuged at 25,000xg for 20-30 min and the supernant filtered through several layers of cheesecloth to remove particles of fat. Freshly prepared 20% streptomycin sulfate was added dropwise with stirring to a final concentration of 0.8%. The mixture was stirred for 15-20 min, then centrifuged at 20,000xg for 15 min. The streptomycin sulfate treatment gave only a slight purification and no detectable loss of activity. It was included only to reduce interfering ultraviolet absorption due to nucleic acids.

Ammonium sulfate fractionation. Saturated ammonium sulfate solutions were prepared by dissolving ammonium sulfate in water to a concentration of about 4M at room temperature. The pH of the solution was adjusted to pH 7-8 with ammonium hydroxide. The neutralized solution was filtered and then allowed to stand in the cold room at about 4° for several days, at which time crystals had precipitated. Concentrations of ammonium sulfate are expressed as "percent saturation", calculated by simple dilution of this solution. Saturated ammonium sulfate solution was added dropwise to enzyme solutions with vigorous magnetic stirring. After the addition, the mixtures were stirred for an additional 20 min and the precipitates collected by centrifugation at 25,000xg for 15-20 min. The desired fractions were redissolved by covering the precipitates with about one-third the original volume of 0.05M sodium phosphate, pH 7.2, containing 0.01M 2-mercaptoethanol, and allowing them to stand overnight.

A preliminary experiment showed that virtually all of the enzyme activity precipitated between 25% and 65% saturation, with approximately half of the activity precipitating between 35% and 45% saturation. In the first full-scale preparation, the fraction precipitating between 29% and 50% saturation was used. In the light of later experiments, these limits were adjusted to 32% and 49% saturation. Recoveries were never greater than 80%, and were usually about 60%.

Heat treatment in the presence of substrate and cofactor. In their preparation of decarboxylase from rat liver, Awapara, Sandman, and Hanly (1962) heated the crude homogenate at 55° for three min. By this procedure, these workers achieved only a 2.4-fold purification (little better than would have been obtained by simply centrifuging the homogenate) and 55% yield. Preliminary experiments showed that substantially better recoveries were obtained when the enzyme was heated in the presence of 2×10^{-4} M 5-hydroxytryptophan and 7×10^{-5} M pyridoxal phosphate. Approximately 1.5-fold purification was obtained after heating for three min at 50°, 55°, or 60°; there was no purification at 65° and a substantial loss of activity was recorded at 70°. The optimum conditions giving significant purification with acceptable recovery of activity were 55° for less than 10 min. The presence of 0.6 M KCl enhanced the purification, probably by salting out denatured proteins, but had no effect on the yield. The presence of 0.02 M 2-mercaptoethanol had no effect.

The above results were obtained using small test aliquots of enzyme. When the procedure was "scaled up" to preparative quantities, the results were disappointing. By reducing the temperature and time of heating to 50° and six min, a marginal purification and 70-80% yield was obtained

when the batch was divided into small aliquots. It was decided to retain the step in the final procedure, however, since it is necessary to reduce the total amount of protein before the later chromatographic steps.

In the final procedure, the redissolved "32-49% ammonium sulfate fraction" was made up to contain 0.067 M sodium phosphate, pH 7.2, 0.02 M 2-mercaptoethanol, 0.6 M potassium chloride, and 7×10^{-5} M pyridoxal phosphate. Aliquots of 6ml of this solution were added to 16x150mm tubes containing 0.6ml of 2×10^{-4} M 5-hydroxytryptophan. The tubes were then placed in a 50° water bath for six min and immediately cooled in an ice bath. When cold, the precipitated protein was removed by centrifugation at 30,000xg for 20 min. The supernatant (about 280 ml) was dialyzed overnight against two changes of 4 l each of 0.005 M sodium phosphate, pH 7.2, containing 0.01 M 2-mercaptoethanol.

Alumina Cγ gel adsorption. Clark, Weissbach, and Udenfriend (1954) and Lovenberg, Weissbach, and Udenfriend (1962) achieved an approximately four-fold purification by adsorption of the guinea pig kidney enzyme to alumina Cγ gel (about 1 mg gel/mg protein) at pH 5.8 and elution with 0.1 M phosphate buffer pH 6.3. In the present study, the procedure was somewhat modified. Preliminary experiments showed that no activity was lost when the ammonium sulfate fraction was carefully titrated to pH 5.8. At this pH, the enzyme was quantitatively adsorbed to the gel at a ratio of 0.8 mg gel (dry weight)* per A_{280} unit (measured prior to the titration). The adsorbed enzyme was not eluted from the gel by washing with water. Buffers of 0.1, 0.2, and 0.5 M sodium phosphate, pH 6.3 and pH 7.2 were tested for their ability to remove

* The concentration of gel in the commercial suspension was estimated by drying weighed samples in tared beakers overnight at 110°. The commercial preparations were found to contain 3-4% solids.

the enzyme from the gel. About 90% of the enzyme activity was recovered with a single elution with 0.1 M sodium phosphate, pH 7.2. Purification was about 3.6-fold over the ammonium sulfate fraction. Somewhat better purification with somewhat poorer recovery was obtained with 0.1 M sodium phosphate, pH 6.3. The more concentrated buffers gave less purification.

Thus, in the final procedure, the dialyzed "heat supernatant" (about 310 ml having an absorbance at 280 nm of about 20) was adjusted to pH 5.8 with 0.2 N acetic acid and centrifuged at 20,000xg for 20 min. To the clear supernatant was added a 3% suspension of alumina Cγ gel to a final concentration of 0.4 mg dry weight of gel per A_{280} unit. This was stirred for 20 min, then centrifuged and the supernatant discarded. The sedimented gel was resuspended in about 150 ml of 0.01 M 2-mercaptoethanol, centrifuged, and the supernatant discarded. The washed gel was then resuspended in about 200 ml of 0.1 M sodium phosphate buffer, pH 7.2, containing 0.01 M 2-mercaptoethanol, centrifuged, and the clear, yellow "alumina Cγ gel eluate" was retained.

Polyethylene glycol-6000 fractionation. Polson et al. (1964) have investigated the use of polyethylene glycols in the fractionation of serum proteins. They found polyethylene glycol-6000 the most suitable for this purpose and Janssen and Ruelius (1968) used this material as the only reagent in their elegant purification of an alcohol oxidase from Basidiomycete. These workers used polyethylene glycol in the solid form and carried out the fractionation at room temperature. However, at the temperatures used in the present procedure, the material was found to dissolve very slowly, so it was added as a 40% (w/v) solution.

The enzyme was found to be rather soluble in polyethylene glycol solutions, only about half the activity being precipitated at a concentration of 20% polyethylene glycol. At this concentration, the solution was quite viscous

and it was difficult to sediment the precipitated proteins at forces attainable in the Sorvall RC2-B centrifuge (about 40,000xg). Since most proteins are precipitated at 10% polyethylene glycol, it was decided to use this method as a negative precipitation procedure, contaminating proteins being precipitated with polyethylene glycol and the enzyme recovered from the supernatant.

At first, it was attempted to precipitate the enzyme activity from a 20% polyethylene glycol supernatant by the addition of a saturated solution of ammonium sulfate. Unexpectedly, this led to the separation of the mixture into two phases without precipitation of the enzyme. The phases were separated by centrifugation and assayed for enzyme activity. The enzyme was found to partition between the phases with a distribution coefficient of about 1.5 (concentration in upper phase/concentration in lower phase). While this raised the interesting possibility of purifying the enzyme by a countercurrent distribution procedure, it was decided to investigate more direct methods of reisolating the enzyme. It was found that the enzyme could be efficiently adsorbed to DEAE-cellulose and then eluted with a salt solution in a batch process. When the DEAE-cellulose was sedimented in a swinging bucket rotor (Sorvall HB-4), the surface of the pellet was level so that the supernatant could be withdrawn without disturbing the rather loosely packed material.

In the final procedure, then, a 40% solution of polyethylene glycol-6000 was added dropwise to the alumina Cγ gel eluate to a final concentration of 12.5%. After stirring for 20 min, the precipitated protein was sedimented at 40,000xg for 45 min. To the 290 ml of supernatant was added 140 ml of a suspension of DEAE-cellulose (about 27 mg/ml). After stirring for 20 min, the DEAE-cellulose was isolated on a coarse sintered glass funnel and washed with 100 ml of 0.01 M sodium phosphate buffer, pH 7.2, containing 0.01 M

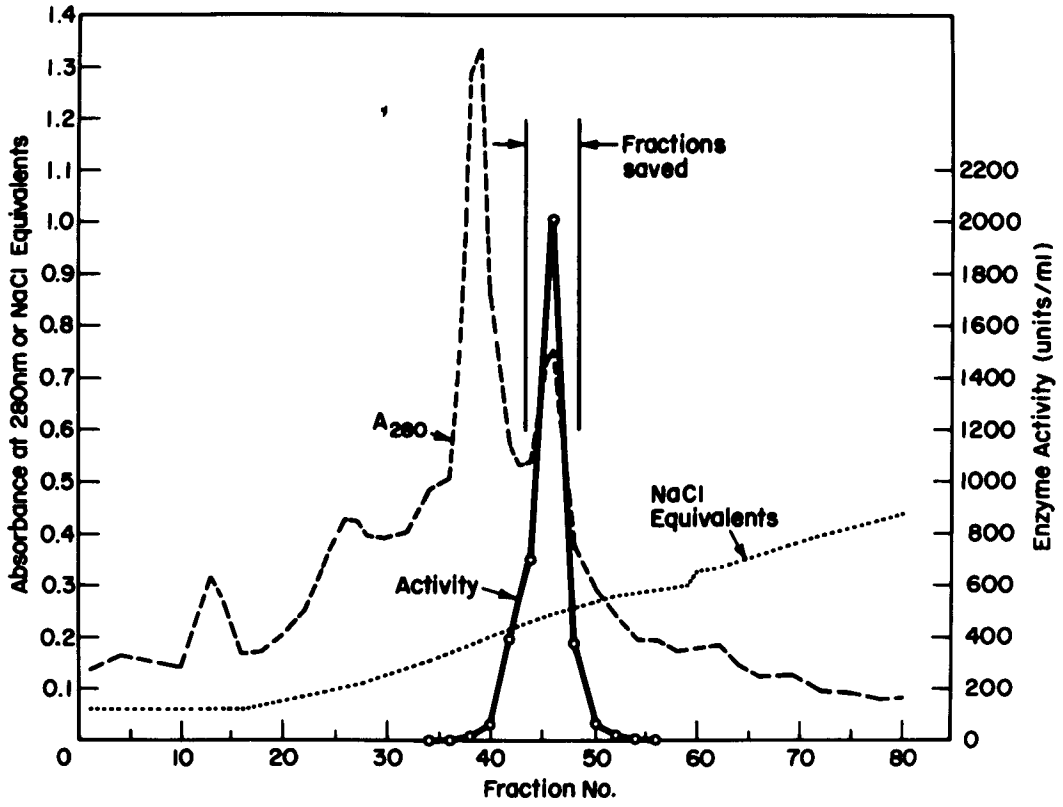
2-mercaptoethanol. The adsorbed material was then eluted from the DEAE-cellulose by suspending it in 100 ml of 0.05 M sodium phosphate buffer, pH 7.2, containing 0.6 M sodium chloride and 0.01 M 2-mercaptoethanol, then sedimenting the DEAE-cellulose at 25,000xg for 20 min in the HB-4 rotor. The "PEG-DEAE eluate" was dialyzed against two changes of 2 l each of 0.05 M sodium phosphate buffer, pH 7.2, containing 0.01 M 2-mercaptoethanol.

DEAE-Sephadex fractionation. Awapara, Sandman, and Hanly (1962) used a DEAE-Sephadex A-50 column and eluted with a stepwise gradient of sodium phosphate buffer in their preparation of the enzyme from rat liver. It seemed likely that a better purification could be obtained by using a linear gradient. Accordingly, the dialyzed PEG-DEAE eluate was loaded on a 2.5x30-cm column of DEAE-Sephadex A-50, which had been equilibrated with 0.05 M sodium phosphate buffer, pH 7.2, containing 0.01 M 2-mercaptoethanol, and rinsed on with two small aliquots of the same buffer. The column was developed at a flow rate of 8-10 ml/hr with a linear gradient of 0.05 M sodium chloride in a total volume of 1000 ml of the same buffer. Fractions of about 10 ml were collected.

As shown in Figure 1, the enzyme activity eluted as a single sharp peak at about 0.25 M NaCl, just following the major A_{280} peak. A peak of yellow color usually eluted just before the major A_{280} peak. The fractions containing peak activity, except those fractions within the major A_{280} peak, were combined and saved.

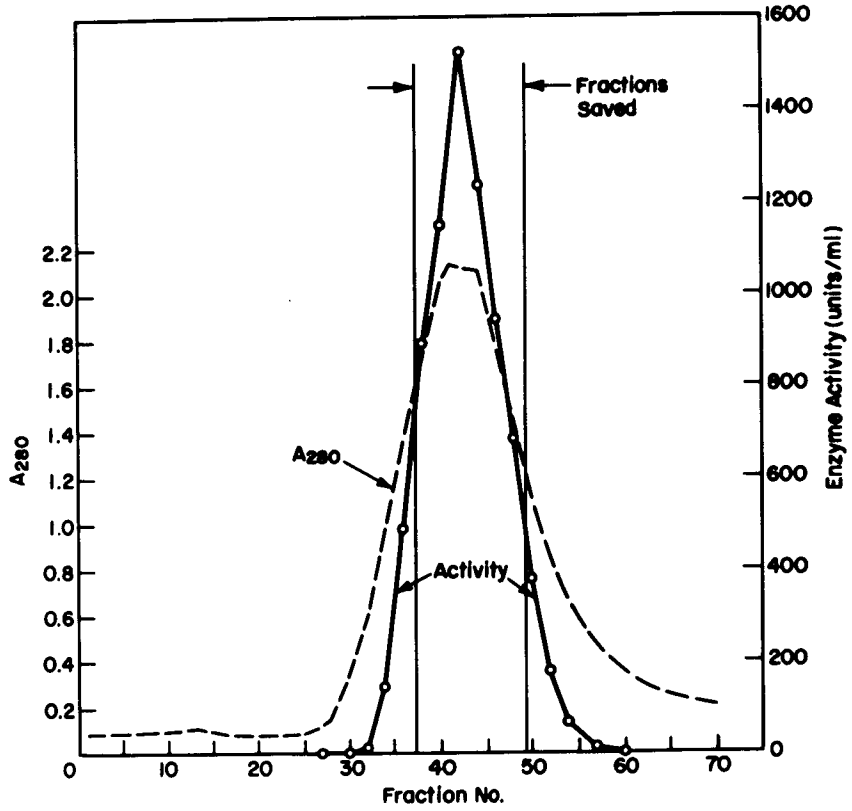
Sephadex gel filtration. Trial runs on Sephadex G-200 and G-100 were made both before and after the DEAE-Sephadex step. A typical run on G-100 is shown in Figure 2. It may be seen that the peak of enzyme activity coincided with the major peak of A_{280}. Thus, the slight purification achieved was offset by a small loss of enzyme activity. Since this procedure is rather

FIGURE 1



Chromatography on DEAE-Sephadex A-50. Chromatography was carried out as described in the text. "NaCl Equivalents" were determined conductimetrically on small aliquots of selected fractions and converted by reference to standard solutions of sodium chloride.

FIGURE 2



Gel Filtration on Sephadex G-100. The DEAE-cellulose eluate (about 30 ml) from the polyethylene glycol-6000 fractionation was concentrated to about 25 ml by ultrafiltration. This material was then loaded onto a column (2.5 x 90 cm) of Sephadex G-100 which had been equilibrated with 0.05 M sodium phosphate buffer, pH 7.2, containing 0.01 M 2-mercaptoethanol, and adapted for upward flow. The column was developed with the same buffer at a flow rate of 8-10 ml/hr, using a Mariotte flask at a hydrostatic pressure head of about 7 cm. Fractions of 6 ml were collected.

time-consuming, it was omitted from the final purification scheme, although it was included in the first preparation of essentially homogeneous enzyme (see Figure 4).

Hydroxylapatite column chromatography. The use of hydroxylapatite column chromatography in the purification of this enzyme has not been reported previously. Fellman (1959) used calcium phosphate gel in his preparation of the enzyme from bovine adrenal medulla. However, Fellman's procedure was a negative adsorption, i.e., contaminating proteins were adsorbed to the gel while the enzyme was not.

Preliminary experiments using hydroxylapatite in a batch procedure showed that the enzyme was adsorbed to the gel from dilute sodium phosphate buffer and could be eluted with 0.05 M sodium phosphate, pH 7.2. However, in a column procedure, using a linear gradient from 0.05 to 0.1 M sodium phosphate, pH 7.2, the enzyme was not eluted from the column. Upon washing the column with 0.2 M sodium phosphate, the enzyme activity was recovered in 90% yield. A concave gradient, formed by a variable gradient maker, was found to elute most of the contaminating proteins in the earlier, shallow portion of the gradient and to elute the enzyme in the later, steeper portion.

Thus, in the final procedure, the pooled DEAE-Sephadex fractions were concentrated by ultrafiltration to about 10 ml and dialyzed overnight against 1 l of 0.01 M sodium phosphate buffer, pH 7.2, containing 0.01 M 2-mercaptoethanol. The dialyzed material was then loaded on a freshly prepared 0.5 x 27 cm hydroxylapatite column, rinsed in with two or three small aliquots of starting buffer, and eluted with a concave gradient. The chambers of the gradient mixer contained 90 ml each of the following concentrations of pH 7.2 sodium phosphate buffer: chamber 1, 0.01 M; chambers

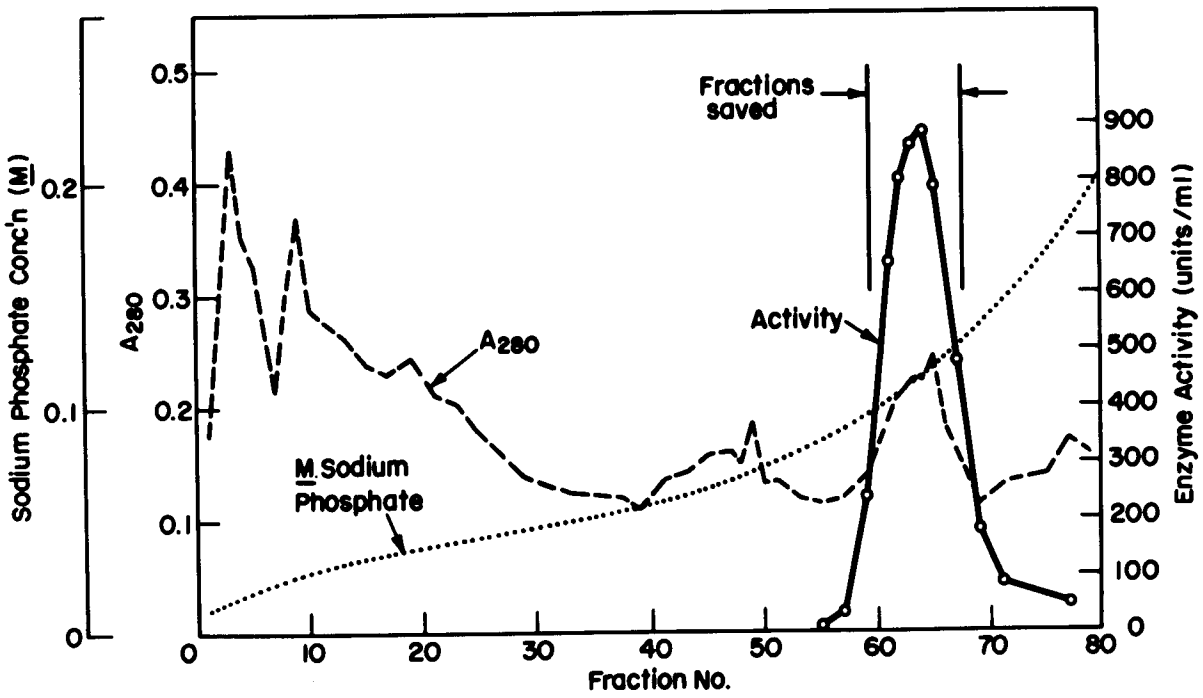
2, 3, 4 0.05 M; chamber 5, saturated (made up to 0.3 M at room temperature and allowed to crystallize in the cold room; the actual concentration of the mother liquor was about 0.2 M). All buffers contained 0.01 M 2-mercaptoethanol. Fractions of about 5 ml were collected. The fractions containing peak activity, which eluted at about 0.09 M sodium phosphate, were combined and saved.

A typical hydroxylapatite column profile is shown in Figure 3. As can be seen, the enzyme activity eluted as a single, symmetrical peak at about 0.09 M sodium phosphate. The associated peak of A_{280} was somewhat asymmetric and in some cases a definite shoulder was observed, suggesting that the enzyme was not completely homogeneous at this stage. Examination of the staining pattern after polyacrylamide gel disc electrophoresis as described in Materials and Methods revealed one or two contaminants constituting three to five percent of the total. The amounts of the impurities could be reduced by repeating the hydroxylapatite column chromatography.

Purity of the enzyme. The results of a typical purification are summarized in Table 2. Purifications averaged about 300-fold with recoveries from 5 to 14%. The final specific activities ranged from 7500 to 9500 units/mg. The purified enzyme was relatively stable during storage, having a half-life of about six weeks at 0°. Activity was lost upon freezing.

The enzyme prepared as described was 97-100% homogeneous, as estimated by polyacrylamide gel disc electrophoresis, staining, and scanning as described in Materials and Methods. Typical electrophoresis patterns are shown in Figure 4. In the purification of Preparation A, gel filtration on Sephadex G-100 was included in the procedure, just before the DEAE-Sephadex chromatography step. Note that the slow contaminant present in Preparation A was

FIGURE 3



Chromatography on Hydroxylapatite. Chromatography was carried out as described in the text. The plot of sodium phosphate concentration was calculated according to Peterson and Sober (1959) and shows the concentration at the outlet of the gradient maker. The actual concentration in each fraction is delayed by the void volume of the column plus the volume of connections, etc. (approximately 10 fractions). The maximum concentration was estimated conductimetrically.

TABLE 2

SUMMARY OF PURIFICATION OF DECARBOXYLASE FROM HOG KIDNEY

Step	Volume (ml)	Total Protein (mg)	Total Activity (units x 10^{-3})	Specific Activity (units/mg)	Purification (Fold)	Recovery (%)
Crude supernatant	987	2.31×10^4	604	26.1	---	---
32-49% Ammonium sulfate fraction	183	5.07×10^3	353	69.6	2.7	58
Heat supernatant	283	3.04×10^3	214	70.3	2.7	35
Alumina Cγ gel eluate	176	1.22×10^3	209	171	6.6	35
Polyethylene glycol-6000 precipitation DEAE-cellulose eluate	95	396	138	373	14.3	23
Chromatography on DEAE-Sephadex	50	34.7	68	1960	75.0	11
Chromatography and rechromatography on Hydroxylapatite	45	4.1	36	8670	332	5.9

These figures represent average values obtained with several preparations, based on 350 g wet weight of defatted hog kidney as starting material. The assays were carried out with L-DOPA as substrate under the usual conditions.

FIGURE 4

LEGEND TO FIGURE 4

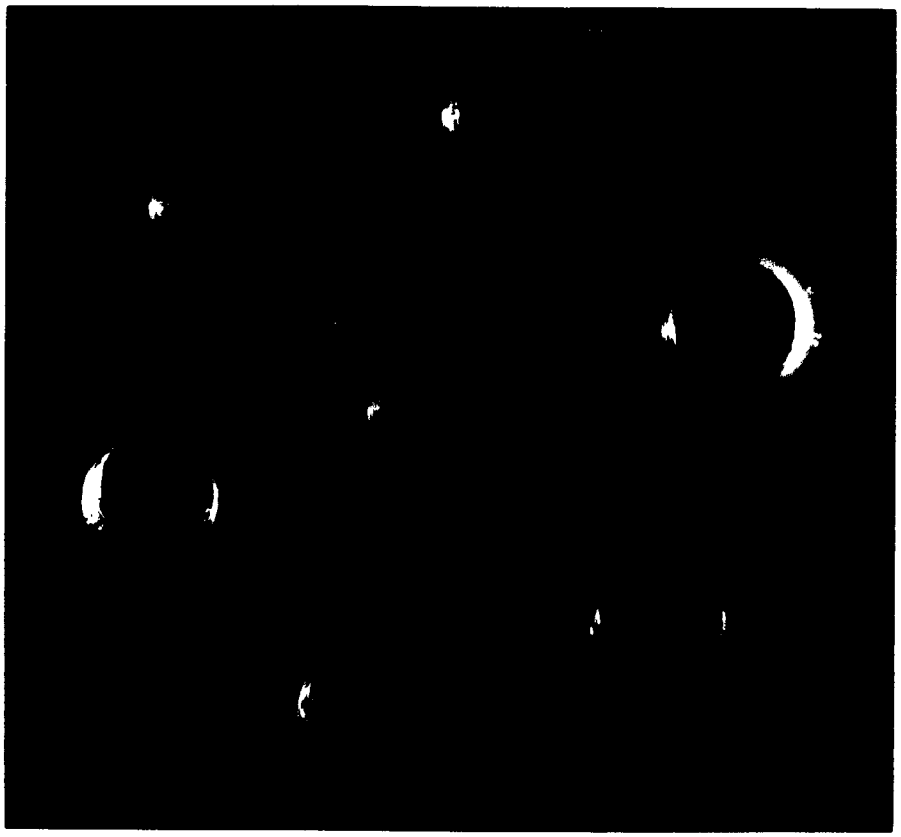
Polyacrylamide gel disc electrophoresis of purified aromatic L-amino acid decarboxylase. Direction of migration was from top to bottom (anode). From left to right: (1) 20 µg of Preparation A at pH 8.3; running time, 2 hr 20 min. The contaminant running just behind the major band represents about 3% of the total protein. (2) 10 µg of Preparation B at pH 8.3; running time 1 hr 30 min. The two contaminants running just ahead of the major band together represent less than 1% of the total protein. The mark near the lower end of the gel shows the position of the tracking dye. (3) 20 µg of Preparation A at pH 7.5; running time 1 hr 20 min. (4) 30 µg of Preparation B at pH 7.5; running time 1 hr 20 min. The opaque material at the top of the pH 7.5 gels is the stacking gel, which adhered tightly in this system. It was not stained by the dye.

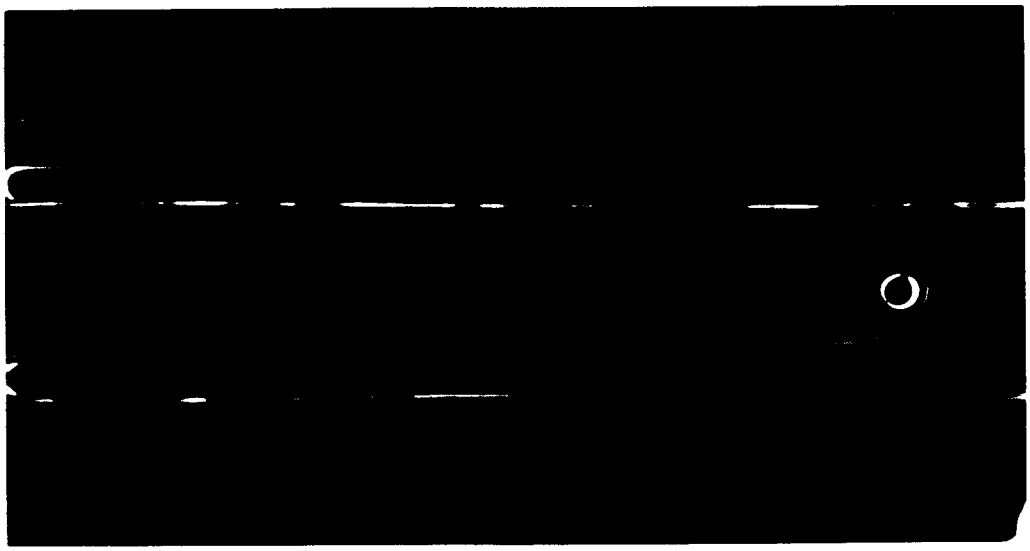
absent in Preparation B and that the two fast contaminants present in B were absent in A. This suggests that the major band represents the enzyme. Although only a few percent of the enzyme activity could be recovered after gel electrophoresis, the activity which was recovered coincided with the major band shown in Figure 4. The presence or absence of a given contaminant probably depends upon minor differences in the choice of fractions to be saved.

Although the pure enzyme appeared as a rather broad band in polyacrylamide gel disc electrophoresis, it yielded antibody which gave a single precipitin line against even the crudest preparation on Ouchterlony double diffusion (Figure 5). If the pure enzyme had contained more than a single protein, one would have expected to find two or more precipitin lines or to see precipitin lines which crossed, rather than merged. A control experiment using normal goat serum instead of antiserum showed no reaction with any of the antigen preparations.

On velocity sedimentation at 52,000 rpm and 13° or 33°, a single, symmetrical boundary of optical density was obtained, as shown in Figure 6. Equilibrium sedimentation at 4° resulted in a linear plot of ln c vs. r^2, as shown in Figure 7. The molecular weight determined from the slope of this plot was 112,000 daltons. Details of the sedimentation equilibrium experiments are included in a subsequent section. Early in the equilibrium run, 128 min after reaching speed, estimates of the molecular weight at the meniscus and at the bottom of the cell were made by the Archibald (1947) technique, as described in Materials and Methods. The molecular weights so determined were 108,000 and 107,000, daltons, respectively, in good agreement mutually, and with the molecular weight obtained from sedimentation equilibrium.

FIGURE 5A AND 5B



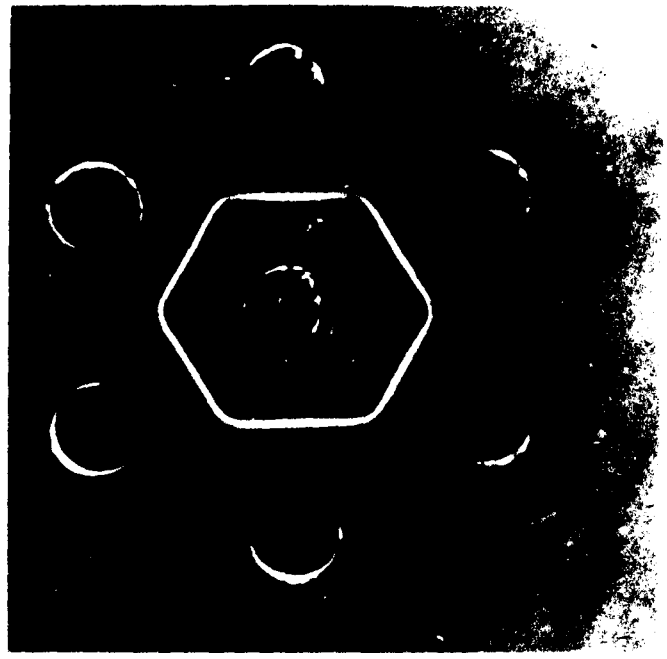


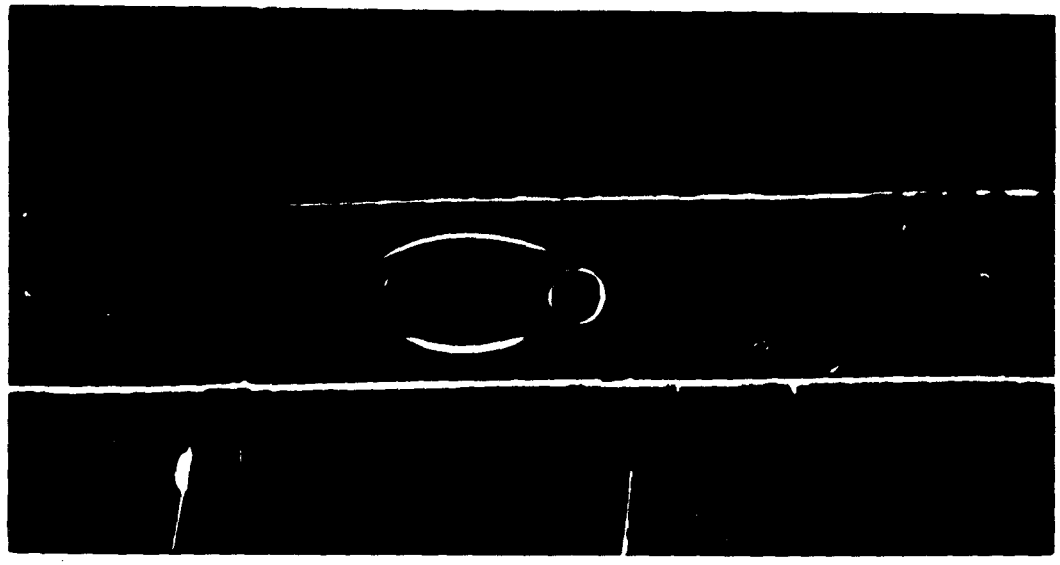
LEGEND TO FIGURE 5A AND 5B

A. Immunodiffusion experiment using antiserum obtained after first injection. The center well contained 50 µl of goat anti-enzyme serum obtained three weeks after the first injection. The outer wells contained, clockwise: 1 o'clock, early fractions from a DEAE-Sephadex column containing protein, but no enzyme activity (about 12 µg of protein); 3 o'clock, 400 µg of heat-treated supernatant; 5 o'clock, 200 µg of alumina Cγ gel eluate; 7 o'clock, DEAE-Sephadex fractions containing peak enzyme activity (about 20 µg of protein); 9 o'clock, about 100 µg of polyethylene glycol-6000 supernatant, eluted from DEAE-cellulose; 11 o'clock, 4 µg of purified enzyme, estimated to be 97% homogeneous. Development was for approximately 40 hr at 5°.

B. Immunoelectrophoresis of antiserum obtained after the first injection. The well contained 5 µg of an enzyme preparation estimated to be about 20% pure; antiserum was the same as that in Figure 5A. Electrophoresis was performed in 1% agar and 0.05 M sodium barbital buffer, pH 8.8, for 60 min, cathode at the right. The upper trough contained 25 µl of antiserum and the lower 50 µl. Both were diluted to 200 µl with 1% NaCl.

FIGURE 5C AND 5D



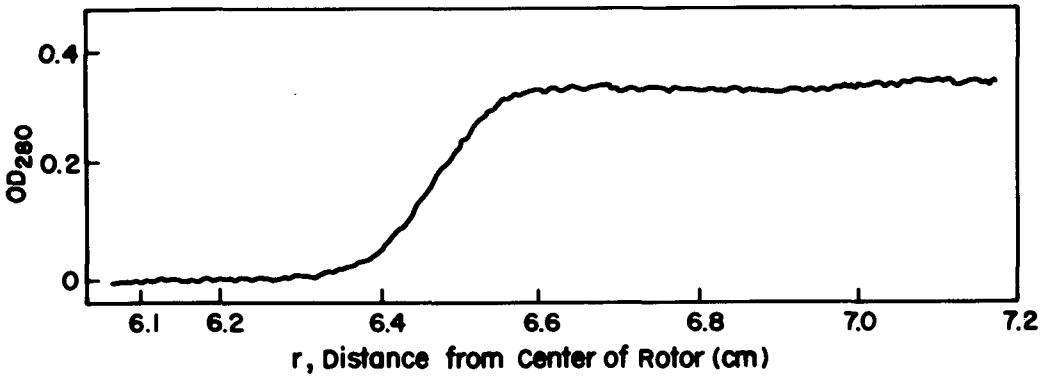


LEGEND TO FIGURE 5C AND 5D

C. Immunodiffusion experiment using antiserum obtained after the first and second injections. The center well contained 75 µg of an enzyme preparation estimated to be 20% pure. The outer wells contained, clockwise: 12 o'clock, 75 µl of antiserum obtained three weeks after the first injection; 2 and 4 o'clock, 75 and 50 µl of antiserum obtained five days after the second injection; 6,8, and 10 o'clock, 75, 50, and 25 µl of antiserum obtained seven days after the second injection. Development was for approximately 48 hr at room temperature.

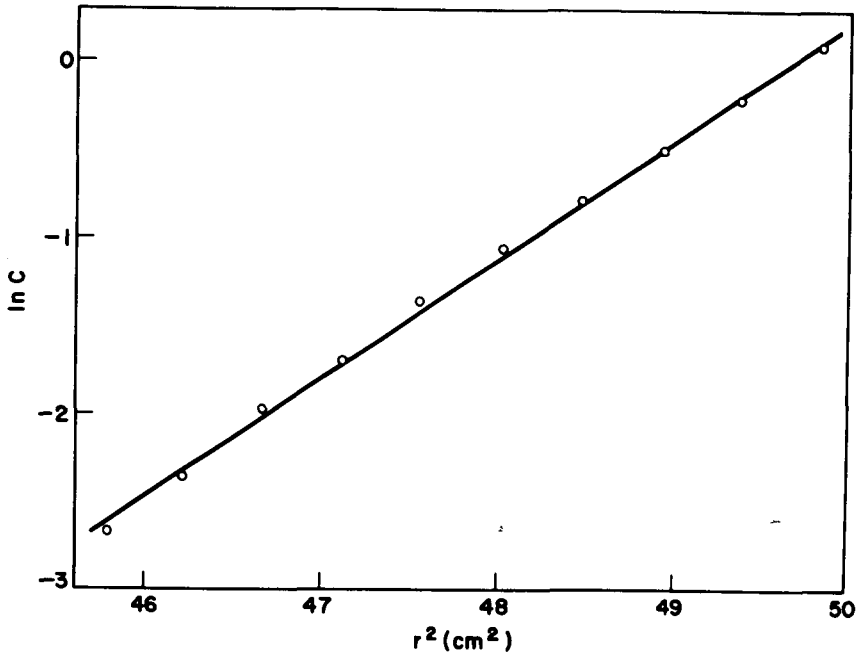
D. Immunoelectrophoresis of antiserum obtained after the second injection. The well contained about 80 µg of hog kidney protein, purified only through the heat step. The upper trough contained 25 µl of antiserum obtained nine days after the second injection and the lower 50 µl. Electrophoresis was performed as in the experiment shown in Figure 5B, but run for 75 min.

FIGURE 6



Rapid sedimentation of aromatic L-amino acid decarboxylase. Rotor speed was 52,000 rpm, temperature 13°. Other conditions were as specified in Materials and Methods. The enzyme sample was estimated to be 97% homogeneous by disc gel electrophoresis.

FIGURE 7



Equilibrium sedimentation of aromatic L-amino acid decarboxylase. Rotor speed was 10,000 rpm, temperature 4°. Other conditions were as specified in Materials and Methods. The enzyme sample was estimated to be 99% pure by disc gel electrophoresis. These data were obtained 50 hr after reaching speed.

Test for supplementation of purified enzyme by crude material. Since the degree of purity obtained seemed surprisingly high, it was necessary to consider the possibility that some activating substance had been removed or that some inhibitor had been accumulated during the course of purification. To test this, aliquots of purified enzyme and a crude supernatant from hog kidney containing approximately equal amounts of activity were assayed separately and also when mixed together. If either preparation had contained inhibitors or activators, the activity of the two mixed and assayed together would have been greater or less than the sum of the two activities measured separately. The purified enzyme and crude supernatant assayed separately yielded 366 and 416 nmoles of CO_2, respectively, and 791 nmoles when assayed together. It was concluded that neither preparation contained inhibitors or activators.

II. PROPERTIES OF THE PURIFIED ENZYME

Requirement of a free sulfhydryl group. Previous studies have shown that, when crude or partially purified enzyme preparations from other sources were incubated in the presence of sulfhydryl reagents, decarboxylase activity is inhibited (Buzard and Nytch, 1957a; Fellman, 1959; Lovenberg, Weissbach, and Udenfriend, 1962; Vogel, Snyder and Hare, 1970). The effects of pretreatment of the hog kidney enzyme with some sulfhydryl reagents and thiol-containing compounds are summarized in Table 3. This study differs from the previous studies in that excess sulfhydryl reagents were destroyed before assaying to exclude possible side reactions with the cofactor or substrate. That p-chloromercuribenzoate resulted in only partial inhibition may be due to the reversibility of mercaptide formation in the presence of excess 2-mercaptoethanol.

TABLE 3

EFFECTS OF SOME SULFHYDRYL REAGENTS AND SULFHYDRYL COMPOUNDS ON THE DECARBOXYLATION OF DOPA

Treatment	CO_2 Evolved (nmoles)
Control (incubated with H_2O)	255
2-Mercaptoethanol	261
Dithiothreitol	271
p-Chloromercuribenzoate	61
Iodoacetamide	18
N-Ethylmaleimide	6

A sample of the enzyme was dialyzed free of 2-mercaptoethanol. Aliquots of the dialyzed enzyme were then incubated in the presence of the compounds shown in the Table for 1 hr at room temperature in 8×10^{-3} M sodium phosphate buffer, pH 7 (except p-chloromercuribenzoate, in which case the buffer was 0.012 M sodium phosphate, pH 7.8). The incubation mixtures were then chilled on ice and a 10-fold excess of cold 0.05 M 2-mercaptoethanol was added to destroy unreacted sulfhydryl reagents. The treated enzyme was then assayed by the usual procedure except that no further 2-mercaptoethanol was added.

Although the sulfhydryl compounds did not result in significant stimulation of activity, it was thought that thiols might stabilize the enzyme to autoxidation. Accordingly, all buffers used in this work contained 0.01 M 2-mercaptoethanol, unless otherwise stated. This may explain the relative stability of the enzyme in these studies compared with the findings of previous workers.

It was also found that, in the presence of 0.01 M 2-mercaptoethanol, the "no enzyme" blank for the assay averaged about 50 cpm compared with about 200 cpm (including environmental background and machine noise) in the absence of reducing agents. The blank value was also reduced by the presence of 10^{-4} M ascorbate, raising the possibility that the nonenzymatic decarboxylation described by Vogel (1969) depends upon an oxidizing environment. Still, even in the absence of reducing agents, the blank values obtained in this work do not approach the rates reported by Vogel.

Presence of pyridoxal phosphate in the purified enzyme. The purified enzyme was found to contain 0.7 to 1.1 mole of pyridoxal phosphate per 112,000 g of protein. The excitation and emission spectra of the fluorescent derivative agreed well with those of the pyridoxal phosphate standards and with published spectra (Adams, 1969). Table 4 shows that pyridoxal phosphate was purified concomitantly with the enzyme. Although this particular preparation was somewhat atypical in that the initial and final specific activities were rather low, the final material appeared at least 97% homogeneous by polyacrylamide gel disc electrophoresis. The final value of 8.3 nmoles of pyridoxal phosphate per mg of protein is equivalent to about 0.9 mole per 112,000 g.

Effect of pyridoxal phosphate. The enzyme catalyzed significant decarcarboxylation without exogenous pyridoxal phosphate, but the addition of

TABLE 4

PURIFICATION OF PYRIDOXAL PHOSPHATE

DURING THE COURSE OF ENZYME PURIFICATION

Step	Enzyme Specific Activity (units/mg)	Pyridoxal Phosphate	
		pmoles/unit	nmoles/mg protein
Crude supernatant	10	16.7	0.17
32-49% Ammonium sulfate fraction	40	5.7	.23
Heat supernatant	55	5.1	.28
Alumina Cγ gel eluate	169	3.4	.57
Polyethylene glycol-6000 precipitation DEAE-cellulose eluate	405	2.4	.99
Chromatography on DEAE-Sephadex	2550	1.9	4.7
Chromatography and rechromatography on hydroxylapatite	5910	1.4	8.3

Pyridoxal phosphate was assayed by the method of Adams (1969). Enzyme activity was measured as described in Materials and Methods, with DOPA as substrate. Protein was assayed by the method of Lowry et al. (1951).

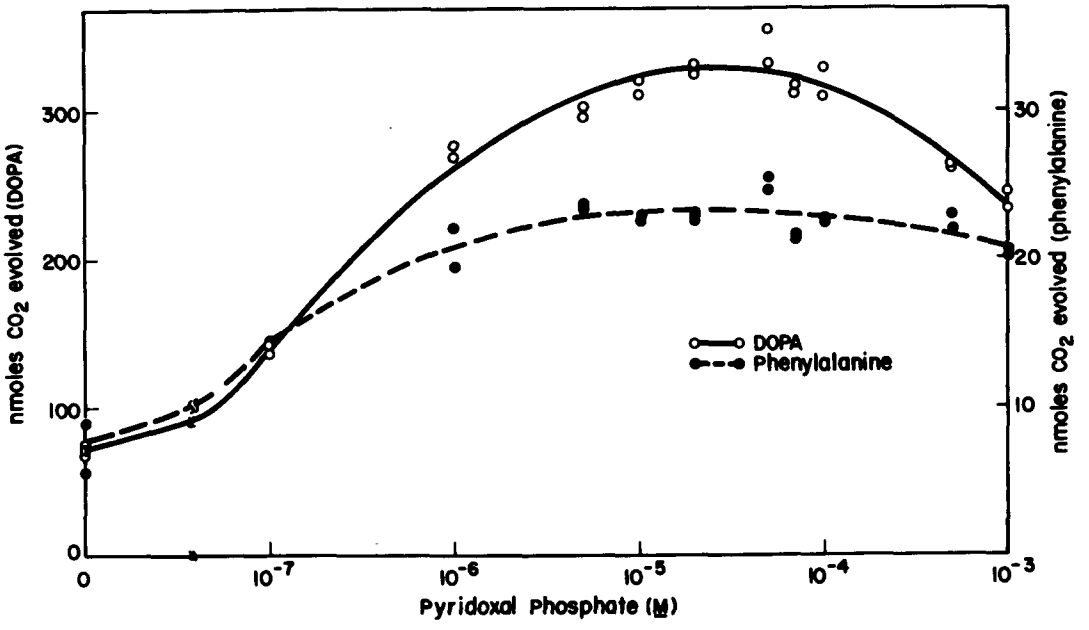
$1x10^{-7}$ to $1x10^{-3}$ M pyridoxal phosphate stimulated the activity two-to five-fold, with a broad maximum in the range of $5x10^{-6}$ to $1x10^{-4}$ M (Figure 8). To test whether inhibition of DOPA decarboxylation by pyridoxal phosphate at concentrations above $1x10^{-4}$ M was due to the formation of the tetrahydroisoquinoline cyclization product (Schott and Clark, 1952), the effect of pyridoxal phosphate upon the decarboxylation of phenylalanine was also tested. The latter does not form a cyclic product with pyridoxal phosphate. With phenylalanine as substrate, there was two-to three-fold stimulation throughout the range of $1x10^{-7}$ to $1x10^{-3}$ M pyridoxal phosphate, reaching a plateau at approximately $1x10^{-6}$ M. Thus, inhibition of the decarboxylation of DOPA by the higher pyridoxal phosphate concentrations is probably due to formation of the tetrahydroisoquinoline derivative. The experiment with phenylalanine also shows that the sites responsible for activation by exogeneous cofactor approach saturation at 10^{-6} M pyridoxal phosphate.

Attempts to resolve the holoenzyme complex have not been successful. An attempt to bind more cofactor to the enzyme by incubation with pyridoxal phosphate followed by dialysis increased the pyridoxal phosphate content of the enzyme by about 50%, but there was no significant change in the stimulation afforded by exogenous pyridoxal phosphate.

pH Dependence. The effect of pH on the decarboxylation of phenylalanine and tryptophan is shown in Figure 9. In view of the general agreement that the decarboxylation of DOPA is optimal at about pH 7 (see Introduction), this point was not reinvestigated and DOPA decarboxylation was always assayed at pH 7.0.

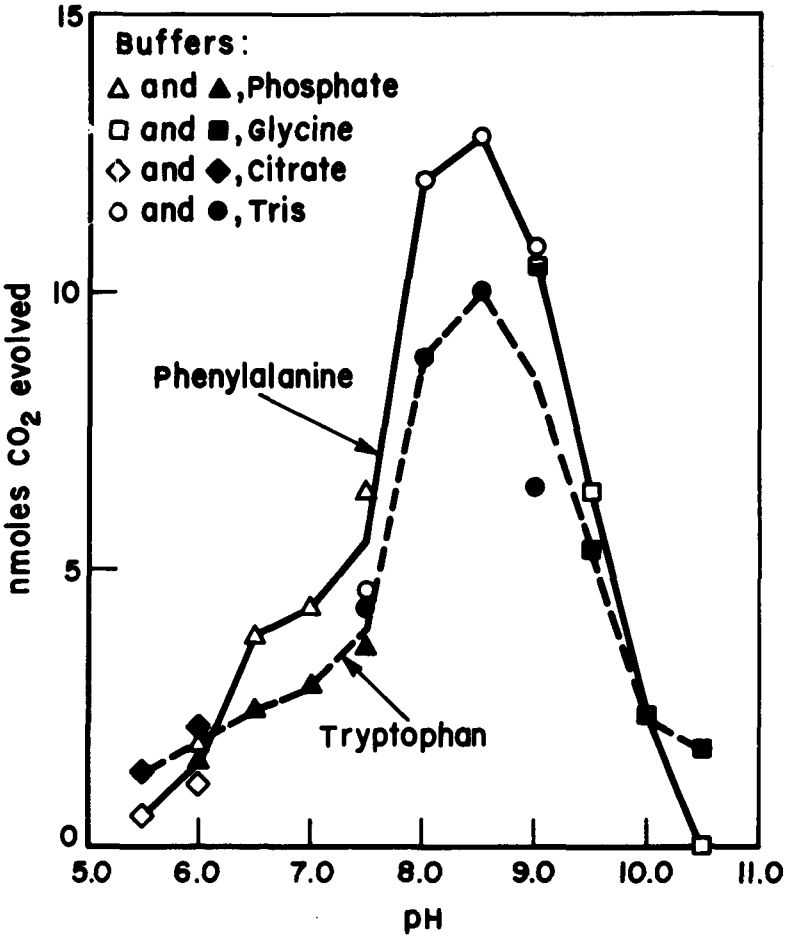
Substrate specificity and kinetics. The purified enzyme was found to decarboxylate DOPA, 5-hydroxytryptophan, phenylalanine, tryptophan, and tyrosine at readily measurable rates. A summary of the kinetic parameters

FIGURE 8



Dependence upon exogenous pyridoxal phosphate concentration of the decarboxylation of DOPA and phenylalanine. The concentrations of DOPA and phenylalanine were 0.003 and 0.017 M, and the incubation times were 15 and 25 min, respectively.

FIGURE 9



Dependence upon pH of the decarboxylation of phenylalanine and tryptophan. The enzyme preparation used was estimated to be about 90% homogeneous by disc gel electrophoresis. Asays were performed in the presence of 0.08 M buffer and either 0.01 M tryptophan containing 0.2 μCi of [1-^{14}C] tryptophan or 0.017 M phenylalanine containing 0.5 μCi of [1-^{14}C] phenylalanine. Incubation time was 25 min. The buffers used were sodium citrate for pH 5.5 and 6.0; sodium phosphate for pH 6.0-7.5; Tris-HCl for pH 7.5-9.0; and sodium glycinate for pH 9.0-10.5.

obtained for these substrates is given in Table 5. The data for 5-hydroxytryptophan were somewhat erratic with K_m values ranging from 4.8×10^{-5} to 1.08×10^{-4} M and V_{max} values from about 650 to about 950 nmoles x min^{-1} x mg^{-1}, depending upon the type of plot chosen. The activity of the enzyme toward histidine was very low, but detectable by both radiometric and fluorometric assays. Kinetic measurements were not made, but the histidine and tyrosine activities were compared. At substrate concentrations of 2×10^{-3} M, the rate of decarboxylation of tyrosine was 2.0×10^{-2} nmole/min, while the rate of histidine decarboxylation was 2.5×10^{-3} nmole/min.

It is interesting to compare these results with those of Lovenberg, Weissbach and Udenfriend (1962) for the guinea pig kidney enzyme. The order of K_m values may be summarized as follows:

Lovenberg et al. (1962): 5-hydroxytryptophan < DOPA < Trp < Tyr < Phe

Present study: 5-hydroxytryptophan < DOPA < Tyr ∿ Trp < Phe

and the V_{max} values as follows:

Lovenberg et al. (1962): DOPA > 5-hydroxytryptophan > Trp > Phe > Tyr

Present study: DOPA > 5-hydroxytryptophan > Phe > Trp > Tyr

The K_m values for each substrate in the two studies are of the same order of magnitude. Thus, the results are in general agreement.

The V_{max} values reported by Lovenberg, Weissbach, and Udenfriend (1962) for DOPA and 5-hydroxytryptophan are 6400 and 1000 μg of amine formed per hour per mg of protein, respectively (about 650 and 95 nmoles/min per mg in the present units). If it is assumed that the guinea pig kidney enzyme has about the same turnover number and molecular weight as the hog kidney enzyme, their preparation would have been on the order of 10% pure with respect to enzyme protein.

TABLE 5

KINETIC PARAMETERS OF AROMATIC L-AMINO ACID

DECARBOXYLASE FROM HOG KIDNEY

Substrate	K_m (M)	V_{max} (nmoles/min/mg)
DOPA	1.9×10^{-4}	8900
5-Hydroxytryptophan	1×10^{-4}	850
Phenylalanine	4.2×10^{-2}	590
Tryptophan	1.0×10^{-2}	230
Tyrosine	8.4×10^{-3}	30

Kinetic measurements were done in the assay systems described in Materials and Methods, using the serotonin extraction procedure for 5-hydroxytryptophan; DOPA was assayed at pH 7.0, all others at pH 8.5. The enzyme preparation used was essentially homogeneous, over 99% pure. The concentration of carrier amino acid was varied while the amount of radioactive substrate was held constant. K_m and V_{max} were calculated from plots of substrate concentration/velocity vs. substrate concentration fitted by the method of least square using unweighted data. Further details are provided in the text.

Lovenberg, Weissbach, and Udenfriend (1962) reported that the decarboxylation of DOPA by their enzyme preparations was linear with time only for the first three min of reaction. In the present work, production of CO_2 was found to be linear with time for at least 20 min. This discrepancy is not due to relative instability of the guinea pig kidney enzyme, since Lovenberg (1962) found that the rate was linear for at least 20 min with 5-hydroxytryptophan. Another, possibly related, difference was the absence of substrate inhibition at a DOPA concentration of 3×10^{-3} M. Lovenberg (1962) observed pronounced substrate inhibition at this concentration.

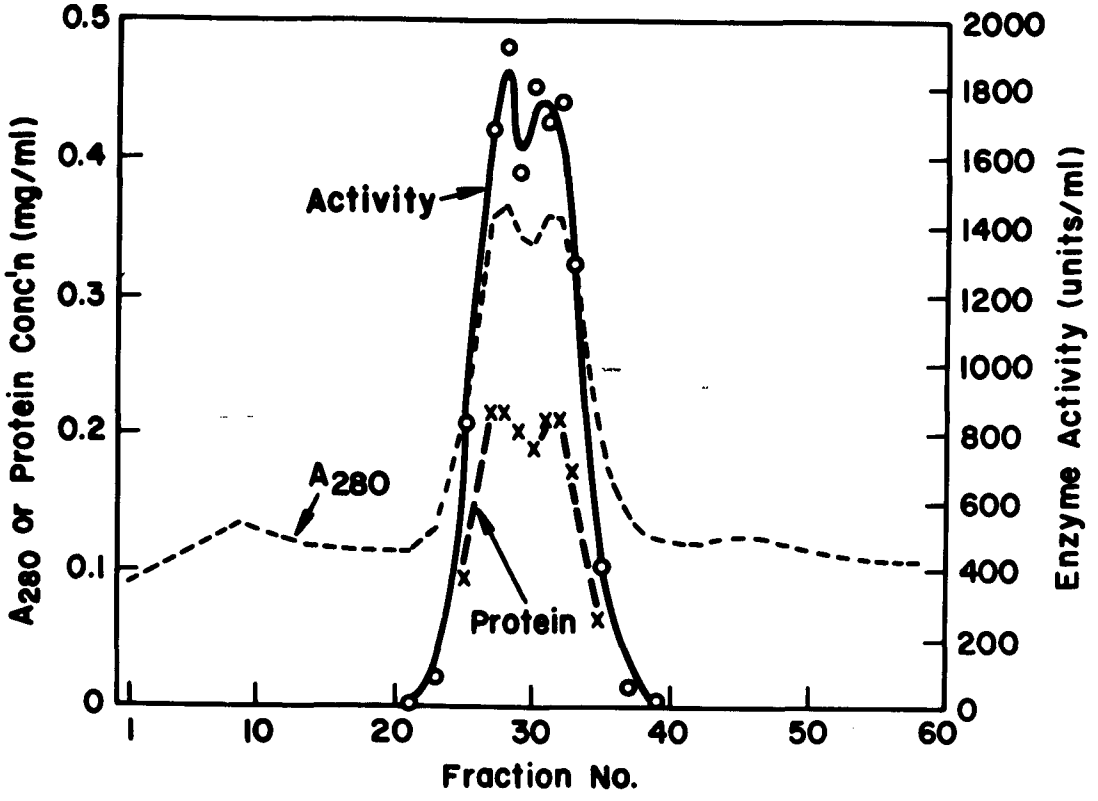
None of the other naturally occurring amino acids was decarboxylated to any detectable extent. Each carboxyl- or uniformly-labeled ^{14}C amino acid was incubated with several relatively large amounts of enzyme for 25 min. In the case of alanine, the radioactivity recovered with NCS was higher than the "no enzyme" blank and was positively correlated with enzyme concentration. To further investigate this apparent decarboxylation of alanine, the incubation was carried out with uniformly labeled alanine. The evolved CO_2 was collected in the usual way from one incubation mixture. Carrier ethylamine was added to two other incubation mixtures and the p-toluenesulfonamide and phenylthiourea derivatives were prepared by standard methods. After three recrystallizations, the radioactivity in the derivatives had decreased to only barely detectable levels and below the amount equivalent to the evolved "CO_2". It was concluded that the enzyme did not decarboxylate alanine and that the original observation may have been due to the presence of some trace contaminant in the radioactive alanine. A turnover number for DOPA of about 1000 mole/min per mole of enzyme may be calculated, based on the average specific activity of homogeneous enzyme. It is interesting to compare this figure with the turnover number of 1060, reported by Friedman

and Kaufman (1965) for dopamine β-hydroxylase, the third enzyme in the norepinephrine biosynthetic pathway.

Effects of cations. No evidence was found that metal ions serve an important function in the decarboxylation of DOPA. At a concentration of 10^{-3} M, the ions Fe^{2+}, Fe^{3+}, and K^{+} were either weakly inhibitory or without effect, while Mg^{2+}, Ca^{2+}, and Al^{3+} enhanced the activity by only 10% or less. Cu^{2+}, Zn^{2+}, and Hg^{2+} were strongly inhibitory. Furthermore, the chelating agents sodium diethyldithiocarbamate, 2,2'-bipyridine, cupferron, 1,10-phenanthroline, sodium EDTA, and 1,5-diphenylcarbohydrazide had no significant effect at concentrations of $1x10^{-4}$ M. These findings are in accord with those of most previous authors (see Introduction).

Separation of the enzyme into two fractions on DEAE-Sephadex. In one preparation, enzyme purified through the second hydroxylapatite chromatography step appeared quite homogeneous on polyacrylamide gel disc electrophoresis, except that the typical broad band in some fractions appeared to have split into two, or possibly three, bands all of which ran very close together. Since contamination was suspected, this material was rechromatographed on a 1.5x30 cm column of DEAE-Sephadex A-50. As shown in Figure 10, the A_{280}, protein, and enzyme activity in the eluate were each split into two peaks. Table 6 summarizes the enzymological characterization of these peaks. It can be seen that there is no significant difference among the fractions in either pyridoxal phosphate content or specific activity for any of the five substrates. Polyacrylamide gel disc electrophoresis of 10-12 μg each of Fractions 26, 28, 30, 32, and 34 gave patterns very similar to those observed with the material before rechromatograhy -- that is, a broad band apparently resolved into two or three components, all running within 4mm of each other.

FIGURE 10



Rechromatography of purified enzyme on DEAE-Sephadex A-50. The column was 1.5 x 30 cm, equilibrated with 0.05 M sodium phosphate, pH 7.2, containing 0.01 M 2-mercaptoethanol. It was developed with a linear gradient of 0-0.5 M NaCl in the same buffer, 600 ml total. Fractions of about 5 ml were collected.

TABLE 6

ENZYMOLOGICAL CHARACTERIZATION OF A SPLIT PEAK OF ENZYME

FOLLOWING RECHROMATOGRAPHY ON DEAE-SEPHADEX

Fraction No.	Protein Conc'n.	Pyridoxal Phosphate Content	Specific Activity (nmoles x min^{-1} x mg^{-1})				
	(mg/ml)	(nmoles/mg protein)	DOPA	5-hydroxytryptophan	Phe	Trp	Tyr
25	0.093	13.7	$8.9x10^3$	---	---	---	---
27	.216	12.6	$7.8x10^3$	835	257	110	9.2
28	.216	12.6	$9.0x10^3$	---	---	---	---
29	.200	12.5	$7.8x10^3$	---	---	---	---
30	.191	12.7	$9.5x10^3$	842	263	118	9.6
31	.209	12.7	$8.2x10^3$	---	---	---	---
32	.210	12.4	$8.4x10^3$	---	---	---	---
33	.172	12.2	$7.5x10^3$	796	267	113	10.2
35	.064	10.7	$6.4x10^3$	---	---	---	---

Enzyme activity for the various substrates was assayed as described in Materials and Methods, using the serotonin extraction procedure for 5-hydroxytryptophan. Pyridoxal phosphate was determined by the method of Adams (1969) and protein by the method of Lowry et al. (1951). The fraction numbers are those shown in Figure 10.

Aliquots of 20 µl of Fractions 26, 28, 30, 32, and 34 were incubated with 20 µl of antiserum -- obtained after the first injection (see section on immunization) -- for 15 min, then centrifuged at 100,000xg for 60 min, and the supernatants assayed for enzyme activity. Virtually quantitative (93-99%) removal of enzyme from solution was observed in each case. Immunoelectrophoresis of aliquots from Fractions 26, 30, and 34 gave precipitin patterns which were not distinguishable from each other nor from a standard of previously prepared enzyme.

It was concluded that the two peaks from the DEAE-Sephadex column represent forms of the enzyme which are identical to the previously studied aromatic L-amino acid decarboxylase, at least by the criteria described in this section. The separation of the two forms is probably due to some conformational change or to hydrolysis of one or more glutamine or asparagine residues or possibly to disulfide exchange. The enzyme used in the second injection for production of antiserum contained equal volumes of Fractions 26-32.

Amino acid analyses. Amino acid analyses were performed as described in Materials and Methods. The results are shown in Table 7. No significant, time-dependent destruction of serine and threonine was observed, although this is generally the case under the conditions of hydrolysis which were used. However, time-dependent losses of proline, lysine, and arginine were observed and such losses have been reported previously (Duggan, 1957). Accordingly, the values for these three amino acids were determined by extrapolation to zero time of hydrolysis, assuming first-order kinetics which the losses fit very well. The recovery of methionine was not improved by the addition of one part per 2000 of 2-mercaptoethanol (Keutmann and Potts, 1969)

TABLE 7

AMINO ACID COMPOSITION OF AROMATIC L-AMINO ACID DECARBOXYLASE

Amino Acid	Grams of residue/100g protein			Residues/ 112,000 g protein: "Best" integral value
	Time of hydrolysis			
	23 hr	48 hr	92 hr	
Aspartic acid[a]	6.80	6.62	6.82	64
Threonine	3.06	3.31	3.22	35
Serine	4.29	4.40	4.42	55
Proline[b]	4.28	4.04	3.82	50
Glutamic acid[a]	12.46	12.50	12.91	107
Glycine	4.36	4.58	4.96	89
Alanine	6.87	7.19	7.46	110
Valine[c]	5.74	5.88	5.77	64
Half-Cystine[d]	2.08	--	--	21
Methionine	2.62	2.67	2.85	23
Isoleucine[c]	3.92	4.14	4.20	41
Leucine[c]	11.98	12.06	12.93	125
Tyrosine	4.30	3.94	4.09	28
Phenylalanine	7.63	7.86	6.84	55
Lysine[b]	5.28	4.89	4.35	48
Histidine	3.06	3.29	2.92	25
Arginine[b]	8.20	7.70	7.53	58
Tryptophan[e]	3.07	--	--	18

[a]These figures include both free and amidated residues of aspartic and glutamic acids.

[b]Values for proline, lysine, and arginine were determined by extrapolation to zero time of hydrolysis.

[c]Values for valine, isoleucine, and leucine were taken at the maximum time of hydrolysis.

[d]Cystine and cysteine were determined as cysteic acid as described in Materials and Methods.

[e]Tryptophan was determined fluorometrically from a basic hydrolysate.

or when it was determined as methionine sulfone in the performic acid-oxidized sample. The excitation and emission spectra of the basic hydrolysate agreed well with those of the tryptophan standards and with published results (Duggan and and Udenfriend, 1956).

Recoveries of amino acids after acid hydrolysis accounted for 113-127% of the total protein as estimated by the Lowry method (Lowry et al., 1951). Thus, the enzyme protein concentrations used in this work may be low by a corresponding factor.

Ultracentrifuge studies. The sedimentation constant corrected to standard conditions, $s_{20,w}$, was found to be 5.82 $\pm$ 0.03 S based on four determinations. The sensitivity of the absorption optics is such that this value, determined at a protein concentration of about 0.2 mg/ml, is a good estimate of the sedimentation constant at infinite dilution.

The molecular weight of the enzyme was calculated from sedimentation equilibrium using a preparation estimated to be greater than 97% homogeneous by disc electrophoresis. The rotor speed and temperature were 10,000 rpm and 4°. As shown in Figure 7, a linear plot of ln c vs r^2 was obtained. Using a slope of this line, a molecular weight of 112,000 daltons was calculated. The partial specific volume was estimated at 0.742 ml/g by the method of Cohn and Edsall (1943).

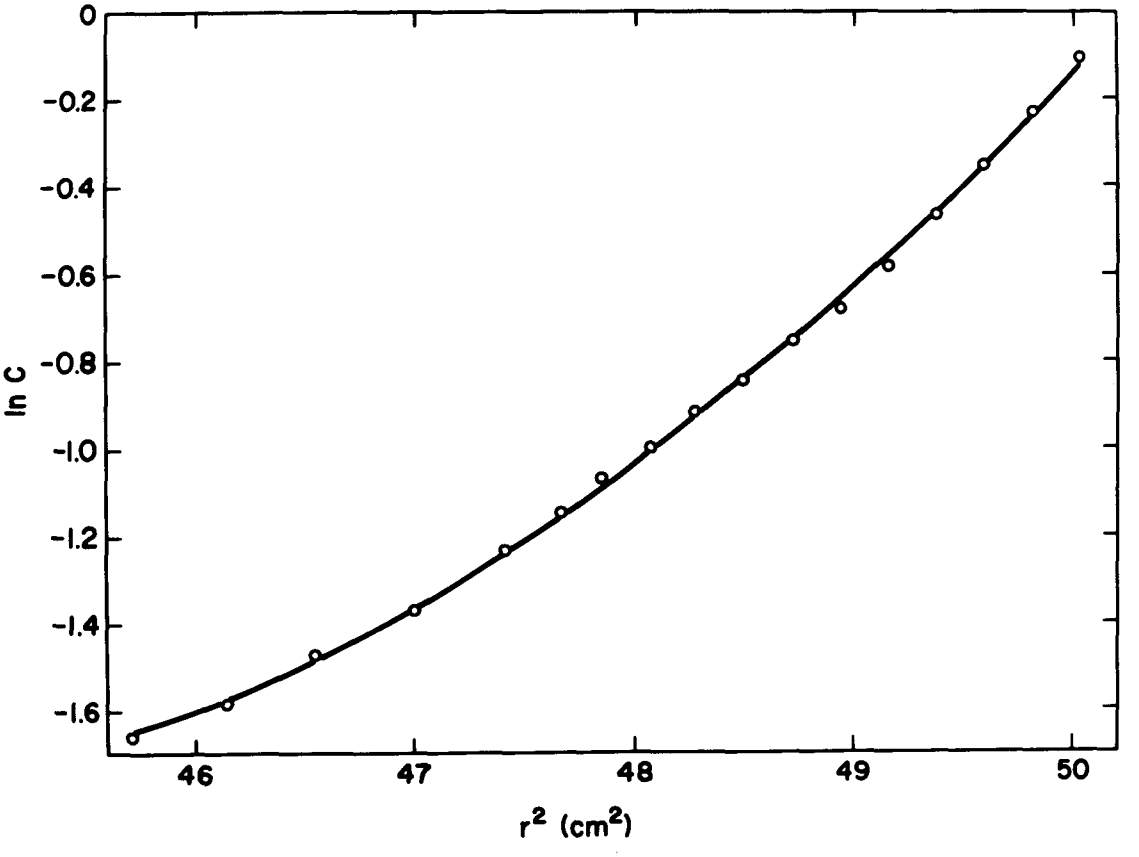
As discussed previously, estimates of the molecular weight at the meniscus and bottom of the cell were made early in the equilibrium run by the method of Archibald (1947). The molecular weights so determined, 108,000 and 107,000 daltons, respectively, were in good agreement with the molecular weight found by sedimentation equilibrium.

Given the molecular weight of 112,000 and the sedimentation constant of 5.82×10^{-13} sec, one may calculate a diffusion coefficient of 4.88×10^{-7} cm^2/sec. This in turn leads to a frictional ratio, f/f_{min}, of 1.36. This ratio is a function both of the shape of the protein and of its degree of solvation. Assuming a reasonable degree of hydration, 0.2 g H_2O/g protein, one may calculate that the frictional ratio due to shape along, f/f_o, is about 1.26. For a prolate or oblate ellipsoid of revolution, this value corresponds to an axial ratio of approximately five. Even for a rather high degree of hydration, say 0.5, the axial ratio is about 3.5. Thus, the enzyme appears to be a relatively asymmetric molecule.

A sedimentation equilibrium run was also made at 10,000 rpm and 22°. As shown in Figure 11, the plot of ln c vs r^2 at 22° was not linear, but concave. Molecular weights calculated from the slope of this curve ranged from 30,000 daltons near the meniscus to greater than 90,000 daltons near the bottom of the cell. This behavior most likely indicates an associating-dissociating system at the higher temperature and suggests that the native enzyme is composed of subunits of lower molecular weight. Further evidence for this hypothesis is presented below.

Polyacrylamide gel disc electrophoresis in the presence of sodium dodecyl sulfate. Shapiro, Viñuela, and Maizel (1967) have shown that polyacrylamide gel electrophoresis in the presence of the denaturing agent sodium dodecyl sulfate may be used to estimate the molecular weights of the subunits of oligomeric proteins. The validity and accuracy of the method has been quite firmly established by Weber and Osborn (1969). In the initial experiment, about 10 μg of the purified enzyme was simply allowed to stand a few minutes at room temperature in 0.1% sodium dodecyl sulfate and 0.01 M 2-mercaptoethanol.

FIGURE 11



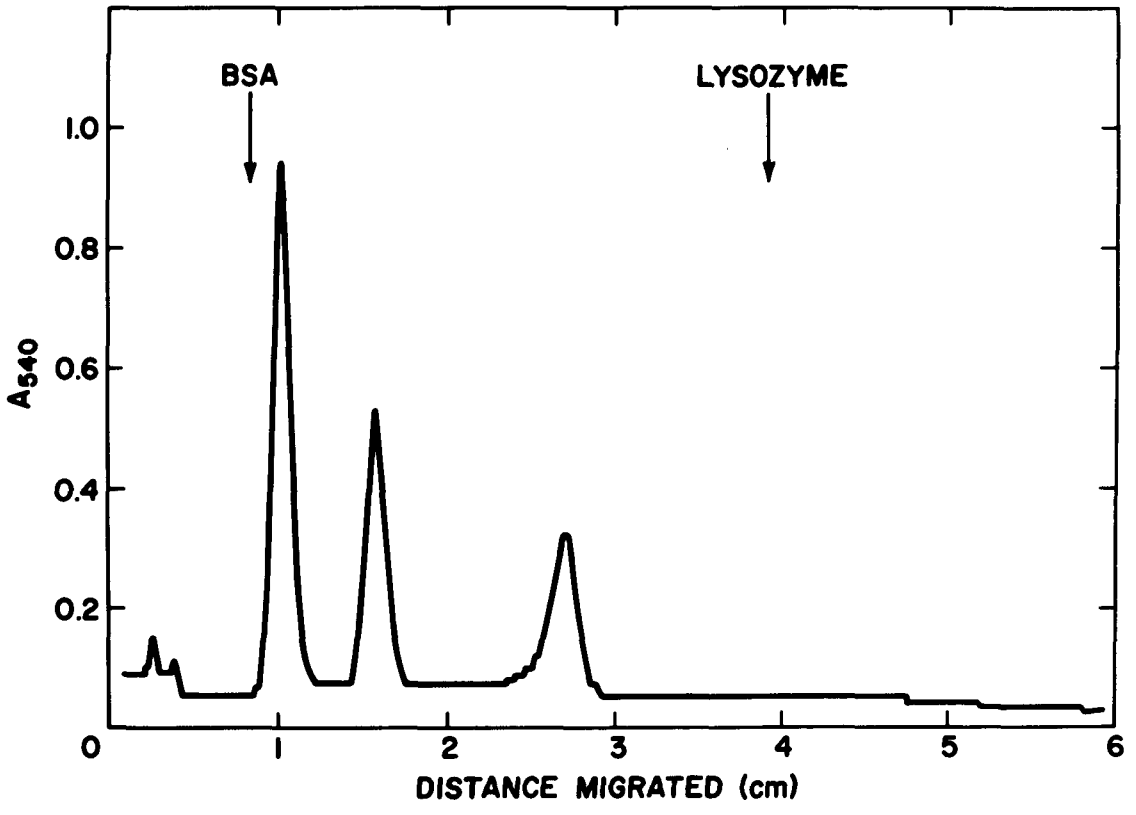
Equilibrium sedimentation of aromatic L-amino acid decarboxylase at 22°. Rotor speed was 10,000 rpm, temperature 22°. Other conditions were as specified in Materials and Methods. The enzyme sample was estimated to be 99% pure by disc gel electrophoresis. The curve was fit to a third order polynomial using a computer program based on the method of least squares.

Electrophoresis and staining essentially by the method of Weber and Osborn (1969) yielded the pattern of bands shown in Figure 12. The calculated molecular weights of these bands were 66,000, 50,000, and 25,000 daltons, containing 40%, 30% and 30%, respectively, of the total stain. Two circumstances mandated caution in interpreting these results. First, the gels were not adequately standardized with respect to mobility as a function of molecular weight. The standards were bovine serum albumin (68,000 daltons) and lysozyme (14,300 daltons), run simultaneously on a separate gel. Since the linearity of the molecular weight function between these extremes was not established, it was thought that the values obtained could easily be in error by as much as 20%. Second, the denaturing and reducing conditions used were much milder than those used by Weber and Osborn (2 hours at 37° in the presence of 1% sodium dodecyl sulfate and 1% 2-mercaptoethanol).

In succeeding experiments, the following standards were used: bovine serum albumin (68,000 daltons), ovalbumin (43,000), pepsin (35,000), chymotrypsinogen (25,700), myoglobin (17,200), lysozyme (14,300), and chrymotrypsin (13,000 and 11,000). In some experiments, ovalbumin dimers (86,000 daltons) were observed, giving a further standard at high molecular weight. Chymotrypsinogen was included as an internal standard, in the same gel in which enzyme was run. All mobilities were expressed relative to chymotrypsinogen and a line was fitted to the standards according to their relative mobilities. Values of the molecular weights of the standards estimated from their mobilities and the fitted line were all within 11% of the values from the literature cited by Weber and Osborn (1969).

Much more severe denaturing and reducing conditions were applied. These conditions included: incubation for 3 hours at 37° in the presence of 0.1% sodium dodecyl sulfate and 0.1 M 2-mercaptoethanol; incubation for 15 min

FIGURE 12



Polyacrylamide gel disc electrophoresis in the presence of sodium dodecyl sulfate. The basic method of Weber and Osborn (1969) was followed. Details are provided in the text. The stained gel was scanned at 540 nm in a Gilford Model 240 recording spectrophotometer equipped with a Model 2410 linear transport device.

at 100° in the presence of 1% sodium dodecyl sulfate and 0.2 M 2-mercaptoethanol; incubation at 100° for 15 min in the presence of 0.2% sodium dodecyl sulfate and 0.02 M dithiothreitol. In all cases, the pattern of bands observed was basically similar to that in the original experiment (Figure 12) although the apparent amount of protein in the high molecular weight band was decreased to approximately 20% of the total, while the smaller molecular weight bands were apparently increased to about 40% each of the total. The average values of the molecular weights of the observed bands were 57,000, 40,000 and 21,000 daltons. In one case in which approximately 100 μg of enzyme was used instead of the usual 10 μg, a fourth band (about 10% of the total stain) corresponding to a molecular weight of greater than 100,000 was observed.

A number of models for the quaternary structure of the enzyme are consistent with these results, if it is not assumed that the enzyme was fully denatured and/or reduced. Clearly this cannot be assumed since some material of apparent molecular weight greater than 100,000 still remained after quite vigorous treatment. However, several inferences seem justified. First, the entire molecular weight of the native enzyme is accounted for by the molecular weights of the three bands. Second, the sum of the molecular weights of the faster components is approximately equal to that of the slowest component, suggesting the possibility that this component represents an undissociated dimer of the other two. This is consistent with the fact that the amounts of the two faster components increased relative to the slowest component with more vigorous treatment and that the integrated staining intensities of the faster components were in constant proportion with all treatments, suggesting a stoichiometric relationship. Third, these results are consistent with the ultracentrifugal analysis at 22°, in which material of molecular weight less than 30,000 was present near the meniscus and material of molecular weight greater than 90,000 was present near the bottom of the cell.

III. IMMUNOLOGICAL STUDIES OF THE ENZYME

The preparation of antiserum to the purified enzyme has already been described. The specificity of the antiserum was established by double diffusion and immunoelectrophoresis experiments (see Figure 5) and this specificity was used as evidence for the homogeneity of the enzyme. It was also used in demonstrating the antigenic identity of the two peaks of enzyme activity obtained in the second DEAE-Sephadex fractionation. Antisera have many other important uses, however, such as immunofluorescent localization of enzyme, the estimation of enzyme turnover rates, and the determination of relative or even absolute levels of enzyme protein. An example of this last type of application of this antiserum has been published (Dairman, Christenson, and Udenfriend, 1971). A number of immunological studies of the enzyme were undertaken. Some of these were of a preliminary nature and dealt largely with methodology. The implications of these results will be fully considered in the Discussion.

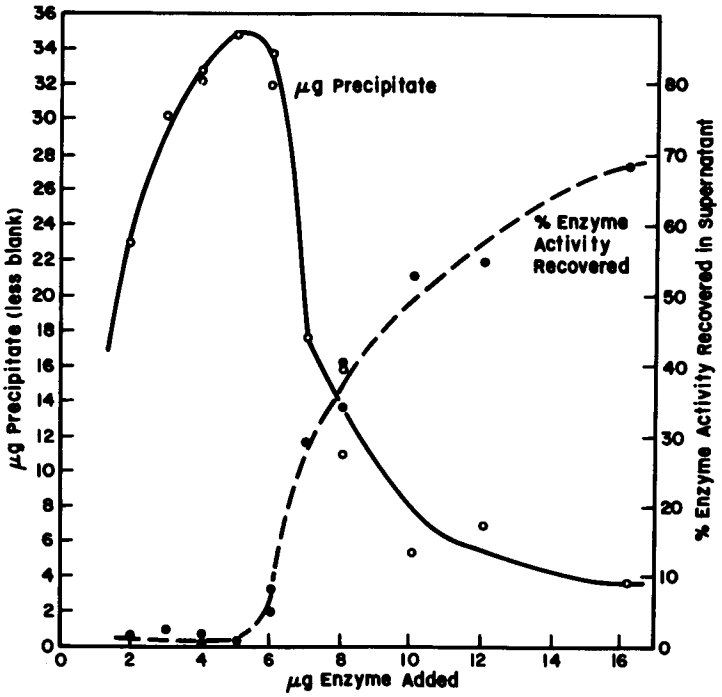
Determination of antibody titer by quantitative precipitin assay. A preliminary experiment was performed to determine the approximate equivalence point, the time required for maximum precipitation, and the values to be expected from various types of blanks. The blanks used contained either antiserum but no enzyme, enzyme but no serum, enzyme and normal goat serum, or enzyme and goat antiserum to another enzyme. An enzyme preparation of about 20% purity was used. The tubes were incubated at room temperature for one hr and at 4° for six days. The tube which contained about 0.4 μg of enzyme per μl of antiserum had the largest amount of precipitate and was assumed to be near equivalence. Precipitates, thought to be due to non-specific aggregation, formed in all tubes which contained serum. The amount

of this nonspecific precipitate appeared to depend upon the particular serum used. Clearly, then, the best blank would contain antiserum but no enzyme.

It was thought that the rate of appearance of the nonspecific precipitate might be slower than that of the immunoprecipitate. If this were the case, it should be possible to choose an optimum incubation time at which the immunoprecipitate would have reached its maximum value while the accumulation of nonspecific precipitate would be minimal. An experiment was performed in which the formation of nonspecific precipitates and immunoprecipitates was followed daily. The enzyme preparation was about 20% pure and the amounts of enzyme and serum were chosen so as to be near the approximate equivalence point determined in the previous experiment. However, in the experiment described below, it was found that this relatively rapid appearance of the specific immunoprecipitate occurred only very near the equivalence point and it was necessary to wait for several days to insure complete precipitation. Under these conditions, it was found that formation of the immunoprecipitates was essentially complete after 60 hrs of incubation at 4° while nonspecific precipitate continued to increase for at least another 24 hrs.

Details of the quantitative precipitin procedure are given in Materials and Methods. The results are shown in Figure 13. Precipitation was maximal at a ratio of 0.51 μg of enzyme per μl of antiserum. At this point, all of the enzyme activity was precipitated, and it was taken as the actual equivalence point. The supernatants were tested for antibody content by determining whether they inhibited added enzyme, but significant inhibition was observed only with the blanks. At the equivalence point, the precipitate, less blank, contained 35 μg of protein. Since the precipitate must contain 5.1 μg of enzyme, the remaining 30 μg must be precipitated antibody. Thus, the absolute

FIGURE 13



Quantitative precipitin assay. Details of the procedure are given in Materials and Methods. The antiserum used was obtained nine days after the second injection. The enzyme preparation was a pool of fractions 28 through 31 shown in Figure 10 and contained no detectable impurities in disc gel electrophoresis.

titer of antiserum was 3.0 mg/ml. It was calculated that the precipitate had a molar ratio of antibody to enzyme of 4:1 at equivalence, assuming a molecular weight of 160,000 daltons for goat γ-globulin. This ratio was as high as 7:1 in antibody excess. Since the amount of antibody precipitated per μg of enzyme increased in the antibody excess region, it is not surprising that antibody could not be detected in the supernatant.

Inhibition of enzyme activity by antiserum. Experiments in which antiserum was simply added to the assay mixture gave erratic results. The extent of inhibition was not clearly related to the amount of antiserum added and in some cases no inhibition whatever was observed. However, when the enzyme was pretreated with antiserum under conditions optimal for the antibody-antigen reaction, then assayed, results such as those shown in Table 8 were obtained. The dashed line represents the equivalence point. Note that no greater than 60% inhibition was observed. In fact, even in extreme antibody excess, up to 60 times equivalence, no more than 72% inhibition has been observed. It is clear from the data in Table 8 that maximum inhibition is approached at the equivalence point. Control sera had no effect on the enzyme activity.

High speed sedimentation of enzyme-antienzyme complexes. The precipitation of antigen by antibody is thought to occur in two steps. The initial antigen-binding reaction is thought to reach completion in a matter of seconds or minutes under ordinary conditions, while the flocculation reaction, in which the original small complexes react to form large, insoluble aggregates, usually requires a matter of days. Feigelson and Greengard (1962) sedimented complexes of tryptophan pyrrolase and its antienzyme at 100,000xg after overnight incubation at 4°. Table 9 shows that aromatic L-amino acid decarboxylase could be quantitatively sedimented after incubation with antiserum for only 20 min at room temperature. As in Table 8, the dashed line divides the data into the

TABLE 8

INHIBITION OF PURE HOG KIDNEY AROMATIC L-AMINO ACID DECARBOXYLASE BY ANTI-ENZYME SERUM

Antiserum (µl)	Enzyme (µg)	Inhibition (%)
2	4.8	13
5	4.8	34
8	4.8	49
10	4.8	54
15	4.8	59

Homogenous enzyme (25 µl) was incubated for 20 min at room temperature with the indicated amount of antiserum in a total volume of 1 ml, containing 0.005 M sodium phosphate, pH 7.2, 0.9% NaCl, and 0.1% bovine serum albumin. Aliquots were then assayed in duplicate. The dashed line divides the data into the regions of antigen excess and antibody excess as determined by quantitative precipitin analysis (Figure 13).

TABLE 9

PRECIPITATION OF DECARBOXYLASE ACTIVITY BY

ANTISERUM USING HIGH SPEED CENTRIFUGATION

Antiserum (µl)	Activity Remaining in 100,000xg supernatant (% of control)	Activity Precipitated (= 100% - % Activity Remaining in the supernatant)
0.5	51	49
1	61	39
2	67	33
5	33	67
7.5	5	95
10	6	94
15	0	100
20	6	94
25	8	92

Homogeneous enzyme (4.8 µg in 25 µl) was incubated for 20 min at room temperature with the indicated amounts of antiserum in a total volume of 1 ml, containing 0.005 M sodium phosphate, pH 7.2, and 0.9% NaCl. The incubation mixtures were then centrifuged at 100,000xg for one hr and duplicate aliquots of the supernatants assayed. The dashed line divides the data into the regions of antigen excess and antibody excess as determined by quantitative precipitin analysis (Figure 13). In the controls, a comparable amount of non-immune goat serum was substituted for the antiserum.

zones of antigen excess and of antibody excess as determined by quantitative precipitin assay. Note that maximum precipitation occurs near the equivalence point. Unfortunately, however, there is no clear and simple relation between the amount of antiserum added and the extent of precipitation. For example, 0.5 µl of antiserum appears to precipitate more enzyme than does 2 µl. In later studies, with relatively crude enzyme preparations and thus high total protein concentrations, this difficulty was not encountered. The addition of greater amounts of antiserum consistently resulted in greater extent of precipitation (for example, see Figure 18).

Preparation of radioactively-labeled enzyme. Enzyme bearing a radioactive label is a prerequisite for a radioimmunoassay for the enzyme and would be useful for other purposes, such as tracing inactive enzyme. For a practical radioimmunoassay, a specific activity of at least 10,000 cpm per µg of enzyme would be necessary. At the same time, minimal modification of antigenic determinants is necessary for a good tracer in immunological studies. Attempts to react the sulfhydryl groups of the enzyme with [^{14}C] iodoacetamide and N-ethyl-[^{14}C]maleimide (Amersham/Searle) resulted in the incorporation of less than 1000 cpm/µg, far below the desired specific activity, even after reduction of the enzyme by dialysis against 1×10^{-3} M dithiothreitol.

Labelling of tyrosine residues with radioactive iodine has been used successfully in the radioimmunoassay of proteins (see, for example, Utiger, Parker and Daughaday, 1962; Kolb and Grodsky, 1970; Temler and Felber, 1971). The enzyme was treated with ^{125}I essentially according to the procedure of Hunter and Greenwood (1962). Details of the method are given in Materials and Methods. The specific radioactivity of the labelled enzyme was about 3.6×10^7 cpm/µg. Since the efficiency of the counter was not known, the

extent of reaction could not be calculated exactly. Assuming an efficiency of 30%, it can be shown that approximately one tyrosine residue in ten had reacted, if only monoiodinated tyrosines were produced.

Preparation of a solid-phase immunoadsorbent. An antibody bound to a solid phase is a highly specific reagent for studying the antigen. Antigen may be bound to the solid phase rapidly and separated from components in solution by simple, low speed centrifugation and washing. Since proteins may be non-specifically bound to the immunoadsorbent, it is desirable to separate the antibodies from other serum components before coupling to the solid support. Preliminary experiments showed that the enzyme-precipitating and enzyme-inhibiting activities of the antibody were precipitated from serum by adding one-half its volume of saturated ammonium sulfate. This is the behavior expected of a γ-globulin. About 1/3 to 1/4 of the A_{280} of the serum was recovered in this fraction.

The procedure of Cuatrecasas (1970) was investigated as a method for coupling protein to Sepharose 4B. The procedure involves two steps: "Activation" of the Sepharose by reaction with cyanogen bromide at pH 11, and the reaction of the activated Sepharose with the protein at pH 10. Twenty ml of 50% (v/v) Sepharose 4B in water was treated with 5 g of cyanogen bromide. The activated Sepharose was reacted with 200 mg of bovine serum albumin in 10 ml of 0.1 M sodium carbonate, pH 10. The reacted material was washed with thirty volumes of water, eight volumes of 6 M guanidine·HCl, and again with ten volumes of water. From the recovery of A_{280} in the washes, the coupling yield was estimated at 70%.

Cuatrecasas and Anfinsen (1971) point out that with large ligands, such as proteins, better recovery of activity is achieved when the coupling reaction is carried out under suboptimal conditions. Use of 0.2 M sodium

citrate buffer, pH 6.5, was recommended. They attribute this to conformational constraint due to multiple points of attachment, but cite no evidence for this hypothesis. When 20 ml of 50% (^{v}/v) Sepharose 4B was activated with 2.5 g of cyanogen bromide and 200 mg of bovine serum albumin was coupled under these conditions, the coupling yield was estimated at 50%. It was decided that this yield was acceptable, assuming that more antibody activity could be recovered.

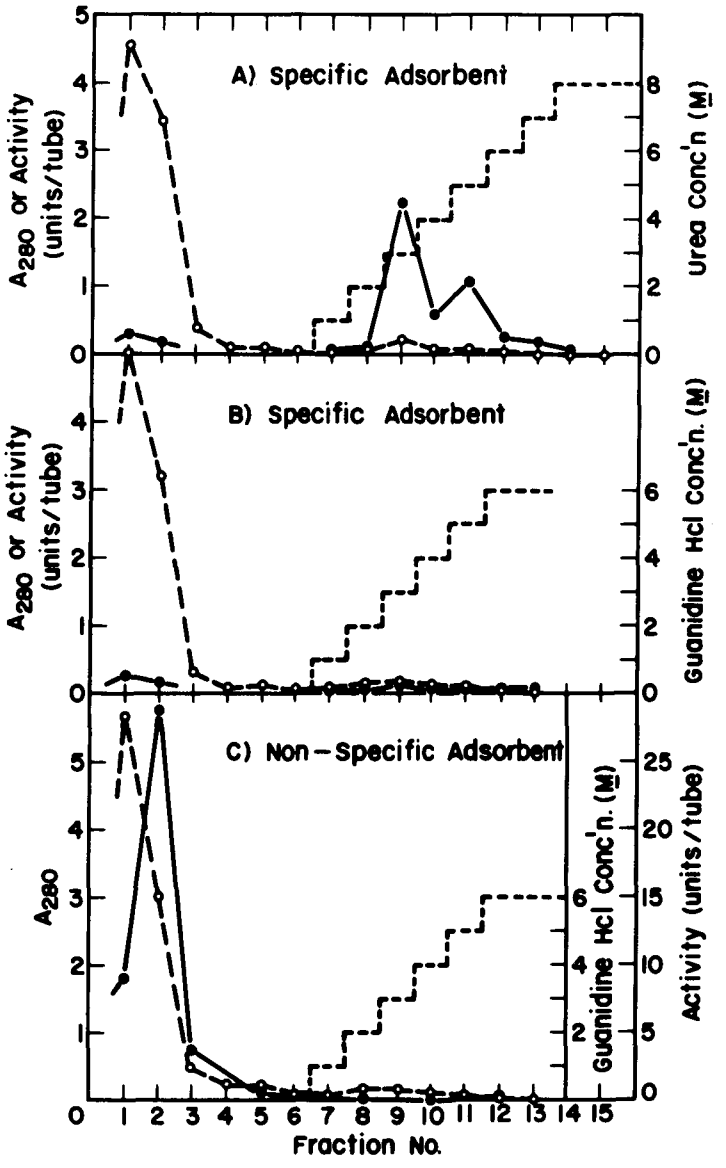
When 80 mg of bovine serum albumin or when 3 g of cyanogen bromide were used, the coupling yield was only marginally improved, if at all. Thus, it is clear that the amount of cyanogen bromide used does not limit the coupling yield under these conditions.

The details of the final procedure used in preparing the immunoadsorbent and a control material of normal goat γ-globulin are given in Materials and Methods. The coupling yields were estimated at 87% and 90%, respectively.

Binding of enzyme by antibody-Sepharose. The enzyme was specifically adsorbed by small columns of antibody-Sepharose, but not by the nonspecific γ-globulin-Sepharose. As shown in Figure 14, most of the protein was eluted from both columns with buffered saline. Most of the enzyme activity was eluted from the γ-globulin-Sepharose column (but not the antibody-Sepharose column) together with the bulk of the protein. The columns were then eluted with 1 ml aliquots of 1-8 M urea or 1-6 M guanidine HCl in 1 M increments. The small amount of activity was recovered in the fractions containing 2-5 M urea. These fractions were dialyzed against 1 l of buffered saline containing 0.01 M 2-mercaptoethanol for four hrs and assayed. There was no increase in activity following dialysis.

To determine the binding capacity of the antibody-Sepharose, the immunoadsorbent was titrated with crude hog kidney enzyme. The enzyme preparation had been precipitated at 50% saturation with ammonium sulfate, redissolved

FIGURE 14


A) Specific Adsorbent
B) Specific Adsorbent
C) Non-Specific Adsorbent
A280 or Activity (units/tube)
A280
Urea Conc'n (M)
Guanidine Hcl Conc'n. (M)
Activity (units/tube)
Fraction No.

LEGEND TO FIGURE 14

Binding of enzyme by columns of antibody-Sepharose. Hog kidney was homogenized and centrifuged as usual, but without 2-mercaptoethanol. The crude supernatant was brought to 50% saturation with ammonium sulfate and the precipitate was redissolved in one half of the original volume of buffered saline, and dialyzed against buffered saline. The columns (0.7 cm diameter) contained 1 ml settled volume of either antibody-Sepharose (Panels A and B) or γ-globulin-Sepharose (Panel C). To each column was added 0.35 ml of the enzyme preparation. The columns were allowed to stand for 20 min at room temperature and were then washed with 6 one ml aliquots of buffered saline followed by 1 ml aliquots of either urea (Panel A) or guanidine hydrochloride (Panels B and C) in concentration increments of 1 M. Plots show absorbance at 280 nm (o---o), enzyme activity (●——●), and concentration of urea or guanidine hydrochloride (---) of the eluant added to the column during collection of the indicated fraction. Note change in scale of enzyme activity in Panel C.

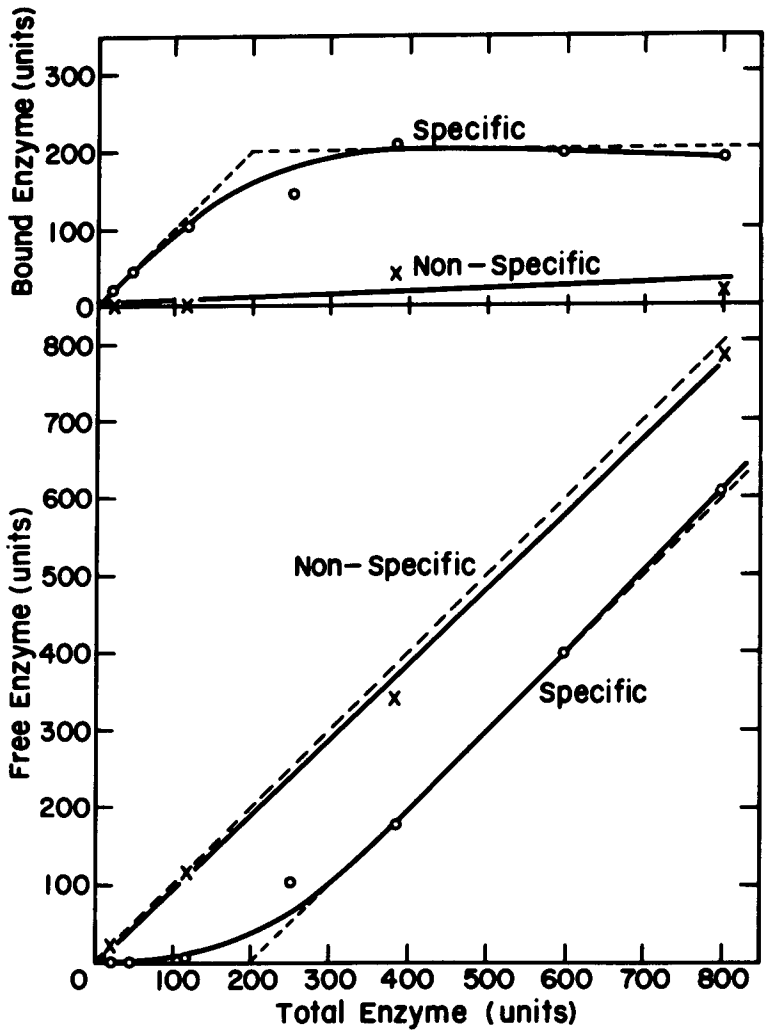
in buffered saline, and dialyzed against buffered saline for 2 hr. Aliquots of 0.010 to 0.40 ml of this preparation were added to 0.05 ml of settled antibody-or γ-globulin-Sepharose in a total volume of 1 ml of buffered saline containing 1 mg of bovine serum albumin. The mixtures were stirred for 30 min at room temperature and the adsorbents separated by centrifugation. The supernatants were then assayed for enzyme activity.

The results are shown in Figure 15. The amount of enzyme bound was calculated as the difference between the enzyme added and the enzyme recovered in the supernatant. The dashed lines show the theoretical behavior for an immunoadsorbent having a binding capacity of 4000 units/ml and a control material which does not adsorb enzyme nonspecifically. It is clear that the actual capacity of the immunoadsorbent is very close to 4000 units/ml.

Assuming that the pure enzyme has a specific activity of 8500 units/mg, the binding capacity may be expressed as 460 μg of enzyme per ml of antibody-Sepharose. It has been shown by quantitative precipitin analysis that the antiserum precipitates 0.51 μg of enzyme per μl of serum at equivalence, so that the 10 ml of serum used in preparing the 25 ml of immunoadsorbent was equivalent to 5100 μg of enzyme or about 200 μg per ml of settled material. It was also shown that the immunoprecipitate had a molar ratio of antibody to enzyme of 4:1 at equivalence. Since the immunoadsorbent presumably follows a 1:1 stoichiometry, the overall recovery of antibody activity was 55%. Taking into account the 87% coupling yield, the recovery would be 63%.

Binding of ^{125}I-enzyme by antibody-Sepharose. In preparing ^{125}I-labelled proteins there is often some damage to the substrate (see Yalow and Berson, 1960; Kolb and Grodsky, 1970) so that some portion of the labeled material may not be antigenically active. This is probably due mostly to side reactions

FIGURE 15



Titration of enzyme-binding capacity of antibody-Sepharose. Details of the experiment are provided in the text. Lower curves show enzyme activity in the supernatant as a function of total enzyme incubated with 0.05 ml of antibody-Sepharose (o——o) or γ-globulin-Sepharose (x——x). Upper curves show bound enzyme determined by difference. Dashed lines are theoretical for a specific adsorbent having a capacity of 4000 units/ml or a control material which does not adsorb enzyme non-specifically.

with the oxidizing agents used to convert radioactive iodide to iodine, but there may also be some radiation damage. To determine how much of the ^{125}I-enzyme was not antigenically active, 3 µl (about 160,000 cpm) of ^{125}I-enzyme was mixed with 0.75 ml of a crude supernatant from hog kidney. Aliquots of 0.25 ml each were applied to small (1 ml) columns of antibody-Sepharose and nonspecific γ-globulin-Sepharose. The material was allowed to run into the column and stand for 20 min. Then the columns were eluted with six 1 ml aliquots of buffered saline, followed by four 1 ml aliquots of 6 M guanidine-HCl. Approximately 95% of the radioactivity was recovered in the first six fractions (saline) from the nonspecific column and another 1% in the next four fractions (guanidine). By contrast, only 10% of the radioactivity was recovered in the saline fractions from the antibody-Sepharose column. Thus, about 10% of the ^{125}I-enzyme has lost immunological activity. It should be noted that only 62% of the radioactivity was recovered in the guanidine fractions; apparently, not all of the enzyme is desorbed from the immunoadsorbent under these conditions.

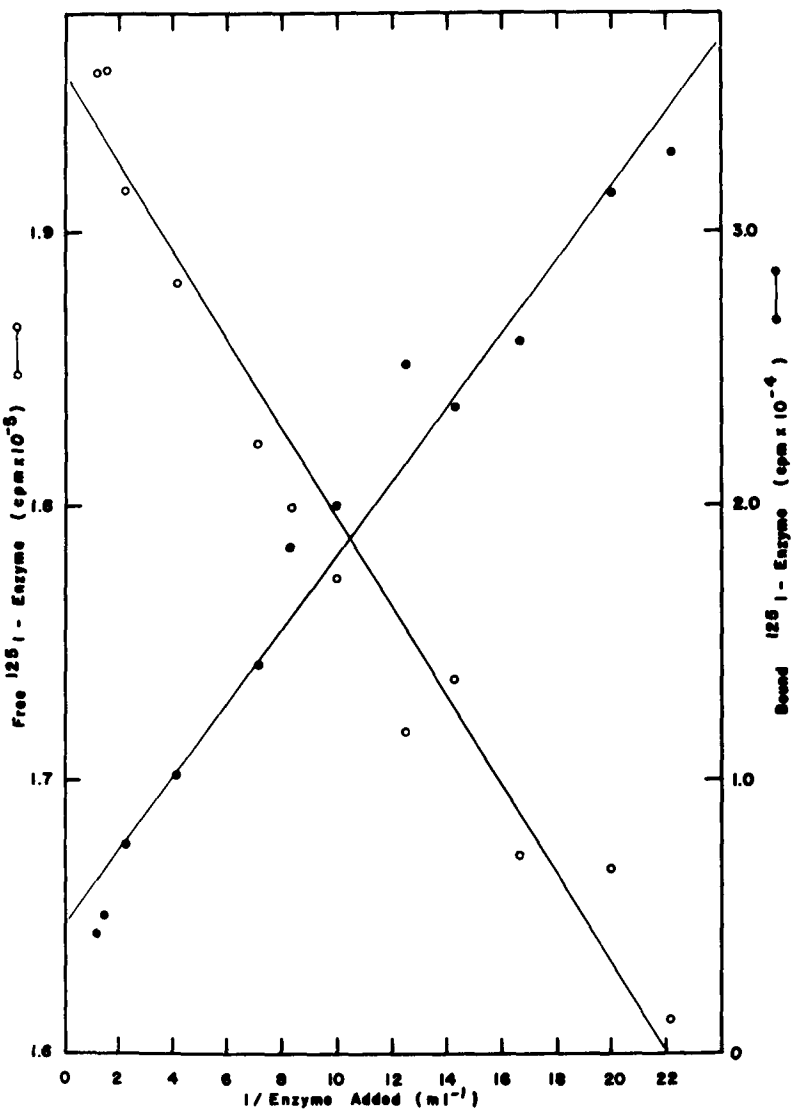
Another method for estimating this figure is to titrate the immunoadsorbent as was previously described (see Figure 15) with a mixture of ^{125}I-enzyme and unlabelled enzyme. The slope of a plot of the ^{125}I cpm bound as a function of enzyme units bound is the specific radioactivity of the immunologically active enzyme in cpm/per unit of enzyme activity. When this value is divided by the overall specific radioactivity of the enzyme, including both immunologically active and inactive forms, the quotient is the fraction of immunologically active ^{125}I-enzyme. A preparation of ^{125}I-enzyme, estimated to contain 20% immunologically inactive enzyme by the column procedure previously described was also tested by this method. The titration method

yielded a value of 24% immunologically inactive enzyme. Since the results are in reasonably good agreement and the column method is considerably easier and faster, it appears to be the method of choice for routine testing.

Competition of ^{125}I-enzyme and unlabelled enzyme for antibody sites on the immunoadsorbent. The displacement of labelled antigens from antibody combining sites, or competition for such sites, by unlabelled antigen forms the basis of all radioimmunoassays. An experiment was performed to determine whether the antibody-Sepharose could be used in a radioimmunoassay for aromatic L-amino acid decarboxylase. As shown in Figure 16, both free and bound radioactivity had a reasonably good, linear correlation with the reciprocal of the amount of unlabelled enzyme added (correlation coefficients > 0.98). This is the expected result if the binding reaction is at equilibrium. The total recovery of free and bound radioactivity was 93.5 $\pm$ 1.0% (S.D.). Although there was considerable scatter and, perhaps, some nonlinearity, this method with some refinement could form the basis for a sensitive radioimmunoassay for the enzyme.

Cross-reactivity of the antiserum with decarboxylase from other species. Hog kidney is an excellent preparative source for the enzyme, but routine use of the hog as a laboratory animal is not generally practical. Therefore, studies of the cross-reactivity of the antiserum with enzyme prepared from common laboratory animals were performed. Quantitative precipitin assays gave unreliable results due to the formation of large amounts of non-specific precipitate. Double diffusion experiments failed to yield precipitin lines over a wide range of enzyme and antiserum concentrations. Complement fixation was not even attempted, since it was found that the antiserum did not fix complement well, even with the enzyme from hog kidney.

FIGURE 16


1.9
1.8
1.7
1.6
3.0
2.0
1.0
0
0 2 4 6 8 10 12 14 16 18 20 22
1/ Enzyme Added (ml⁻¹)
Free 125I - Enzyme (cpm x 10⁻⁵)
Bound 125I - Enzyme (cpm x 10⁻⁴)

LEGEND TO FIGURE 16

Competition of unlabelled enzyme and ^{125}I-enzyme for immunoadsorbent sites. The unlabelled enzyme preparation was prepared as in the experiment described in Figure 14. The ^{125}I-enzyme was diluted 1:40 with this preparation (" ^{1}/Enzyme Added" on the abscissa includes both the unlabelled enzyme used in this dilution and that added later). A suspension of 50% (v/v) antibody-Sepharose was diluted 1:10 with a 50% (v/v) suspension of washed Sepharose 4B. To each tube was added in the following order: 40 µl of diluted ^{125}I-enzyme (about 200,000 cpm), a sufficient volume of 1% bovine serum albumin in buffered saline to bring the final volume to 1 ml, aliquots of unlabelled enzyme (0.005 to 0.8 ml), and 0.1 ml of the Sepharose suspension. The mixtures were stirred at room temperature for 1 hr, then centrifuged. The supernatants were removed to counting tubes and the pellets washed with two 1 ml aliquots of 2 M NaCl in 0.005 M sodium phosphate buffer, pH 7.2, which were combined with the supernatants. The Sepharose pellets were then washed into counting tubes with two 0.5 ml and one 1 ml aliquot of buffered saline. Both phases were counted.

Perhaps the simplest and fastest method of determining whether the antiserum reacts with the enzyme from a given species is merely to mix them and measure the inhibition of enzyme activity. It has already been shown that the antiserum inhibits the enzyme from hog kidney up to about 70% (Table 8). When a similar experiment was performed using rat kidney as the enzyme source, a similar degree of inhibition was observed.

Another method of following the antibody-enzyme reaction is the high speed centrifugation method (see Table 9). Similar experiments were performed with enzyme from rat kidney (Table 10); guinea pig kidney, brain and adrenal; rabbit kidney, brain, and adrenal; human liver; and frog heart, kidney, and brain. The results are shown in Table 11. It was concluded that the antiserum cross-reacts with the enzyme from all the mammalian tissues and species studied, but not with the enzyme from frog tissues.

Neither of the techniques discussed above is likely to be useful in determining quantitatively the extent of structural similarity between the homologous enzyme (i.e., the one used as antigen) and another, heterologous enzyme. This is because they depend upon very limited regions of homology to give an observable effect. Two other methods, which should require extensive homology, were investigated. The binding capacity of the immunoadsorbent for the guinea pig kidney was determined in a manner similar to that used for the hog kidney enzyme (Figure 15), except that the ratio of antibody-Sepharose to enzyme was much higher. A curve similar to that in Figure 15, but strongly shifted toward the origin, was obtained. The binding capacity calculated for the guinea pig kidney enzyme was about 100 units per ml of antibody-Sepharose (cf. 4000 units/ml for the homologous enzyme).

The competition of the rat kidney enzyme with ^{125}I-enzyme from hog kidney for sites on the immunoadsorbent was also tested. Aliquots (0.05 to 0.8 ml) of a crude supernatant from rat kidney were added to tubes containing about

TABLE 10

CROSS-REACTIVITY OF ANTISERUM WITH RAT KIDNEY ENZYME AS INDICATED BY INHIBITION AND BY HIGH SPEED SEDIMENTATION

Antiserum (µl)	Activity Remaining in 100,000xg Supernatant (% of control)	Activity Precipitated (100% - % Activity Remaining)	Inhibition (%)
10	28	72	16
50	5	95	45
100	4	96	60

Aliquots of a crude supernatant from rat kidney (8.5 units of activity in 50 µl) were incubated with the indicated amount of antiserum under the conditions described in Table 9. One set of incubation mixtures was then centrifuged at 100,000xg for 60 min, while the other was held on ice. Both sets were assayed in duplicate; the latter set of data is expressed as inhibition. In the controls, a comparable amount of non-immune goat serum was substituted for the antiserum.

TABLE 11

CROSS-REACTIVITY OF ANTISERUM WITH ENZYME FROM

GUINEA PIG, RABBIT, HUMAN, AND FROG TISSUES

Species	Tissue	Enzyme (units)	Antiserum (µl)	Activity Remaining in 100,000xg Supernatant (% of control)
Guinea Pig	Kidney	63	10	84
			100	19
	Brain	3.4	1	100
			10	40
	Adrenal	1.3	1	78
			10	29
Rabbit	Kidney	42	10	29
			100	2
	Brain	4.2	1	52
			10	9
	Adrenal	2.4	1	37
			10	15
Human	Liver	9.2	2	53
			5	33
			10	26
			25	20
Frog	Heart	0.34	1	112
			10	121
			100	106
	Kidneys and Adrenal Bodies	14	1	111
			10	103
			100	109
	Brain	4.8	1	102
			10	101

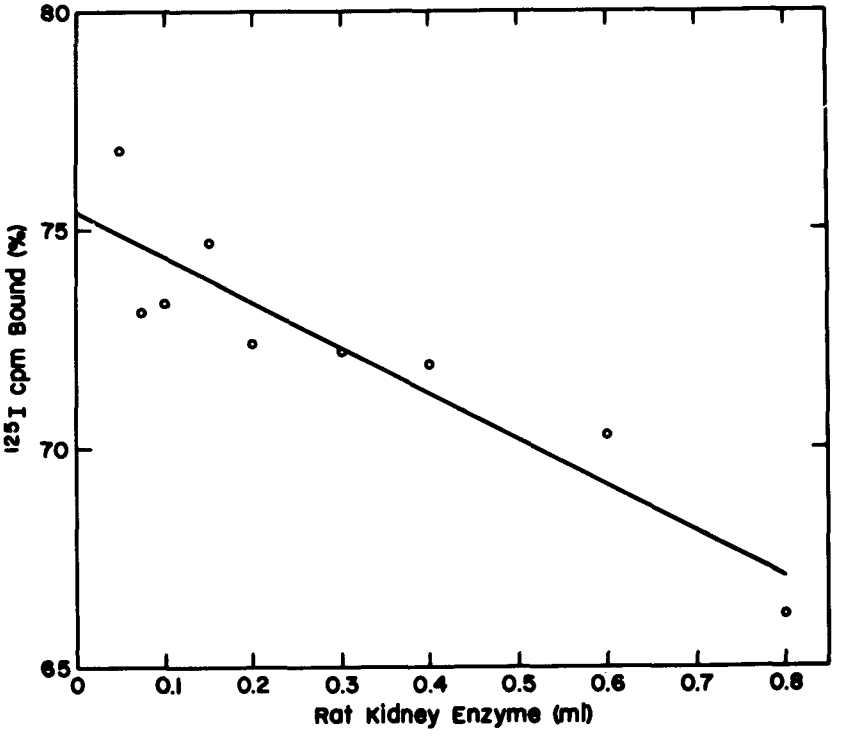
Enzyme sources were as follows: one male guinea pig, 850 g, killed by cervical dislocation; one male rabbit, 3.6 kg, killed by injecting air into ear vein; one female human, 56 years of age, died of stroke, portion of liver obtained at autopsy; four female frogs (Rana pipiens), about 12 cm long, killed by decapitation. Tissues were homogenized in three volumes of 0.005 M sodium phosphate buffer, pH 7.2, and centrifuged at 25,000xg for 20-40 min. The guinea pig extracts were brought to 60% saturation with ammonium sulfate, the precipitates were redissolved and used as the enzyme preparation. In all other cases, the enzyme preparation was simply the 25,000xg supernatant. Incubation and centrifugation were carried out as described in Table 9, except in the case of human liver, where incubation was for 60 min. In the controls, a comparable amount of non-immune goat serum was substituted for the antiserum.

1μl of ^{125}I-enzyme. As shown in Figure 17, there was some apparent displacement of ^{125}I-enzyme from the immunoadsorbent by the heterologous enzyme. The hypothesis that the correlation coefficient is zero was tested (Bowker and Lieberman, 1959, p. 274) and rejected at the 99.9% confidence level. However, it is not clear whether this correlation reflects true competition by the rat enzyme or some nonspecific effect. This may be contrasted with the very effective competition shown by the homologous enzyme (Figure 16).

Simultaneous immunological titration of DOPA and 5-hydroxytryptophan decarboxylase activities. The availability of a specifc antiserum to aromatic L-amino acid decarboxylase made it possible to test the hypothesis that both DOPA and 5-hydroxytryptophan are decarboxylated by a single enzyme. The rationale of the experiment is as follows. If there were a single enzyme acting on both substrates, the activities should be precipitated proportionately by the antiserum; that is, removal of some fraction of one activity from solution should entail the removal of the same fraction of the other activity. Complete precipitation of one activity would imply complete loss of the other as well. On the other hand, if there were more than one enzyme, it should be possible to remove one activity without precipitating the other.

The most convenient approach to the problem would be the use of antibody-Sepharose to adsorb the enzyme. However, when this was attempted with brain tissue, it was found that complete precipitation of either activity was never obtained. It was noted that in the previous studies, in which virtually complete adsorption had been achieved (see p. 91), the enzyme source was kidney and the amounts of enzyme used were very high relative to the amounts of brain enzyme which were available. To test whether the incomplete adsorption

FIGURE 17



Competition of rat kidney enzyme and ^{125}I-enzyme from hog kidney for immunoadsorbent sites. The unlabelled enzyme preparation was a crude supernatant from rat kidney prepared in the usual way. The ^{125}I-enzyme was diluted 1:100 with buffered saline containing 1% bovine serum albumin. The dilution of antibody-Sepharose and incubation conditions were similar to those of the experiment described in Figure 16, except that 100 µl of diluted ^{125}I-enzyme (about 200,000 cpm), 0.05 to 0.8 ml aliquots of unlabelled enzyme, and an incubation time of 90 min were used. The solid phase was isolated by filtration and washed. Both the solid phase and the filtrate were counted.

of the brain enzyme was due merely to the low concentration of enzyme, kidney enzyme at high and low levels was treated with antibody-Sepharose. It was found that about 0.5 to 2 units of activity remained in the supernatant at either enzyme concentration. This was negligible when the original amount of enzyme was 50 units or more, but very substantial when the original amount of enzyme was only ten units or less. At low enzyme levels, the adsorption was still incomplete even when the enzyme and immunoadsorbent were incubated for as long as 2.5 hr.

Another method available was incubation of enzyme and antibody in solution, followed by centrifugation at 100,000xg (see p. 86). The time-course of this reaction was followed using low levels of kidney enzyme. The reaction was essentially complete after 1 hr at room temperature.

Since this experimental procedure requires high speed centrifugation, it was necessary to show that both activities are soluble under the conditions used. As shown in Table 12, virtually all of the decarboxylase activity for both substrates was found in the supernatant fractions. These soluble activities did not sediment under the conditions used as controls in the immunological titrations. Recoveries after centrifugation at 100,000xg for 60 min (in the presence of control serum) were 98% and 97%, for DOPA and 5-hydroxytryptophan decarboxylase activities, respectively.

The enzyme levels of the various tissues to be examined were determined and preliminary immunological titrations were performed to determine approximately the amount of antiserum required to precipitate completely the DOPA decarboxylase activity (Figure 18). In the case of the rat brain and rabbit pineals, some activity remained in the supernatant, even in extreme antibody excess. If soluble, active antibody-enzyme complexes were present, it

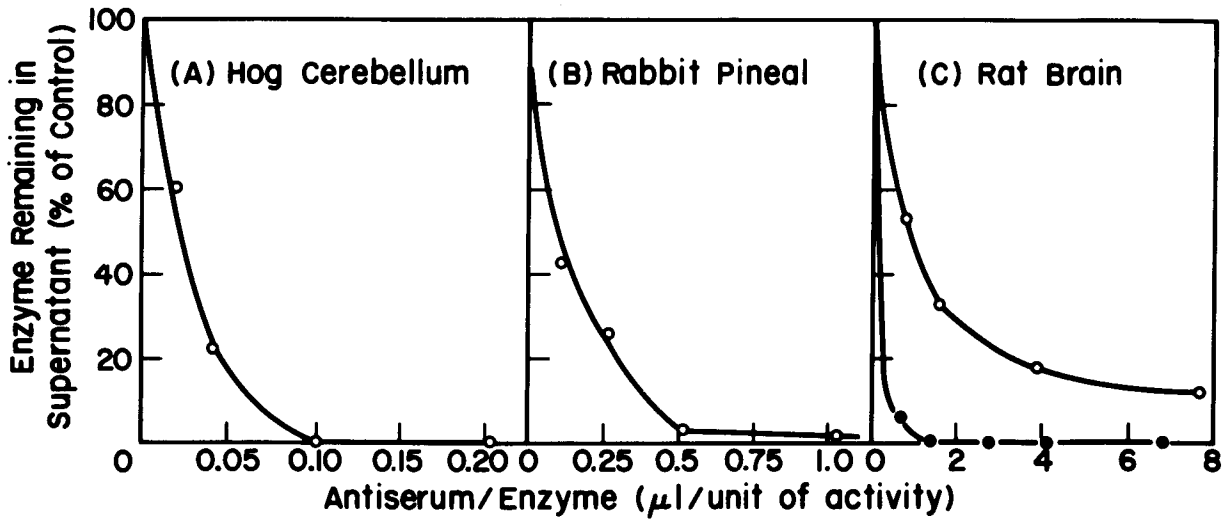
TABLE 12

SOLUBILITY OF DOPA AND 5-HYDROXYTRYPTOPHAN DECARBOXYLASE ACTIVITIES FROM BRAIN

Tissue	Fraction	DOPA Decarboxylase		5-Hydroxytryptophan Decarboxylase	
		Activity (units/g of tissue)	Recovery (%)	Activity (nmole/min per g of tissue)	Recovery (%)
Hog Brain	Crude homogenate	10.6	115	1.05	
	Crude supernatant	12.2	24	1.02	97
	First wash supernatant	2.6	3	0.24	23
	Second wash supernatant	0.3	3	0.06	6
	Washed precipitate	0.4		0.10	9
Rat Brain	Crude homogenate	37.7		1.19	
	Crude supernatant	32.7	87	1.04	87
	First wash supernatant	3.9	10	0.11	9
	Second wash supernatant	1.0	3	0.03	2
	Washed precipitate	1.3	4	0.07	6
Rabbit Pineal	Crude homogenate	2770		276	
	Crude supernatant	2350	85	238	87
	First wash supernatant	40	1	5	2
	Second wash supernatant	3	0.1	0.7	0.3
	Washed precipitate	39	1	6	2

Whole brains were homogenized in three volumes of 0.005 M sodium phosphate, pH 7.2; pineals in ten volumes of the same buffer. Aliquots (60 ml, hog; 5 ml, rat; 1 ml, rabbit pineal) of the homogenates were centrifuged at 27,000xg for 15 min. The precipitates were washed twice with the same buffer (80 ml, 5 ml, and 1 ml, respectively). The washed precipitates were then resuspended in the same buffer (45 ml, 3.75 ml, and 1 ml, respectively). Each fraction was assayed for both enzyme activities, using the $^{14}CO_2$ method for 5-hydroxytryptophan. Recoveries are based on the activity of the crude homogenates.

FIGURE 18


Enzyme Remaining in Supernatant (% of Control)
100
80
60
40
20
0
(A) Hog Cerebellum
(B) Rabbit Pineal
(C) Rat Brain
0.05
0.10
0.15
0.20
0
0.25
0.5
0.75
1.0
0
2
4
6
8
Antiserum/Enzyme (μl/unit of activity)

LEGEND TO FIGURE 18

Immunological titration of DOPA decarboxylase activity from various tissues. Crude supernatants were prepared as in the experiment described in Table 12. Equal aliquots of each crude supernatant were incubated with various amounts of antiserum (obtained nine days after the second injection) or a control serum (obtained from a goat immunized against another enzyme) at room temperature for 1 hr. The incubation mixtures contained, in a final volume of 1 or 2 ml: 0.9% NaCl, 0.005 M sodium phosphate buffer (pH 7.2), 3 to 20 units of enzyme, and 0.1 to 100 µl of antiserum or control serum. After incubation, the mixtures were centrifuged at 100,000xg for 1 hr and the supernatants were assayed in duplicate with DOPA as substrate. In a separate experiment with rat brain (Panel C), an amount of rabbit anti-goat γ-globulin equal to twenty times the volume of antiserum was added after the 1 hr incubation; incubation was continued an additional 30 min before centrifugation (o, antiserum only; •, antiserum plus 20 volumes of rabbit anti-goat γ-globulin).

should be possible to precipitate the remaining activity by adding antiserum to goat γ-globulin. In a separate experiment, aliquots of rat brain crude supernatant were incubated for 60 min at room temperature with a constant amount of antiserum sufficient to give maximum, though incomplete, removal of the enzyme (about 8 μl per unit of activity). Then various amounts of rabbit anti-goat γ-globulin were added, incubated a further 20 min, and centrifuged at 100,000xg for 60 min. It was found that a volume of anti-goat γ-globulin equal to twenty times the volume of antiserum was sufficient to precipitate the enzyme activity completely. A fixed amount of rat brain supernatant was then titrated with antiserum and twenty volumes of anti-goat γ-globulin serum in a similar manner. As shown in Figure 18C, 1.5 μl of antiserum plus 30 μl of anti-goat γ-globulin per unit of activity resulted in complete removal of the enzyme from the supernatant.

Knowing the approximate endpoints for the enzymes of the various species, it was possible to perform titrations and assay for both enzyme activities. Amounts of antiserum were chosen such that several points would be in antigen excess and one point in antibody excess for complete removal of enzyme activity. The "single enzyme" hypothesis would predict that loss of a given fraction of one activity should be associated with loss of that same fraction of the other activity. When one activity is completely lost, the other should be lost as well. The results shown in Table 13 are just those predicted by the hypothesis. The recoveries of enzyme activities in the controls were 98 $\pm$ 8% and 97 $\pm$ 9% (S.D.) for DOPA and 5-hydroxytryptophan decarboxylase, respectively.

Essentially the same data are presented in Figure 19, in which the percentage of 5-hydroxytryptophan decarboxylase activity recovered in the supernatant has been plotted as a function of the percentage of DOPA

TABLE 13

SIMULTANEOUS IMMUNOLOGICAL TITRATION OF DOPA

AND 5-HYDROXYTRYPTOPHAN DECARBOXYLASE ACTIVITIES

Tissue	Antiserum/ Enzyme (µl/unit activity)	Decarboxylase Activity Remaining in the Supernatant	
		DOPA (% of control)	5-Hydroxy-tryptophan (% of control)
Hog Kidney	0.022	89	81
	0.041	64	62
	0.045	68	62
	0.082	36	39
	0.102	20	22
	0.134	7	7
	0.307	0	0
Hog Liver	0.180-0.9	0	0
Hog Brain (whole)	0.013	82	79
	0.025	63	65
	0.190	0	0
cerebellum	0.016	78	83
	0.031	69*	70
	0.078-0.23	0	0
midbrain	0.007	92	84
	0.014	75	83
	0.016	79	78
	0.021	75	75
	0.039	47	59
	0.063	28	33
	0.106-0.24	0	0
hypothalamus	0.014	85	83
	0.024	67	68
	0.048	37	40
	0.120	0	0
Rat Brain (whole)	0.190	88	85
	0.635	56	50
	0.952	41	40
	1.90 †	2	1
Rabbit Pineal	0.028	93	94
	0.055	84	87
	0.138	61	62
	0.277	5	5

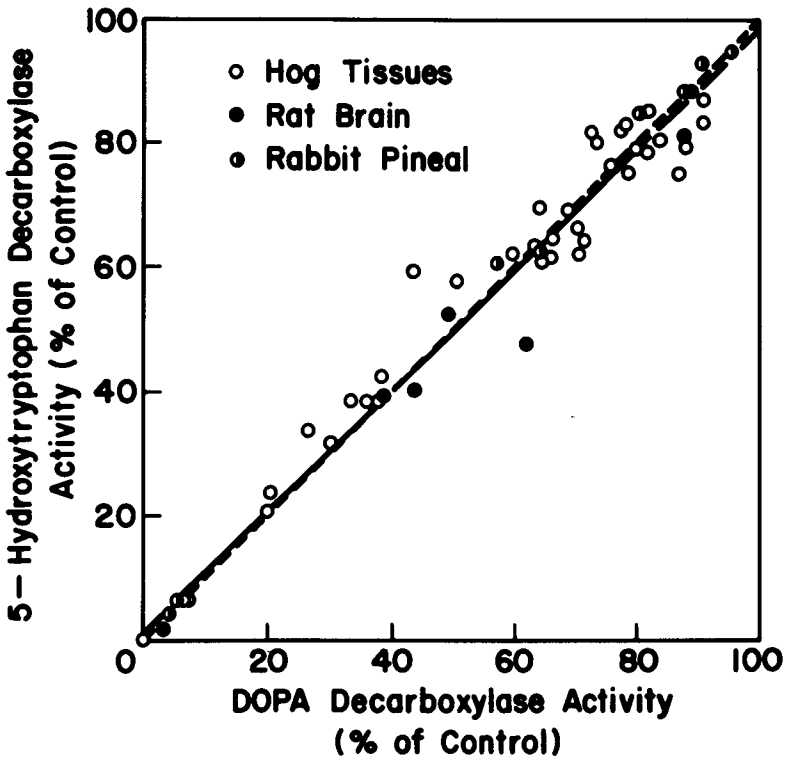
The experimental conditions were similar to those of the experiment in Figure 18. The amount of enzyme used ranged from 5 to 120 units and the amount of antiserum from 0.1 to 30 µl. Incubation mixtures were

prepared in duplicate and each supernatant was assayed only once with each substrate, using the $^{14}CO_2$ method for 5-hydroxytryptophan. The data are presented as averages of the duplicate experiments.

* Based on a single experiment.

† Twenty volumes of rabbit anti-goat gamma globulin were added after one hr of incubation; the mixture was incubated for an additional 30 min before centrifugation.

FIGURE 19



Simultaneous immunological titration of DOPA and 5-hydroxytryptophan decarboxylase activities. The data from the experiments described in Table 13 are presented graphically, with 5-hydroxytryptophan decarboxylase activity remaining in the 100,000xg supernatant plotted as a function of the DOPA decarboxylase activity remaining. Each point represents a single assay for each substrate.

decarboxylase activity. If both activities were associated with a single antigenic entity (that is, with aromatic L-amino acid decarboxylase), the plot should be a straight line with a slope of 1 and an intercept of 0 (dashed line). The line fitted by linear regression analysis to these data (solid line) has a slope of 0.970 and an ordinate intercept of 1.4% with a correlation coefficient of 0.989.

DISCUSSION

The most significant findings of these investigations are that a single homogeneous enzyme is capable of decarboxylating all the naturally occurring aromatic amino acids and that DOPA and 5-hydroxytryptophan decarboxylase activities are not distinguishable by a monospecific antiserum to this enzyme. In view of these results, the name of the enzyme, "aromatic L-amino acid decarboxylase", originally proposed ten years ago by Lovenberg, Weissbach, and Udenfriend (1962), is fully appropriate. Moreover, the distinction between L-3,4-dihydroxyphenylalanine and L-5-hydroxytryptophan carboxy-lyases (EC 4.1.1.26 and 4.1.1.28) in the Enzyme Commission nomenclature should be reviewed.

Although Fellman (1959) has previously reported the preparation of a homogeneous decarboxylase with similar properties from bovine adrenal medulla, serious questions must be raised concerning the purity of Fellman's preparation. For example, his most highly purified preparation was less than 20-fold purified from a high-speed supernatant, compared with over 300-fold purification in the present study. Furthermore, Fellman reported a specific activity of only 158 µl of CO_2/hr per mg of protein, or about 120 nmoles/min per mg, compared with over 8600 nmoles/min per mg in the present study. Fellman's criteria of purity were the presence of a single schlieren peak during velocity sedimentation and a single protein peak in paper and free boundary electrophoresis. The staining procedures used were not described. By current standards, none of these is a particularly reliable or sensitive indicator of homogeneity. The following evidence for the homogeneity of the preparation reported here is cited: presence of a single band in polyacrylamide gel disc electrophoresis in two different buffer systems;

demonstration that the electrophoretically pure enzyme contains only a single antigenic species by Ouchterlony double diffusion and immunoelectrophoresis; presence of a single symmetical boundary of optical density during velocity sedimentation; linearity of plots of ln c as a function of r^2 at sedimentation equilibrium; and agreement of molecular weights determined at the meniscus and at the bottom of the cell by the Archibald method.

Although the enzyme preparations were homogeneous by all the above criteria, it was surprising to obtain such homogeneity after only 300-fold purification of a mammalian enzyme. The possibility that the degree of purification is misleading due to the presence of an activator in the crude material or an inhibitor in the purified enzyme was investigated. This was ruled out by showing that the activities of purified enzyme and crude supernatant were additive. Taken at face value, the data suggest that the decarboxylase represents approximately 0.3% of the soluble protein of hog kidney. Kidneys from other species are also known to be rich in this enzyme (Clark, Weissbach, and Udenfriend, 1954; Awapara, Sandman and Hanly, 1962). The significance of such a high concentration of this enzyme in the kidney is not apparent.

It has long been accepted that pyridoxal phosphate is the cofactor for the enzyme (see Introduction), but the nature of the binding of the cofactor is not yet clear. The facts that the coenzyme is purified concomitantly with the enzyme and that the pure enzyme contains approximately one mole of pyridoxal phosphate per mole of protein suggest that the cofactor is rather tightly bound. The presence of pyridoxal phosphate in the pure enzyme has been confirmed by Voltattorni, Minelli, and Turano (1971). These authors found 0.94 mole of coenzyme per 112,000 g of protein, in excellent agreement with the present results.

A puzzling aspect of the requirement for pyridoxal phosphate is the fact that, although the pure enzyme contains tightly bound pyridoxal phosphate and is active in the absence of exogenous cofactor, the addition of the coenzyme results in a large stimulation of the activity. One explanation is that the coenzyme dissociates from the apoenzyme during the course of the reaction. The presence of exogenous pyridoxal phosphate would, of course, promote reassociation. Another possibility is that the enzyme contains at least two types of binding site, one of high affinity and another of low affinity, the latter being the more numerous or the more active in catalysis. It is conceivable that pyridoxal phosphate has a structural role in the decarboxylase, as it has in glycogen phosphorylase (Shaltiel, Hedrick and Fischer, 1966; Shaltiel et al., 1969; DeVincenzi and Hedrick, 1970), as well as a catalytic role. Since Voltattorni, Minelli, and Turano (1971) were able to resolve the decarboxylase, this question may be investigated by studying the interaction of apoenzyme and coenzyme.

Although it is clear that at least one sulfhydryl group is required for activity, the mechanism of its involvement is not known. While sulfhydryl groups have been implicated in the binding of coenzyme in certain pyridoxal phosphate-dependent enzymes (for example, aspartate aminotransferase; see Turano, Giartosio, and Fasella, 1964), this may be due to stabilization of a favorable conformation, as was concluded by Tate and Meister (1968) in the case of aspartate β-decarboxylase, rather than to a direct interaction with the cofactor. Experiments using the apoenzyme and sulfhydryl reagents can be designed to investigate this point.

As noted in the Introduction, the finding of a lower pH optimum for DOPA than for the other substrates is common in the literature on the enzyme.

The difference in pH optima may be due merely to the inherent instability of catechols at higher pH's or it may suggest that the decarboxylation of DOPA actually occurs by a different mechanism. Suitable experiments on this problem may now be possible.

No differences in specific activity, cofactor content, substrate specificity, or immunological reactivity could be detected in the two peaks of activity separated by rechromatography on DEAE-Sephadex. The separation was, therefore, attributed to an artifact. However, the possibility remains that the two peaks do indeed represent different forms of the enzyme. The observations that the enzyme consistently yields broad bands in disc gel electrophoresis and that it has subunit structure lend some plausibility to the argument that multiple forms do exist. The finding of forms of the enzyme from rat liver with different electrophoretic mobilities (Coulson, Bender, and Jepson, 1969; Antonas, Coulson, and Jepson, 1971) also favor this interpretation, although it is possible that these results are also artifactual.

The results of disc gel electrophoresis in the presence of sodium dodecyl sulfate indicate that the enzyme does have quaternary structure. Since the sum of the molecular weights of the three bands found after such electrophoresis accounts for the total molecular weight of the native enzyme, one obvious model for the quaternary structure is a trimer of subunits of unequal molecular weight. However, since it cannot be assumed that the enzyme was fully dissociated under the denaturing conditions used, at least two additional models must also be considered: a tetramer of at least two types of subunit (having molecular weights of about 21,000 and about

40,000 daltons) and a hexamer of subunits of about 21,000 daltons, which may or may not be identical. Experiments involving end-group determinations, peptide mapping, and sedimentation analyses could distinguish among these models.

If the trimer model is confirmed, it will be a unique event in biochemistry. Only one other enzyme, 2-keto-3-deoxy-6-phosphogluconate aldolase from Pseudomonas putida, composed of three subunits is known (Hammerstedt et al., 1971; Robertson et al., 1971). However, in this aldolase, there is very substantial evidence that the subunits are identical, whereas in aromatic L-amino acid decarboxylase, they would necessarily be non-identical.

Of the various immunological techniques examined, the most useful proved to be sedimentation of early antigen-antibody complexes at high speed following a relatively short incubation period. This method was used in the demonstration that DOPA decarboxylase and 5-hydroxytryptophan decarboxylase are manifestations of a single enzyme and in the study of the decrease of decarboxylase in rat liver following administration of DOPA (the latter study has not been described here; see Dairman, Christenson, and Udenfriend, 1971). The principle benefit of the method is its speed, which obviates any necessity of prolonged incubations during which enzyme activity may be lost for other reasons and non-specific precipitation may become a problem. A further advantage is the fact that the method works with cross-reacting enzyme from other species, even when the cross-reactivity was not apparent by other methods which depend upon the formation of a measurable or visible precipitate, such as double diffusion. Thus, it was possible to use the same antiserum in the study of the enzyme from three different species.

The most serious disadvantage of the technique is that the fraction of the activity precipitated depends upon the absolute as well as the relative concentrations of antigen and antiserum. More complete precipitation is obtained at higher concentrations of either antigen or antibody, even though the relative concentration (in μl of antiserum per unit of enzyme activity) is the same. This is probably due to the fact that the extremely complex precipitation reaction is not allowed to come to equilibrium. Thus, in comparing two enzyme samples, it is necessary to adjust the enzyme concentrations so that they are equal by the criterion of enzyme activity and then determine whether the antiserum precipitates the same amount of activity from both samples. If it does, the relative concentration of enzyme protein is presumably equal to the dilution required to equalize the concentrations of enzyme activity. If this is not the case, it can be said that the enzyme protein concentration is higher or lower in one of the samples, but the relative levels cannot be determined quantitatively from these data. In other words, one is merely testing the hypothesis that the ratio of enzyme activity to antigen is the same in the two samples. The possibility of compensating errors is a very real problem.

A solid-phase immunodisplacement assay, such as that examined above in a preliminary fashion, would retain, or even improve upon, the advantage of speed and convenience and yet provide quantitative results. Unfortunately, because of the poor competition of the rat enzyme, the method did not prove useful for studies with this common laboratory animal. The immunodisplacement assay apparently requires a very high degree of homology and may be limited to studies of a single or very closely related species.

In fact, the immunoadsorbent, which appeared to have such great potential as a specific and practical reagent, was somewhat disappointing. Since enzyme activity could not be recovered from the antibody - Sepharose in good yield, it was not useful as an adsorbent in purification of the enzyme. Nor did it quantitatively bind the enzyme at low levels, so it was not useful in the simultaneous titration of DOPA and 5-hydroxytryptophan decarboxylase activities. Of course, the potential use of such an adsorbent in radioimmunoassay of homologous enzyme could be of great value.

The estimation of structural homology between enzymes from different species by studies of immunological cross-reactivity is fraught with difficulty. However, it does have an advantage over such techniques as peptide mapping and sequence determination in that it does not require that all of the enzymes to be studied must be purified. Several methods of determining cross-reactivity were studied, but, without much more extended effort, only qualitative results can be expected. Conceivably, only a single region of partial homology would be required in order to observe an interaction by an effect on enzyme activity (usually inhibition) or precipitation of enzyme-antibody complexes. Cross-reactivity was observed with enzyme from rat, guinea pig, rabbit, and human tissues by these methods. Methods which would be expected to require a high degree of structural homology were also examined. The binding capacity of antibody-Sepharose was shown to be about 2.5% as great for the decarboxylase from guinea pig as for the hog kidney enzyme, based on binding of enzyme activity. To compare the capacities for the two enzymes on a mole or weight basis would require knowledge of the turnover numbers of both enzymes, or the assumption that they are equal.

Competition of the enzyme from rat kidney with the ^{125}I-enzyme from hog kidney for sites on the immunoadsorbent was also tested. As previously noted, it was found that the rat enzyme competed only very poorly, if at all. This method probably would be useful only with very closely related enzymes.

Because the inhibition and precipitation methods require only limited homology in order to give observable results, they are really only useful in a qualitative sense. The binding capacity and competition assays might be capable of yielding quantitative results, but they also have disadvantages of their own. The binding capacity method requires either (1) purification of the second enzyme as well as the first, in which case more straightforward methods of determining homology are available, or (2) the not necessarily valid assumption of equality of the turnover numbers. Even if this assumption is valid, one has only one reference point -- the structure of the pure enzyme originally used as antigen. As an example, let us assume that we have an antiserum to an enzyme from species A. Further, assume that the analogous enzymes from species B, C, and D cross-react with the antiserum to varying degrees. Now, if immunological cross-reactivity is proportional to structural homology, the B, C, and D enzymes may be ranked relative to their structural homology with A, but not relative to each other, since we have only one reference point in a presumably multi-dimensional system. In order to do so, at least one additional reference point -- i.e., antiserum -- would be necessary. Even then, other interpretations would be possible. Thus, only very limited information about enzyme structure may be derived from any of the methods described.

Previous to the results reported herein, the evidence that DOPA and 5-hydroxytryptophan were decarboxylated by the same enzyme was rather indirect

(see Introduction). The inhibition results favoring the "single enzyme" hypothesis would also be consistent with the (albeit less likely) hypothesis that there exist two enzymes which are subject to inhibition by the same compounds. DOPA might be a competitive inhibitor of a 5-hydroxytryptophan decarboxylase, and vice versa. The concomitant purification of the two activities was not entirely convincing, since contrary evidence had also appeared. Even the isolation of a single protein with both activties did not exclude the possibility that a second enzyme was also present *in vivo*, at least in some tissues, and was removed in the course of purification. Thus, conclusive evidence for the "single enzyme" hypothesis would require a method which is not dependent upon kinetic studies or enzyme purification.

In the simultaneous immunological titration study reported herein, only a single enzyme was found in crude preparations of both central and peripheral tissues from three species. If one were to speculate on a tissue likely to contain a specific 5-hydroxytryptophan decarboxylase, one might well choose the pineal gland, in which the synthesis of serotonin and its derivative, melatonin, is a major metabolic pathway. The levels of norepinephrine, serotonin, and melatonin in the pineal and in the nerve endings impinging upon it undergo rhythmic diurnal fluctuations (Wurtman and Axelrod, 1966; Snyder *et al.*, 1965; Lynch, 1971). Since these fluctuations are large (from 2- to 12- fold) and are out of phase, one might expect the enzymes involved in the synthesis of these compounds to be under strict and independent control, yet only a single decarboxylase was found in the rabbit pineal.

It is conceivable that a second enzyme does exist, but its level is too low to be detected by the present assay. However, the sensitivity of the assay is sufficient to detect as little as 1% of the original activity

if it did not react with antiserum (except in the case of the hog cerebellum, where 2% could have been detected). It is unlikely that such a low activity would be physiologically significant.

Finally, it could be argued that a second enzyme exists but that it cross-reacts strongly with the antiserum. This possibility cannot be completely excluded by the present results. However, the results do impose the constraint that the second enzyme, if it exists, must be so closely related structurally to aromatic L-amino acid decarboxylase as to be identical by the immunological criteria used in these studies. This structural homology, together with the kinetic findings suggesting that DOPA and 5-hydroxytryptophan compete for a single active site, provides convincing evidence that these two amino acids are decarboxylated by a single enzyme.

One of the predictions of the "single enzyme" hypothesis is that DOPA should be decarboxylated in cells that make serotonin. The dopamine thus produced might then displace serotonin from storage. In fact, this has already been shown (Bartholini, DaPrada, and Pletscher, 1968; Ng et al., 1970; Butcher, Engel and Fuxe, 1970). A corollary of this prediction is that 5-hydroxytryptophan should be decarboxylated in cells that make catecholamines. To the author's knowledge, this has not yet been tested. However, it is suggestive that, in early studies of the pharmacology of 5-hydroxytryptophan, the administration of this amino acid to animals resulted in accumulation of serotonin in tissues where this amine is normally absent, including some sympathetically innervated tissues. (Udenfriend, Weissbach, and Bogdanski, 1957) It would be interesting to reexamine these tissues to see if a decrease in norepinephrine accompanies the increase in serotonin. Similar experiments could be devised for catecholamine-rich areas of the brain.

The existence of a single enzyme catalyzing the same type of reaction in different biosynthetic pathways is an unusual circumstance in mammalian biochemistry. Examples that have been well established would include transketolase and transaldolase and the purine phosphoribosyltransferase, which catalyzes the formation of both inosinate and guanylate from the corresponding bases and 5-phosphoribosyl-1-pyrophosphate (see Henderson et al., 1968, and references therein). The finding of the same genetically determined enzyme in the noradrenergic and serotonergic pathways indicates that the two pathways must be more closely related with respect to their evolution and function than has been supposed.

It is interesting to speculate on just how such pathways may have arisen in evolution. One might envision a primordial pathway producing either a catecholamine or serotonin or even some other related amine. Duplication of the hydroxylase structural gene and mutation followed by the pressure of natural selection could then have given rise to separate pathways. The decarboxylase of the archetypical pathway is assumed to have had sufficiently broad specificity to act on either substrate so there would be no necessity for the evolution of separate decarboxylases. This assumption is, of course, consistent with our knowledge of the present enzyme. This hypothesis would suggest that tyrosine and tryptophan hydroxylase may be related structurally. Confirmation of this prediction must await the purification of at least one of the enzymes. Efforts to this end are now proceeding in several laboratories. In this connection, it is interesting to note that tryptophan is also a substrate for liver phenylalanine hydroxylase (see Renson, Weissbach, and Udenfriend, 1962, and references therein). The

extent to which phenylalanine and tryptophan hydroxylase are related in tissues which are primarily directed toward the formation of 5-hydroxy-tryptophan rather than tyrosine is not yet clear.

Another possible source of evidence concerning the evolutionary relationship between the two pathways is the study of organisms in which the product amines may have roles different from those they seem to have in mammals. One example is the liver fluke, Fasciola hepatica, in which serotonin, rather than epinephrine, increases glycogen phosphorylase activity via activation of adenylate cyclase (Mansour, 1959; Mansour et al., 1969).

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