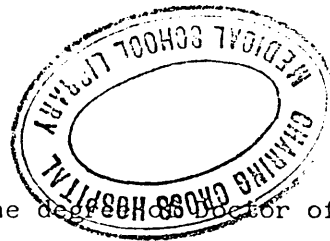


AN EXAMINATION OF THE INFLUENCE OF L-DIHYDROXYPHENYLALANINE (L-DOPA)
AND INHIBITORS OF ITS CATABOLISM, ON METABOLIC PROCESSES IN THE MAMMAL

by

ALBERTO G. RECORDATI



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at the University of London.

Charing Cross Hospital Medical School, London

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ABSTRACT OF THESIS

One of the major catabolic routes of L-dopa and its catecholamine metabolites is by O-methylation. This pathway is catalyzed by the enzyme catechol-O-methyl transferase (COMT) which utilizes S-adenosyl-L-methionine (SAM) as the methyl donor. Synthesis of SAM relies on to the availability of methionine, whose supply to the individual tissues is mainly dependent on transport from the blood and on regeneration from homocysteine, a pathway metabolically linked to the activities of pyridoxal-5'-phosphate (PLP) dependent enzymes. Administered L-dopa competes with the transport of methionine into the brain, and interferes with pyridoxine metabolism.

The catabolic inhibitors of L-dopa used in this study, namely the peripheral decarboxylase inhibitor L- α -(3,4-dihydroxybenzyl)- α -hydrazino propionic acid (carbidopa) and 1-isonicotinoyl-2-isopropyl hydrazino phosphate (iproniazid), also interfere with pyridoxine metabolism.

The present work is an investigation on the effect of chronic treatment with L-dopa, in conjunction with either carbidopa or iproniazid, on methyl group metabolism and on the activities of some PLP dependent enzymes.

Experimental observations were carried out on control rats and on rats treated daily with either carbidopa, L-dopa plus carbidopa, iproniazid or L-dopa plus iproniazid for a period of 15 days. Body weight, food and water intake were recorded daily.

Blood haemoglobin and protein concentrations in the blood plasma and liver were slightly decreased in rats treated with L-dopa plus iproniazid. In this group of animals and in rats treated with iproniazid alone or L-dopa plus carbidopa there was also a decrease in the erythrocyte count. The concentration of blood glucose was increased only by treatment with L-dopa plus iproniazid. Liver glycogen was decreased in rats treated with L-dopa plus iproniazid, but it was increased following treatment with L-dopa plus carbidopa. Hepatic glycogen phosphorylase was not inhibited by either carbidopa alone or L-dopa plus carbidopa, but this activity was slightly decreased by the L-dopa plus iproniazid treatment.

Free amino acid analysis revealed that the concentration of methionine was decreased in the blood plasma but not in liver and brain of rats treated with L-dopa in conjunction with either inhibitor. Both combination treatments affected the concentration of amino acids closely related to methionine metabolism in most tissues.

None of the treatments affected the activity of brain COMT. In rats treated with L-dopa plus carbidopa there was a slight decrease in erythrocyte COMT activity.

Brain concentration of SAM was decreased in the rat by treatment with L-dopa in conjunction with either inhibitor; in this tissue the concentration of S-adenosylhomocysteine (SAH) was not affected in relation to control rats by either of the L-dopa treatments. In the liver of rats treated with L-dopa plus carbidopa there was an increase in the SAH concentration, while the hepatic level of this metabolite was decreased in rats treated with L-dopa and the monoamine oxidase inhibitor.

Analysis of the base composition of brain and liver transfer RNA revealed no overall differences in the methylated base content between treated and untreated rats. Some changes in the base composition of transfer RNA molecules from the brains of rats treated with L-dopa plus iproniazid were observed.

No marked differences in brain glutamate aminotransferase (GOT) or tyrosine aminotransferase (TAT) activities or in their stimulation by addition of PLP in vitro, were observed between treated and untreated rats.

Liver, blood plasma and erythrocyte GOT activities were decreased by treatment with either carbidopa alone or L-dopa plus carbidopa; addition of PLP in vitro restored the activities toward normal levels, although some inhibition was still observed particularly in the erythrocytes. In the tissues examined, L-dopa plus iproniazid treatment had no marked effect on GOT activities when compared to control rats.

Liver TAT activity was depressed by treatment with either carbidopa or iproniazid; inhibition was released by addition of PLP in vitro.

This activity was also inhibited following L-dopa plus carbidopa administration, but addition of PLP stimulated the enzyme to levels above control. Hepatic TAT was significantly stimulated by treatment with L-dopa plus iproniazid, but stimulation by PLP was similar to the one observed in control rats.

TAT activity was significantly decreased in the blood plasma of rats treated with L-dopa plus iproniazid, an effect which was reversed by addition of PLP in vitro.

The results reported in this thesis suggest that the interaction between pyridoxal phosphate and L-dopa and carbidopa may be of clinical significance. The tRNA methylation studies clearly indicate that, under the conditions used in these experiments, changes in base methylation are unlikely to be a significant factor in the inhibitory action of L-dopa/dopamine on tissue protein biosynthesis which has been recorded in the literature.

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CHAPTER 1) PARKINSON'S DISEASE AND LEVODOPA TREATMENT

1.1 HISTORICAL BACKGROUND

Parkinson's disease was first described by James Parkinson in 1817, under the name of "Paralysis agitans". In his original description he drew specific attention to the increasing tremor at rest, which, he noted, sometimes subsided during voluntary movement, and also to the festinant gait, to the flexed posture, to the dysarthria and dysphagia, to insomnia and to severe constipation. The only cardinal features which he did not mention, and which would now be regarded as essential for a diagnosis, were the muscular rigidity with slowness of initiation of movement and the facial immobility.

It was Charcot, some forty years later, who regarded the term "Paralysis agitans" as a misnomer, when muscular strength was well preserved and tremor might be lacking, and who proposed that the disorder should be known as Parkinson's disease.

1.2 MAIN BIOCHEMICAL AND PATHOLOGICAL FEATURES OF PARKINSON'S DISEASE

The first evidence of a biochemical defect in the brain of Parkinsonian patients came from the original observation that dopamine concentrations were depressed in the neostriatum (1), the region of the CNS which contains most of the cerebral dopamine (2). The axons of this area of the brain originate from the substantia nigra (3). This neuronal system, known as the nigrostriatal pathway, is an integral part of the extrapyramidal motor system, probably exerting a modulating influence on neuronal activity in the striatum. In this region of the CNS dopaminergic neurones are generally considered inhibitory, whereas cholinergic

neurones are believed to be excitatory (4). Under normal conditions, it seems that a balance between the two neurotransmitters exists in the striatum (5).

The main pathological feature of Parkinson's disease is the degeneration of cell bodies in the substantia nigra, which results in marked biochemical changes in the striatum and also in the pallidum, another region of the CNS which receives fibers from the substantia nigra (6). The degenerative changes were particularly noticeable in the zona compacta of the substantia nigra (3).

This cellular breakdown is reflected in low levels of dopamine and of its major metabolite homovanillic acid. The activities of tyrosine hydroxylase, the rate limiting enzyme in the synthesis of catecholamines, and of aromatic amino acid decarboxylase, the enzyme catalyzing the decarboxylation of levodopa into dopamine are also decreased (Fig.1). In Parkinson's disease, striatal cholinergic neurones are generally present in normal numbers and can function normally (7). Thus, the pharmacological theory explaining Parkinsonism rests on the opposite effect of dopamine and acetylcholine and the shift in balance towards acetylcholine once the breakdown of striatal dopaminergic neurones sets in (8). A role for the neurotransmitter acetylcholine in the development of the symptoms of the disease was suggested by the observation that anticholinergic agents were useful in the treatment of the disease (9). The central role of dopamine in the pathology of Parkinsonism is supported by a study showing the good correlation between striatal dopamine deficiency and the severity of akinesia (10).

However, the concept of Parkinsonism as solely a striatal dopamine deficiency syndrome is now no longer tenable, because of the accumulating evidence of extrastriatal dopamine deficiency (11,12,13) and of alteration in the metabolism of other neurotransmitters like noradrenaline (14), acetylcholine (15), serotonin (10), γ -aminobutyric acid (16) and neuropeptides (17,18) in the striatum as well as in other regions of the CNS.

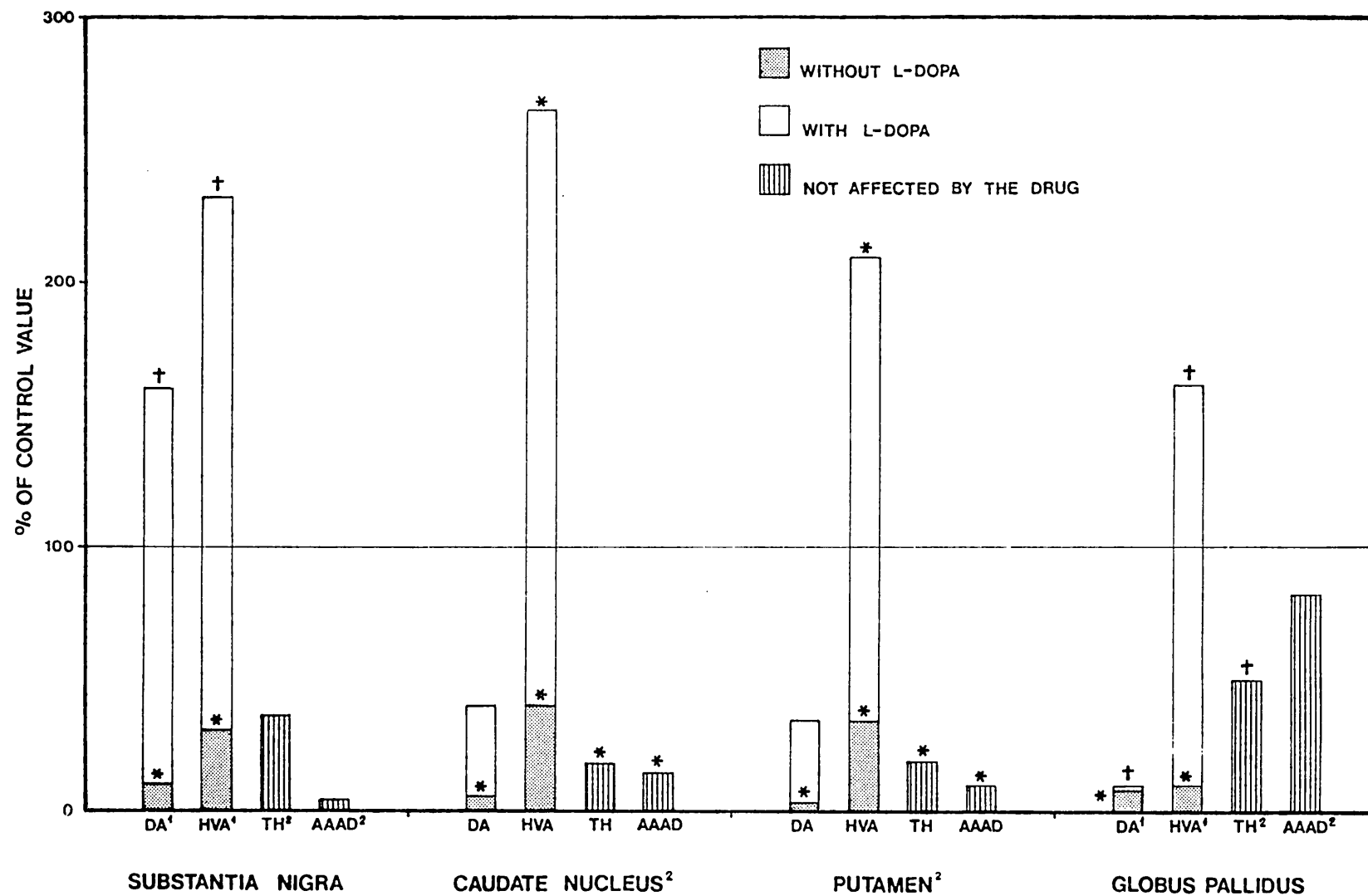


Fig. 1 Changes in dopamine metabolism in the nigrostriatopallidal pathway of Parkinson's disease.

(1: see Ref. 19; 2: see Ref. 20; *: significantly different from control; +: significance not determined)

1.3 TREATMENT OF PARKINSONS'S DISEASE WITH LEVODOPA

Following the observation that dopamine concentration was depressed in the basal ganglia of Parkinsonian patients the amino acid precursor of dopamine L-3,4-dihydroxyphenylalanine (levodopa; L-dopa) which, unlike dopamine, penetrates the blood-brain barrier, was successfully introduced as a therapeutic agent (21). Administration of levodopa is still the best therapy available for Parkinson's disease, with 90% of patients responding to treatment (3).

The mechanism of the pharmacological actions of levodopa ~~is~~ supported by the observations that treatment with this amino acid restores dopamine levels towards normal (Fig.1) and by the good correlation between successful response to treatment and the level of striatal dopamine observed in post-mortem analysis of Parkinsonian patients (20). The therapeutic effect of levodopa also suggests that, although striatal decarboxylase activity is markedly reduced during the progress of the disease, enough activity remains in the striatum to convert exogenous levodopa to dopamine (Fig.1).

Subsequent to its administration, levodopa can be metabolized in a variety of tissues. In man, it is mainly metabolized in the gastrointestinal tract with about 30-50% reaching the systemic circulation (22) and less than 5% the CNS (23). In primates only 20% of the levodopa that enters the brain is made available to the neurones (24). Thus, because of its extensive metabolism and unfavourable distribution, levodopa had to be administered in large doses (25).

The introduction of inhibitors of levodopa metabolism in the treatment of Parkinson's disease was aimed to reduce the therapeutically effective dose of the amino acid, by decreasing its peripheral catabolism or by blocking the catabolism of dopamine. For example, treatment with levodopa and an inhibitor of peripheral aromatic amino acid decarboxylase decreased by 75% the therapeutically effective dose of the amino acid (26). This treatment blocks only the peripheral decarboxylation of levodopa, thereby increasing the proportion of the administered amino

acid available for transport into the brain; this approach has the additional advantage of reducing some side effects of levodopa treatment, presumably due to the peripheral decarboxylation of the amino acid (25). Another approach is treatment with levodopa and a monoamine oxidase inhibitor which inhibits the oxidative deamination of dopamine, the main catabolic route of catecholamines in the brain (27).

Levodopa treatment is a replacement therapy and thus it does not stop the progress of Parkinson's disease (28); therefore, administration of the amino acid has to be carried on continuously from the onset of the clinical symptoms. Long-term treatment with levodopa is marred by a variety of side effects and by a progressive decrease with time of the beneficial response to therapy. In man "on-off" phenomena, instability in posture, and unresponsiveness increase with time, whereas peripheral side effects decrease with time (29). Furthermore some side effects are more or less prominent, depending on the catabolic inhibitor jointly administered with the levodopa (30,31); this suggests an additional role of the inhibitors in the complications arising during chronic levodopa treatment. The reason for the side effects or for the loss in response are not known: they are apparently related to the length of the treatment, a suggestion further supported by the fact that "drug holidays" are generally beneficial (26).

It has been suggested that metabolism of exogenous levodopa may lead to the formation of toxic substances like quinones (32), 2,4,5-trihydroxyphenylacetic acid (33), 3-O-methyldopa (34), tetrahydroisoquinoline derivatives (35) and hydrogen peroxide (33); indeed most of these are formed in vivo following L-dopa administration, however it is not known if they reach toxic levels. Alternatively, the relatively large doses of levodopa used in treatment may interfere in the metabolism of other nutrients.

For example, it has been suggested that chronic administration of this amino acid may induce a partial deficiency of vitamin B6 (37) whose biologically active form, pyridoxal-5'-phosphate, is the cofactor of about

50 enzymes (38). It has also been suggested that catabolism of large doses of exogenous levodopa via O-methylation, may interfere with the methylation of endogenous substrates, by decreasing the availability of the methyl donor S-adenosyl-L-methionine (39).

Similarly, the progressive loss of the therapeutic response to levodopa can be attributed to many factors. Parkinson's disease patients who are more disabled, with a longer history of the disease and a declining response to therapy have a decreased number of post-synaptic dopamine D_2 -receptors; this would suggest that the progress of Parkinsonism and the loss of therapeutic response is also due to post-synaptic changes and not just to the progressive degeneration of the nigro-striatal pathway (40). Another possible reason may be a change in levodopa pharmacokinetics (3). Indeed chronic levodopa treatment affects the activity of various enzymes (37), and it induces morphological and biochemical changes in the gut of the experimental animal which may become irreversible with time (41).

In this work we studied the effect of chronic treatment with levodopa in conjunction with either carbidopa, a peripheral aromatic amino acid decarboxylase inhibitor, or iproniazid, an irreversible monoamine oxidase inhibitor, on certain aspects of methylation and on the activities of enzymes requiring pyridoxal-5'-phosphate as cofactor. Moreover the activities of some enzymes were studied in relation to adaptive metabolic changes which may occur during chronic drug treatment.

CHAPTER 2) CHRONIC TREATMENT WITH LEVODOPA IN CONJUNCTION WITH EITHER A PERIPHERAL DECARBOXYLASE INHIBITOR OR A MONOAMINE OXIDASE INHIBITOR: EFFECTS ON METABOLIC PROCESSES REQUIRING VITAMIN B6 DEPENDENT ENZYMES AND ON THE PHARMACOKINETICS OF LEVODOPA

2.1 L-DOPA AND CATECHOLAMINE METABOLISM

The main physiological role of L-dopa is as an intermediate in the biosynthetic pathway of catecholamines, as shown in Fig.2.

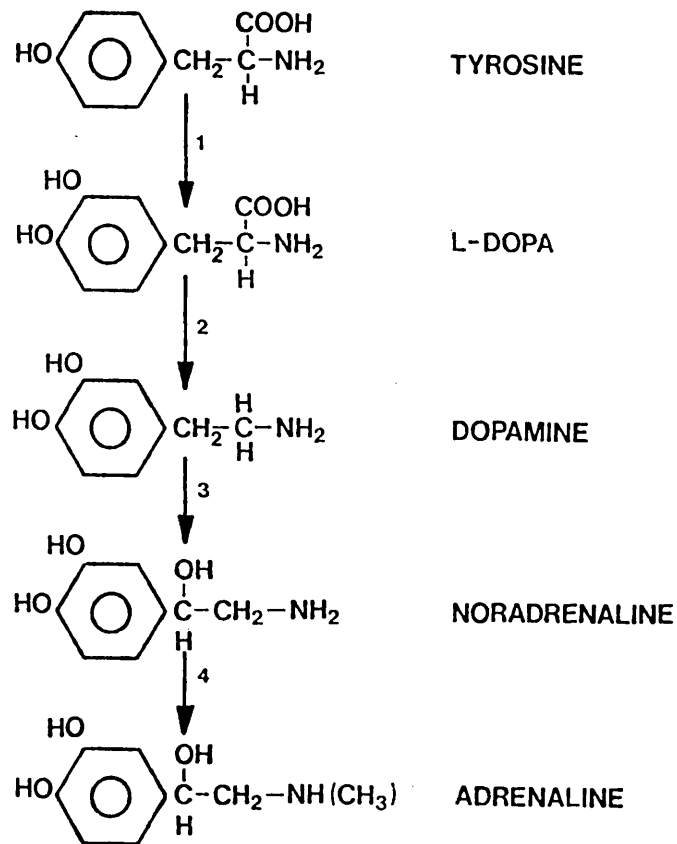


Fig.2 The biosynthesis of catecholamines.

(1: tyrosine hydroxylase; 2: aromatic amino acid decarboxylase; 3: dopamine- β -hydroxylase; 4: phenethanolamine-N-methyltransferase).

In this pathway the rate limiting enzyme is tyrosine hydroxylase (42), a step which is bypassed by L-dopa administration.

Exogenous L-dopa is mainly metabolized by four enzymes: aromatic amino acid decarboxylase (AAAD), catechol-O-methyltransferase (COMT), tyrosine aminotransferase (TAT) and phenolsulphotransferase (PST) (43,44).

Decarboxylation, catalyzed by AAAD, a pyridoxal phosphate dependent enzyme, is the major metabolic pathway of L-dopa (43). AAAD activity is found in many tissues with the highest activities present in liver, kidney and adrenal gland (45); because of its wide distribution, exogenous L-dopa is decarboxylated to dopamine throughout the body (46). Moreover AAAD is not specific for L-dopa, but it is active on other aromatic amino acids (47); thus in the CNS this enzyme is not confined to catecholaminergic neurones, but it is found also in other cells where it plays a different physiological role (48). This may partly explain why in the Parkinsonian brain enough dopamine is formed from exogenous L-dopa, although the activity of AAAD in the basal ganglia is severely affected by the progress of the disease (see Fig.1).

The synthesis of the other catecholamines, noradrenaline and adrenaline, is dependent on the activities and cellular distributions of dopamine- β -hydroxylase (DBH) and phenethanolamine-N-methyltransferase (PNMT) in the different tissues. In the CNS, these enzymes are localized within noradrenergic and adrenergic nerve terminals, and their activities are generally low compared to AAAD (49). This probably explains why L-dopa administration does not markedly affect the concentrations of either noradrenaline (50) or adrenaline (51) in the brain. Another major factor regulating the steady state of catecholamines is the activity of the catabolizing enzymes. In the brain these enzymes are monoamine oxidase (MAO), COMT and PST (27); thus the catecholamines and their precursor L-dopa are catabolized by two common enzymes (i.e. COMT and PST). In the periphery a fourth enzyme, glucuronyltransferase catalyses the

conjugation of catecholamines (and mainly dopamine) to glucuronide (46,52). The metabolic relationship between L-dopa and the catecholamines in the brain is summarized in Fig.3.

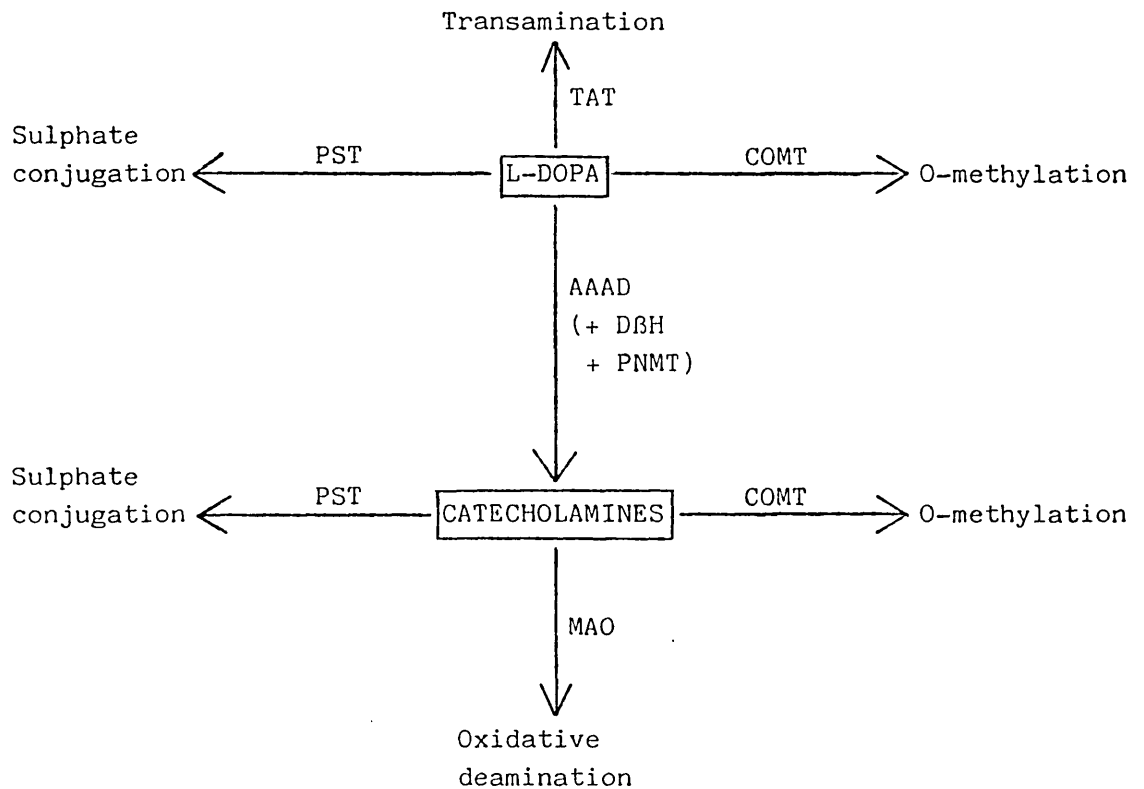


Fig.3 Main metabolic routes of L-dopa and the catecholamines.

In this study L-dopa is administered either with a peripheral decarboxylase inhibitor (L-3,4-dihydroxybenzoyl)- α -hydrazino propionic acid; carbidopa), or with a monoamine oxidase inhibitor (1-isonicotinoyl-2-isopropylhydrazine; iproniazid). Administration of these inhibitors together with L-dopa, markedly changes the metabolism of the amino acid and of the catecholamines. When decarboxylation is inhibited, transamination and O-methylation become the major catabolic routes of L-dopa (43). When MAO, the major catabolic enzyme of catecholamines is inhibited, a higher proportion of catecholamines is presumably O-methylated or undergoes some other form of conjugation. Finally, administration of L-dopa and inhibitors of its catabolism interferes with the metabolism of

pyridoxal-5'-phosphate, and might affect the activities of many vitamin B6 dependent enzymes involved in L-dopa metabolism and methyltransferase reactions, as explained in the following sections.

2.2 THE STRUCTURE AND MAIN METABOLIC FUNCTIONS OF PYRIDOXAL-5'-PHOSPHATE

The biologically active form of vitamin B6 is pyridoxal-5'-phosphate (PLP) whose structure is shown in Fig.4.

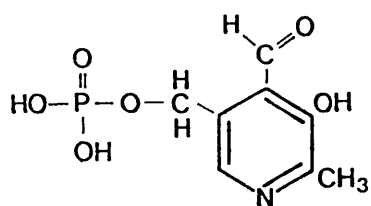


Fig.4 Molecular structure of pyridoxal-5'-phosphate.

The functional portions of the molecule are the aldehyde and the phosphate groups.

PLP is required in many of the reactions involved in amino acid metabolism (e.g. decarboxylation and transamination), in porphyrin synthesis (it is the cofactor of δ -aminolaevulinic acid synthetase, the rate limiting enzyme of this pathway), in glycogen metabolism (it is the cofactor of glycogen phosphorylase) and in metabolism of the methyl group (see section 2.4.2). It has been suggested that PLP plays a role in the regulation of phenotypic expression of genes in eukaryotic cells, by reacting with cytosolic steroid-receptor complexes and inhibiting their binding to DNA or cell nuclei (53). Liver cytosol of rats fed on vitamin B6 deficient diet have more activated corticoid-receptor complexes able to bind to DNA and these have a faster rate of nuclear binding than normal.

For example, it is known that a PLP dependent enzyme like tyrosine aminotransferase is induced more rapidly by hydrocortisone in vitamin B6 deficient rats than in normal rats (54).

Other non-enzymatic roles of PLP are its ex vivo inhibition of platelet aggregation and blood coagulation (55), and the in vivo property of the vitamer and some of its derivatives (e.g. the Schiff base formed by PLP and some amino acids) to act as iron chelators (56).

Vitamin B6 takes part in many biological processes; the highly reactive groups of the PLP structure, which account for its versatility, are responsible for its interaction and/or binding to different macromolecules in vivo. Similarly, some chemicals, which are used as therapeutic agents, may bind to PLP, and this may result in a partial deficiency of the vitamer which may lead to the unwanted side effects observed with certain treatments (57,58).

L-Dopa, carbidopa and iproniazid, or some of their major metabolites, can form complexes with PLP in vitro; however, in vivo, it is not clear if complexes are formed, since in physiological conditions, PLP is mainly bound to macromolecules through the aldehyde group (59). Moreover the high levels of catecholamines, resulting by treatment with L-dopa together with either carbidopa or iproniazid, may interfere with PLP metabolism. The mechanisms by which each drug can interfere with the availability of PLP in different tissues and decrease the activities of PLP dependent enzymes are described in the next sections.

2.2.1 L-Dopa and pyridoxal-5'-phosphate

The effects of L-dopa on vitamin B6 metabolism, can be divided into chemical and biochemical ones.

At a chemical level, the non-enzymatic condensation between PLP and L-dopa, as shown in Fig.5, can be easily achieved in vitro (60).

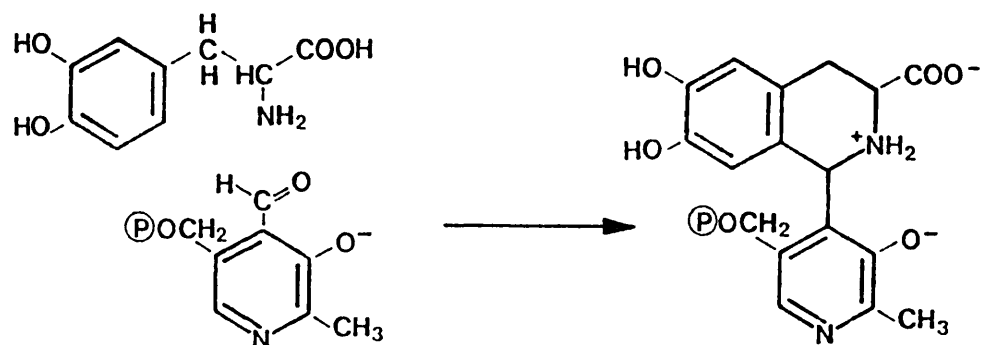


Fig.5 Condensation of L-dopa and PLP.

Similar reactions can occur between PLP and other catechol amino acids or with catecholamines; the condensation products formed in these reactions are known as isoquinoline derivatives. Non-catechol amino acids can condense with PLP to form Schiff bases (61).

The condensation reactions are of interest not only for the potential effect on PLP availability, but also for the inhibitory properties that isoquinolines and Schiff bases have on certain enzymes. For example, the stable condensation products formed by PLP and some amino acids are structurally similar to the coenzyme-substrate complex which is formed at the active site of certain PLP-dependent enzymes: thus these compounds have a high affinity for the apoenzyme of rat liver tyrosine aminotransferase (61). They are non-competitive inhibitors of TAT for tyrosine, but competitive for PLP, suggesting that they bind to the coenzyme binding site, and that inhibition is not achieved through displacement of the coenzyme (61). Similarly, the L-dopa-PLP complex has been shown to inhibit purified aromatic amino acid decarboxylase from hog kidney preparations (62).

The condensation products of catecholamines and PLP have similar inhibitory properties on PLP dependent enzymes (63). However catecholamines can condense with other endogenous aldehydes, to form tetrahydroisoquinolines (THIQ) derivatives whose inhibitory properties are more

widespread; for example, formaldehyde and dopamine can form 6,7-dihydroxy tetrahydroisoquinoline, a compound which has been identified as a normal constituent of rat brain (35). Moreover the concentrations of some THIQ derivatives were increased in the brain and urine of rats treated with L-dopa (64,65). Similarly, Parkinsonian patients treated with L-dopa excreted other THIQ derivatives in concentrations higher than normal (66). THIQ(s) may inhibit several enzymes involved in catecholamine metabolism including tyrosine hydroxylase, COMT, AAD, MAO and dopamine- β -hydroxylase (67), or enzymes closely associated with monoamine metabolism such as dihydropteridine reductase (68). Alternatively THIQ may inhibit noradrenaline uptake by synaptosomes (69); they may interfere with dopamine receptors both in the CNS and periphery, and through these effects they may induce hormonal changes (70,71). Furthermore in vitro they bind to opiate receptors and in vivo they induce analgesia (70). In the context of the present study, the above observations, together with the suggestion that conditions which increase the endogenous concentration of formaldehyde (like niacin deficiency) may raise the concentrations of THIQ (35) are of particular interest. Treatment with L-dopa in conjunction with the inhibitors of its catabolism used in this work, increase the concentration of catecholamines; at the same time, L-dopa, carbidopa and iproniazid may have an individual or cumulative effect on the availability of niacin, and thus induce a partial deficiency of this vitamin, as described below (see 2.4.2).

At a biochemical level, L-dopa can interfere with PLP availability via the catecholamines, its main metabolites. Catecholamines have been implicated in the control of Pyridoxal Kinase (PK) activity, the enzyme catalyzing the phosphorylation of pyridoxal to pyridoxal-5'-phosphate. It has been suggested that this enzyme is part of a system which transports vitamin B6 from the blood into the brain (72) and other tissues (73) and that its role is to catalyze the metabolic trapping of unphosphorylated B6 vitamers within the tissues. Although catecholamines, at their physiological level, do not affect PK activity, it is believed

that if their concentrations are increased by drug treatment they may decrease the activity of this enzyme (60). The effect of catecholamines on brain PK activity may be of great significance especially in relation to the role of PLP as cofactor of many enzymes which catalyze the formation of neurotransmitters like γ -aminobutyric acid, dopamine and glycine. The experimental evidence available suggests that acute L-dopa treatment may interfere with vitamin B6 metabolism, although there are some contradictory observations (74,75). More recently it was suggested that a single L-dopa injection to rats significantly decreased the concentration of PLP in the brain (76), but only at higher doses than previously reported (60). Whatever is the effect of acute treatment with L-dopa on the metabolism of vitamin B6, its long term administration does not cause a pyridoxine deficient state as it was originally feared. This has been shown in rats chronically treated with L-dopa, where PLP concentration in the basal ganglia was at control levels throughout the four weeks of treatment and PK activity fluctuated above, below and back to normal after respectively one, three and four weeks from the beginning of the experiment (76). These observations are supported by studies carried out on Parkinsonian patients chronically treated with L-dopa. PLP concentration in the blood of these patients is normal (77), however PK activity in the erythrocytes is higher than normal (37) suggesting that these patients may have a higher than normal requirement for this vitamer (37).

2.2.2 Carbidopa and pyridoxal-5'-phosphate.

The structure of carbidopa is shown in Fig.6.

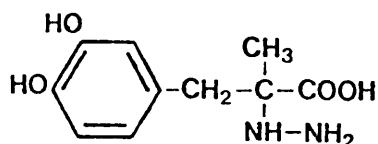


Fig.6 Structural formula of carbidopa.

The main pharmacological property of carbidopa is its potent inhibition of aromatic amino acid decarboxylase (78). Carbidopa, a primary hydrazine derivative, can form a hydrazone with the aldehyde group of PLP as shown in Fig.7.

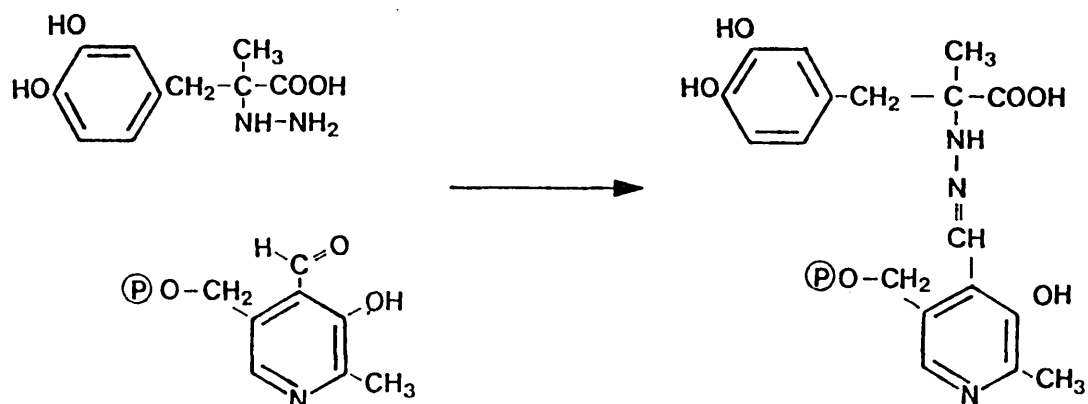


Fig.7 Proposed mechanism of carbidopa binding to pyridoxal phosphate: formation of a hydrazone.

Apparently inhibition is not due to a direct interaction between the hydrazine group of carbidopa and the aldehyde group of PLP of the type described in Fig.7, but it is probably related to the affinity between the holoenzyme and carbidopa, and to some nonspecific binding of the hydrazine derivative to the enzyme bound cofactor (47). Other PLP dependent enzymes are affected by carbidopa, although inhibition is not as marked as for AAAD (79,80,81).

A single i.p. injection of carbidopa to rats decreased PLP concentrations in peripheral tissues and in the hypothalamus, but not in the whole brain; chronic treatment with carbidopa significantly reduced PLP concentrations in both brain and periphery (82). Carbidopa does not cross the blood-brain barrier (83), therefore the reduced concentration of the vitamer in the CNS is presumably a reflection of the reduced availability of PLP in peripheral tissues which is available for transport into the CNS (82). Since carbidopa does not enter the CNS (83), in vivo, the

inhibitory properties of the hydrazine derivative are seen primarily in the periphery (82); however decarboxylation in the hypothalamus was partially inhibited by chronic carbidopa treatment, possibly by the decreased availability of PLP (82).

2.2.3 Iproniazid and pyridoxal-5'-phosphate.

The major metabolic routes of administered iproniazid are shown in Fig.8

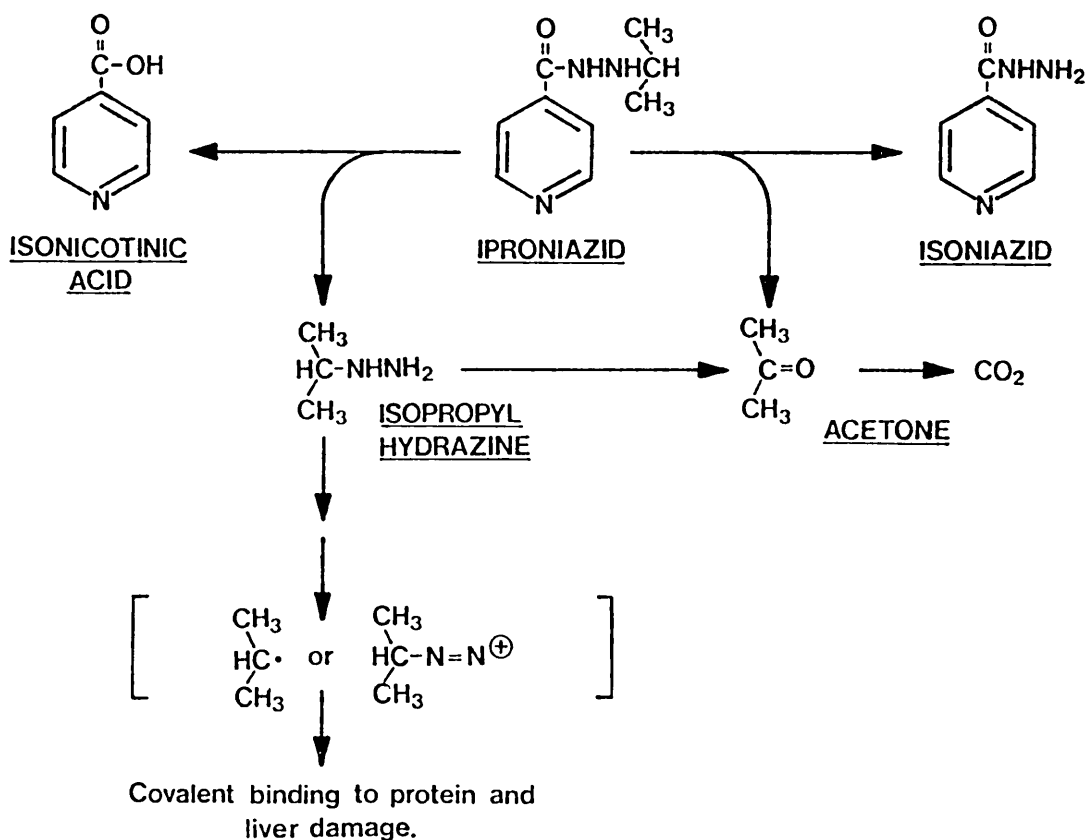


Fig.8 Main metabolic pathways of iproniazid (84).

The main pharmacological property of iproniazid is to irreversibly inhibit monoamine oxidase (85). Although it was the first drug developed for this use, because of its toxic properties it has been withdrawn from clinical practice. The inhibitory and toxic properties of iproniazid arise mainly from its metabolism to hydrazine and hydrazine derivatives (86); within 48 hours of its administration a significant proportion of iproniazid is converted into isoniazid (84), a well known inhibitor of PLP dependent enzymes (87). Similarly isopropylhydrazine, another major metabolite of iproniazid (Fig.8), would be a potential inhibitor of PLP dependent enzymes.

In vivo, it was shown that rats fed on a vitamin B6 free diet, were more susceptible to show toxic symptoms if iproniazid was added to the diet although the activity of PLP dependent enzymes in brain and liver was not affected. If rats were fed with either isopropylhydrazine or isoniazid in place of iproniazid, the activity of brain glutamic decarboxylase, a PLP dependent enzyme, was significantly decreased (88). In another experiment, it was suggested that the high levels of catecholamines following a single iproniazid injection may decrease PK activity in the brain (89).

Finally, iproniazid evokes a wide range of biochemical responses.

For example, it was shown that treatment with iproniazid interfered with blood glucose and liver triglycerides levels, and that these changes were modified if mice were fed on a pyridoxine deficient diet (90).

2.3 AMINOTRANSFERASES; THE ROLE OF TYROSINE AMINOTRANSFERASE IN THE METABOLISM OF EXOGENOUS L-DOPA.

Transamination of the amino acids, which is an important step in their catabolism, is catalyzed by a group of PLP dependent enzymes known as aminotransferases. These enzymes catalyze the removal and transfer of the α -amino group from an amino acid to the carbon atom of an α -keto acid. By the action of these enzymes the amino group of various amino acids are

collected in the form of only one amino acid, usually glutamate. The latter is the specific amino group donor in a final series of reactions in which its amino group is converted into nitrogenous excretory products (e.g. urea).

In previous studies, the activity of glutamate aminotransferase (GOT) has been used as an index of the availability of the cofactor in different tissues (88,91). Under physiological conditions, the brain and liver activities of tyrosine aminotransferase (TAT) are unsaturated by the cofactor (92), suggesting that these activities might be sensitive to a situation of restricted PLP availability. Therefore, in this study, the activities of GOT and TAT are used as an index of PLP availability.

Moreover the measurement of the activity of TAT, the enzyme catalyzing the transamination of L-dopa to dihydroxyphenylpyruvic acid, may be important in understanding the pharmacokinetic handling of L-dopa during chronic treatment. This amino transfer and other reactions closely associated with it are shown in Fig.9.

Liver TAT shows unusual properties, it has a short half-life of about 2-4 hours (93), it exhibits a marked circadian rhythm, and it can be induced many fold over its basal activity, by a number of agents such as hormones (e.g. glucagon and hydroxycortisone) and certain amino acid (e.g. tyrosine) (94).

In the brain there is no enzyme specific for tyrosine transamination (95). However aspartate aminotransferase exhibits some TAT activity, which amounts from 1 to 10% of the activity observed in the liver (95). Furthermore circadian variations have been observed also in the rat brain stem, although they are not as marked as with the liver enzyme (96). In the brain, under normal conditions, transamination of aromatic amino acids is negligible, because of their low intracellular concentrations compared to the high K_m which aspartate aminotransferase exhibits for them (95). However, upon stimulation, TAT may regulate cerebral catecholamine synthesis, by regulating the availability of their precursor tyrosine (97).

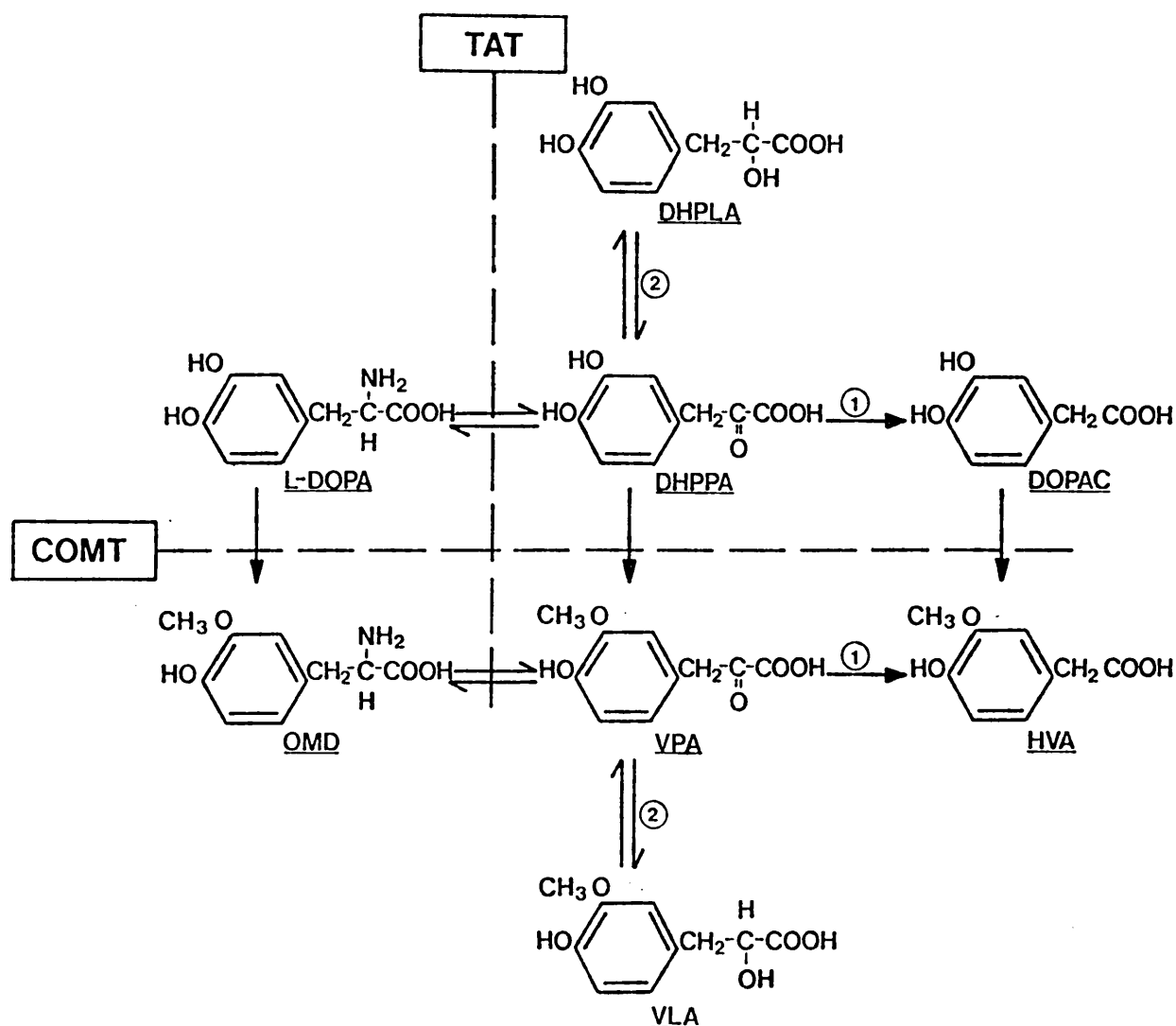


Fig.9 L-Dopa transamination and related metabolic reactions.

(OMD: 3-O-methyldopa; DHPPA: dihydroxyphenylpyruvic acid;

VPA: 3-methoxy, 4-hydroxyphenylpyruvic acid; DOPAC: dihydroxy phenylacetic acid; HVA: homovanillic acid; VLA: 3-methoxy,

4-hydroxy phenyllactic acid; DHPLA: dihydroxyphenyllactic acid;

1: oxidative decarboxylation; 2: aromatic keto acid reductase).

It has been suggested that the activity associated with the cytosolic aspartate aminotransferase is sensitive to stimulation (97) by acute administration of cortisol (98), tyrosine (92) and ethanol; for example, stimulation of brain TAT activity by ethanol was coupled to decreases in the concentrations of tyrosine, dopamine and noradrenaline (97). These

observations are in keeping with the regulatory role of TAT in the synthesis of catecholamines.

Catabolic enzymes are often stimulated by their substrates (99); thus brain (92) and liver (96) TAT activities are increased by treatment with tyrosine. L-Dopa stimulates hepatic TAT activity (100), and thus it may play a similar role in the CNS. The effect of L-dopa administration on brain TAT activity has not been extensively studied. It has been shown that a single injection of L-dopa had a tendency to increase brain TAT activity (75). In another study, it was reported that the decrease in TAT activity induced by reserpine and α -methyltyrosine treatments was reversed by administration of L-dopa, an effect unrelated to catecholamine concentrations (101); this suggests a more direct role of the amino acid, maybe of the kind played by tyrosine.

Stimulation of TAT activity by chronic L-dopa treatment may lead to an accumulation of toxic intermediates. Dihydroxyphenylpyruvic acid, the transaminated product of L-dopa, has been described as a weak inhibitor of dopa decarboxylase (102). Furthermore 2,4,5-trihydroxyphenylacetic acid may be formed by the action of p-hydroxyphenylpyruvate oxidase on dihydroxyphenylpyruvic acid; the distribution of hydroxyl groups of 2,4,5-trihydroxyphenylacetic acid, in relation to the side chain, is identical to that of 6-hydroxydopamine a compound which is highly toxic for catecholaminergic neurones.

Indeed, 2,4,5-trihydroxyphenylacetic acid has been identified in the urine of Parkinsonian patients treated with L-dopa (33). Stimulation of cerebral TAT activity may induce changes in catecholamine metabolism by decreasing the formation of dopamine; moreover, as the half-life of aspartate aminotransferase is considerably longer than that of TAT (103) an increased transamination of aromatic amino acids in the brain, may also affect tyrosine metabolism and thus the synthesis of catecholamines, once the exogenous L-dopa has been metabolized. This metabolic effect would be even more important in Parkinson's disease, as following the degeneration of the nigrostriatal neurones, the firing of the remaining dopaminergic

neurones appears to be regulated by tyrosine availability (104); this is also supported by a study carried out in rats treated with 6-hydroxydopamine (105).

As a pyridoxal phosphate dependent enzyme, TAT is sensitive to hydrazine derivatives in vitro. However, it has been reported that following the administration of carbidopa and L-dopa, the urine level of VLA and HVA were increased and decreased respectively, when these parameters were compared to patients treated with L-dopa alone (43). Similarly, it has been reported that treatment with L-dopa and carbidopa increased the concentrations of VPA and VLA in the cerebrospinal fluid (106). These results suggest that transamination of L-dopa is not decreased by the joint administration of L-dopa and carbidopa (see Fig.9). The apparent lack of effect of carbidopa on TAT activity can be partly explained by the in vitro observation that carbidopa has about 1/10th the affinity for TAT as it does for the decarboxylase enzyme (79); moreover it has been suggested that the mitochondrial activity is relatively resistant to carbidopa inhibition (107).

2.4 METHYLATION OF EXOGENOUS L-DOPA. EFFECTS ON METHYL GROUP METABOLISM

The enzyme catalyzing the O-methylation of L-dopa is active on a variety of other substrates possessing a catechol or a related nucleus, regardless of the substituents on the aromatic ring, hence its name catechol-O-methyltransferase (COMT). The substrates include the catecholamines and their acidic, alcoholic and aldehydic metabolites (108); other substrates include catechols of exogenous origin such as carbidopa and benserazide. In vitro different substrates can compete with one another; for example, the enzyme has a Km for carbidopa comparable to that of L-dopa (109) and O-methylated metabolites of carbidopa have been identified in the urine of Parkinsonian patients treated with the drug (47). In vivo, one of the main role of COMT is to inactivate catecholamines.

This role is supported by observations in vitro, which showed that membrane bound COMT from human brain, is responsible for most of noradrenaline and for a substantial portion of dopamine catabolism when these substrates are present in low concentrations; at higher concentrations the preferred catabolic route of monoamines is by oxidative deamination, catalyzed by MAO (27). O-methylation is also a major catabolic route of L-dopa (110). Since catabolic enzymes are often stimulated by their substrates (99), administration of L-dopa might stimulate COMT activity, and this may be of significance, as the O-methylated product of L-dopa has some toxic properties. Moreover the methyl donor in the reaction catalyzed by COMT in S-adenosyl-L-methionine. Therefore, depletion of the methyl donor in the catabolism of L-dopa may result in decreased methylation of other endogenous substrates.

2.4.1 Potentially toxic effects of an increase in catechol-O-methyl transferase activity induced by chronic treatment with L-dopa and its catabolic inhibitors.

L-Dopa is methylated in the 3 hydroxyl position of the catechol ring, to yield 3-O-methyldopa (OMD). It has been suggested that OMD interferes in the metabolism of L-dopa at different stages; OMD competes with L-dopa for transport across the blood-brain barrier (111), and within the CNS, OMD may compete with the uptake of L-dopa by the neurones, as shown to occur with synaptosomes (112). Moreover as OMD is a substrate for the decarboxylase enzyme, it may competitively inhibit the decarboxylation of L-dopa (112). In vivo, it has been demonstrated that intraperitoneally injected OMD interferes in the uptake of L-dopa by different brain regions (113), and it reduces the number of levodopa induced rotations in rats after unilateral nigrostriatal lesion by 6-hydroxydopamine (114); this suggests a decreased uptake of L-dopa by the neurones as inhibition of the firing rate of dopaminergic neurones by OMD (115) has been ruled out.

The toxicity of OMD is supported by observations in Parkinsonian patients. L-Dopa treated patients with erythrocytes COMT activities and plasma OMD concentrations higher than normal, were those responding less successfully to treatment (112). Dyskinetic crisis and plasma OMD concentrations were shown to be correlated, and a high OMD to L-dopa ratio of their plasma concentrations was shown to be indicative of a poor therapeutic response to L-dopa by the patients (30).

To our knowledge the effect of chronic administration of L-dopa in conjunction with a peripheral decarboxylase inhibitor or a monoamine oxidase inhibitor on brain COMT activity has not been reported. Chronic treatment with L-dopa alone does not affect brain COMT activity but erythrocyte and liver COMT activities are significantly decreased (116). However the joint administration of L-dopa and an inhibitor of its catabolism may induce COMT activities by the following mechanisms.

Carbidopa, the peripheral decarboxylase inhibitor used in this study, blocks the main metabolic route of L-dopa (117); when this inhibitor is administered together with the amino acid, peripheral catabolism of L-dopa proceeds mainly via transamination. However, a significant proportion of exogenous L-dopa and of its transaminated metabolites are catabolized by COMT (Fig.9) (43). Induction of the enzyme in the peripheral tissues may occur as a result of the increased levels of its substrates. Indeed accumulation of 3-O-methyldopa in the plasma is particularly marked only in those patients treated with L-dopa and a peripheral decarboxylase inhibitor (30). This may also occur in the CNS, where the concentration of L-dopa has been shown to increase after treatment with L-dopa plus a peripheral decarboxylase inhibitor (118). Under the more severe conditions used in this work, the high concentration of L-dopa in the CNS, may result in the induction of brain COMT activity.

Iproniazid is the irreversible inhibitor of monoamine oxidase (MAO) (85) used in this study. Because of the relatively long half-life of MAO, chronic treatment with an irreversible inhibitor inactivates most of the hepatic MAO, whereas the cerebral activity is completely inhibited (119). In brain and liver, this enzyme is not induced by either chronic inhibition (119) nor chronic L-dopa administration (120). These observations suggest that the increase in catecholamine concentration, observed after acute treatment with L-dopa and a MAO inhibitor (121), may be maintained for a long time if the treatment with these drugs is chronic. This might induce COMT activity; as the enzyme exhibits broad substrate specificity (108), this would also result in increased catabolism of L-dopa via O-methylation.

2.4.2 The effect of exogenous L-dopa O-methylation on the concentrations of methionine and S-adenosyl-L-methionine and on transmethylation reactions.

One of the major biological roles of methionine is as a precursor of S-adenosyl-L-methionine (SAM), the principal methyl donor in mammals. SAM is the cofactor of COMT the enzyme which catalyses the O-methylation of L-dopa. Thus it has been suggested that chronic administration of L-dopa may deplete the body of free methionine (110). In all tissues, methionine levels are dependent on the uptake from the blood plasma and on intracellular metabolism. (122)

The blood-brain barrier is an effective barrier to most metabolites found in the blood plasma (123). Transport of nutrients across the blood-brain barrier is by specific carriers (123); methionine and L-dopa share the same carrier specific for neutral amino acids (124). The work of others has shown that administration of L-dopa alone did not decrease brain methionine concentration in the rat (125,126). When L-dopa is administered with a peripheral decarboxylase inhibitor the level and the half-life of the amino acid in the plasma are increased when compared to treatment with L-dopa alone (127); thus under such conditions, there

should be higher competition at the blood-brain barrier between L-dopa and methionine.

The observation that brain methionine concentration was decreased only after three successive injections of L-dopa at 45 minutes intervals, suggests that the intracellular regeneration of methionine is an efficient process (122). Moreover, the observation that in folic acid deficient rats a single injection of L-dopa decreased brain methionine, established a link between folic acid and methionine metabolism (122). The intracellular metabolism of methionine is closely linked to the metabolism of S-adenosyl-L-methionine. SAM participates in the methylation, synthesis and catabolism of various neurotransmitters, neuroactive peptides, phospholipids, neuronal membranes and nucleic acids. Furthermore it participates in the synthesis of polyamines (128). The general metabolic role of SAM is outlined in Fig.10.

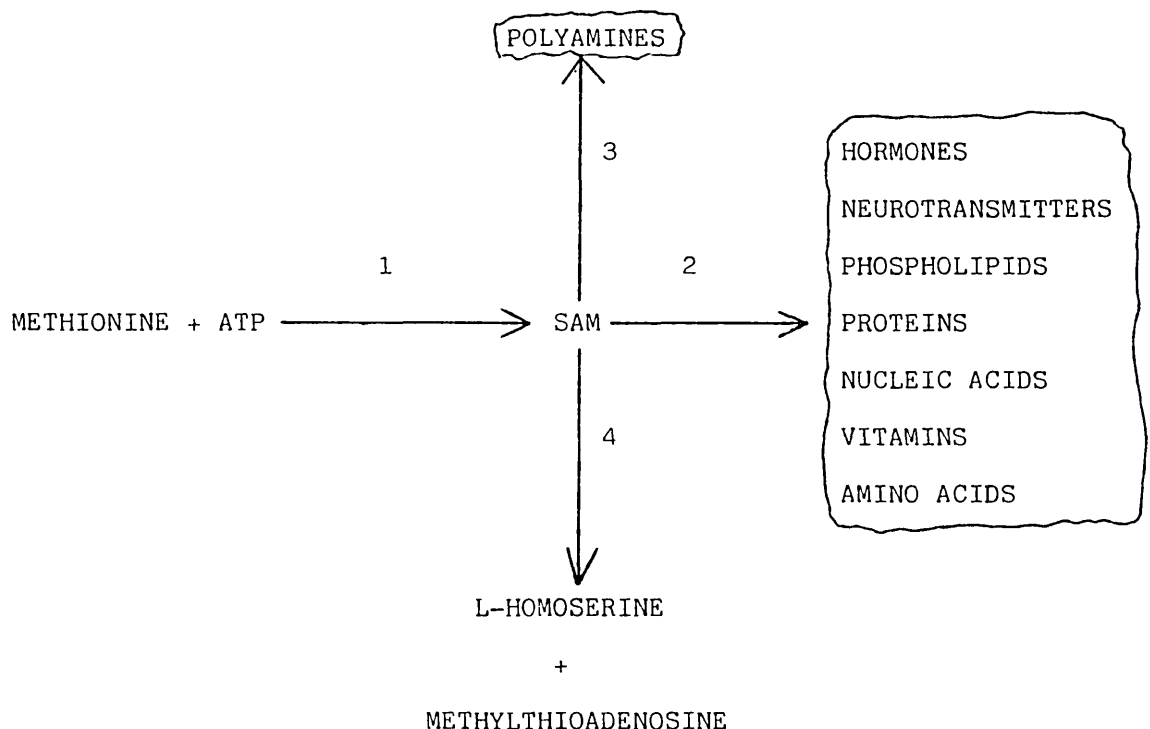


Fig.10 Metabolic routes of SAM

(1: methyl adenosyltransferase; 2: transmethyases;
3: SAM decarboxylase; 4: SAM splitting enzyme).

SAM is the methyl donor in many reactions, thus administration of large doses of L-dopa may decrease its availability for the methylation of other substrates (129). It has been suggested that the lowering of the ratio of the methylated to unmethylated catecholamine metabolites resulting from the administration of L-dopa, may be due to the decreased availability of SAM (130). In keeping with this, it was shown that a single injection of L-dopa decreased SAM levels in brain and kidney, however hepatic SAM concentration fell only after three consecutive L-dopa injection suggesting that in the liver the regeneration of SAM proceeds rapidly (126).

Transmethylations can also be affected by an increase in S-adenosyl-L-homocysteine (SAH) concentration, which is formed from SAM following the transfer of the methyl group to a substrate like L-dopa; SAH is a potent inhibitor of methyl transfer reactions (131). An increase in SAH level, may selectively inhibit different methyltransferase enzymes according to their K_i values (132); among this class of enzymes transfer-RNA methyltransferases have been shown to be sensitive to inhibition by SAH in vitro (133), and in the context of this study, it is noteworthy that dopamine has been reported to inhibit liver transfer-RNA methyltransferases in vitro (134). However a marked increase in SAH concentration may lead to widespread inhibition of methyltransferases, as these enzymes have a higher binding affinity for SAH rather than for SAM (131); this observation has led to the suggestion that transmethylations are influenced more by the ratio of SAM to SAH, rather than by the individual concentration of the two metabolites (135).

SAM is not readily absorbed from the plasma and thus it must be synthesized in the different tissues by methyl adenosyltransferase (136). This enzyme appears to be limited by substrate availability, since methionine administration leads to an increase in brain and liver SAM levels (137); however, since these changes are not reflected in increased methylations of endogenous L-dopa or of other catechols in the rat brain

(137), it has been suggested that SAM exists in different metabolic pools (138). Under normal conditions, after the transfer of the methyl group from SAM to a substrate, SAH is quickly broken down to adenosine and homocysteine by SAH hydrolase, a NAD dependent enzyme (131). Homocysteine may be then catabolized by a PLP dependent enzyme or it may be reutilized in the regeneration of methionine, a reaction catalyzed by 5-methyltetrahydrofolate-homocysteine methyltransferase (methionine synthase). The latter reaction involves the transfer of a methyl group from a folic acid derivative to homocysteine (139); serine and glycine are the main source of the one carbon unit which via folate metabolism is transferred onto homocysteine (139). The metabolic relationship between the folic acid pool and the reactions involved in the regeneration of methionine, and in the synthesis of SAM, are summarized in Fig.11.

The key regulatory step in the regeneration of methionine and thus in the synthesis of SAM is the reaction catalyzed by methylene-THF-reductase (enzyme 7). This rate limiting enzyme is inhibited by SAM in brain and liver, an effect which is partially relieved by SAH; thus the ratio of the concentrations of these two compounds may regulate not only their use, but their synthesis as well (139).

The work of others has shown that administration of L-dopa alone may decrease SAM levels (126) and, under certain conditions, it may also decrease methionine levels in some tissues (122). In our study, administration of L-dopa in conjunction with either one of the two inhibitors of its catabolism, might interfere with the regeneration of methionine since this process is metabolically linked to the activities of niacin and vitamin B6 dependent enzymes.

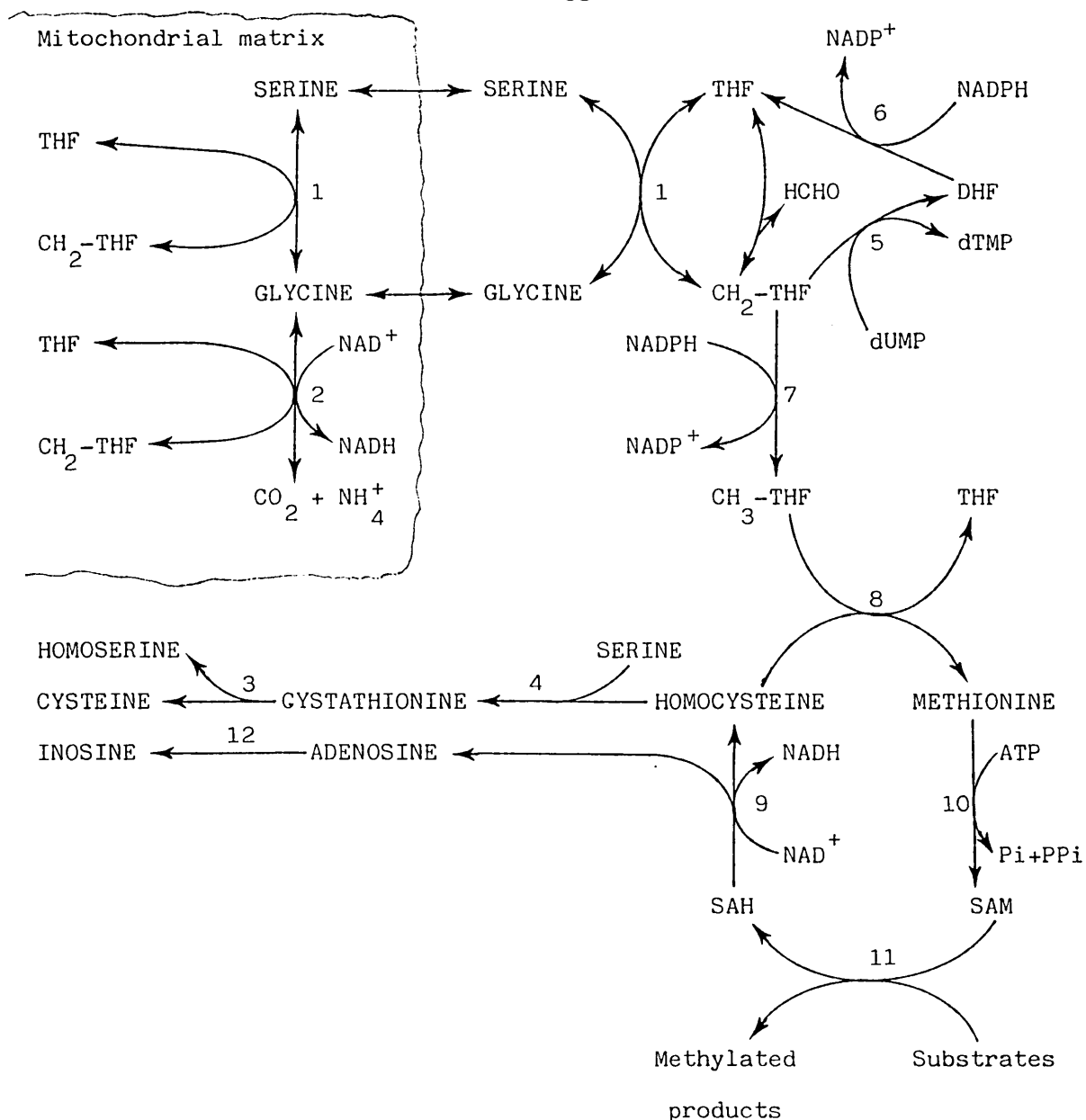


Fig.11 Metabolic relationship between methyltransferase reactions and the regeneration of methionine.

(THF: tetrahydrofolic acid; CH₃-THF: methyl-tetrahydrofolic acid; CH₂-THF: methylene-tetrahydrofolic acid; DHF: dihydrofolic acid

- 1: serine hydroxymethyltransferase;
- 2: glycine cleavage enzyme;
- 3: cystathionine- γ -lyase; 4: cystathionine- β -synthase;
- 5: thymidylate synthase; 6: dihydrofolate reductase;
- 7: 5,10 methenyl-THF reductase; 8: methionine synthase;
- 9: SAH hydrolase; 10: methionine adenosyltransferase;
- 11: methyltransferases; 12: adenosine deaminase).

Carbidopa inhibits two enzymes of the tryptophan oxidative pathway (140), which leads to the formation of NAD and NADP; in another study it was suggested that carbidopa treatment may cause a partial deficiency of niacin in Parkinsonian patients (141). Similarly isoniazid, a major metabolite of iproniazid (see Fig.8), can induce a niacin deficient state, presumably by interfering in the synthesis of nicotinamide nucleotide from tryptophan, an effect which may be related to isoniazid inhibitory properties of PLP dependent enzymes (87).

The effects of L-dopa, carbidopa and iproniazid on vitamin B6 metabolism and PLP availability have been described (see 2.2.1, 2.2.2, 2.2.3). PLP is the cofactor of two enzymes which transfer one carbon unit from either serine or glycine to tetrahydrofolic acid (THF) (enzymes 1 & 2, respectively) (142,143) and it is the cofactor of two enzymes in the catabolism of homocysteine (enzymes 3 & 4) (144). A decreased activity of serine hydroxymethyltransferase and of glycine cleavage enzyme would deprive the folic acid pool of the one carbon unit which is transferred onto homocysteine. Moreover in the CNS the increased methionine turnover, due to exogenous L-dopa catabolism, coupled to a decreased PLP availability, due to chronic carbidopa treatment may bring about another form of toxicity.

It has been suggested that serine is an essential amino acid for the brain (145), where it is a major precursor of glycine (146), since this amino acid is not actively transported into the CNS (147). L-Dopa administration would presumably increase the rate of utilization of serine, owing to the increased requirement for one carbon fragments, and thus increase the rate at which this amino acid enters the CNS. Chronic administration of L-serine raises the glycine concentration in the brain, since the conversion of serine to glycine is faster than that of glycine to serine (148). Accumulation of glycine may then lead to two forms of toxicity: either via its metabolism to glyoxylate which may inhibit glucose oxidation (149), or via its role as a neurotransmitter which may depress the activity of many neurons in the substantia nigra (150).

Moreover an increase in glycine level is even more likely in a situation where the availability of vitamin B6 is restricted, as the deficiency of this vitamin leads to a two- to three-fold increase in brain glycine concentration (151).

A decreased activity of either cystathionine- γ -lyase or cystathionine- β -synthase would ultimately result in an increase in homocysteine level. Accumulation of this amino acid may drive the reaction catalyzed by SAH hydrolase in the synthetic direction, which is greatly favoured in vitro (152). A similar mechanism has been suggested in another work (144), where in rats fed on a pyridoxine deficient diet the hepatic concentration of SAH was increased 4-fold, whereas the SAM concentration was unchanged; this resulted in a marked decrease in the SAM/SAH ratio. The increase in SAH concentration with the resulting change in the SAM/SAH ratio may affect transmethylations, although the SAM concentration is not affected.

Another important route in the metabolism of SAM is its decarboxylation to S-adenosylhomocysteamine, which is the propylamine group donor in the biosynthesis of polyamines (Fig.12). This reaction is catalyzed by SAM decarboxylase, which unlike most decarboxylases is not dependent on PLP as a coenzyme (144). Under normal conditions ornithine decarboxylase (ODC), a B6 dependent enzyme, is the rate limiting enzyme in polyamine biosynthesis.

ODC is stimulated by a variety of hormones in different tissues (153); upon stimulation, ODC activity increases considerably, so that SAM decarboxylase becomes the rate limiting step in the biosynthetic pathway; it will then follow that upon stimulation polyamine synthesis will be dependent on SAM supply. The polyamine biosynthetic pathway is shown in Fig.12.

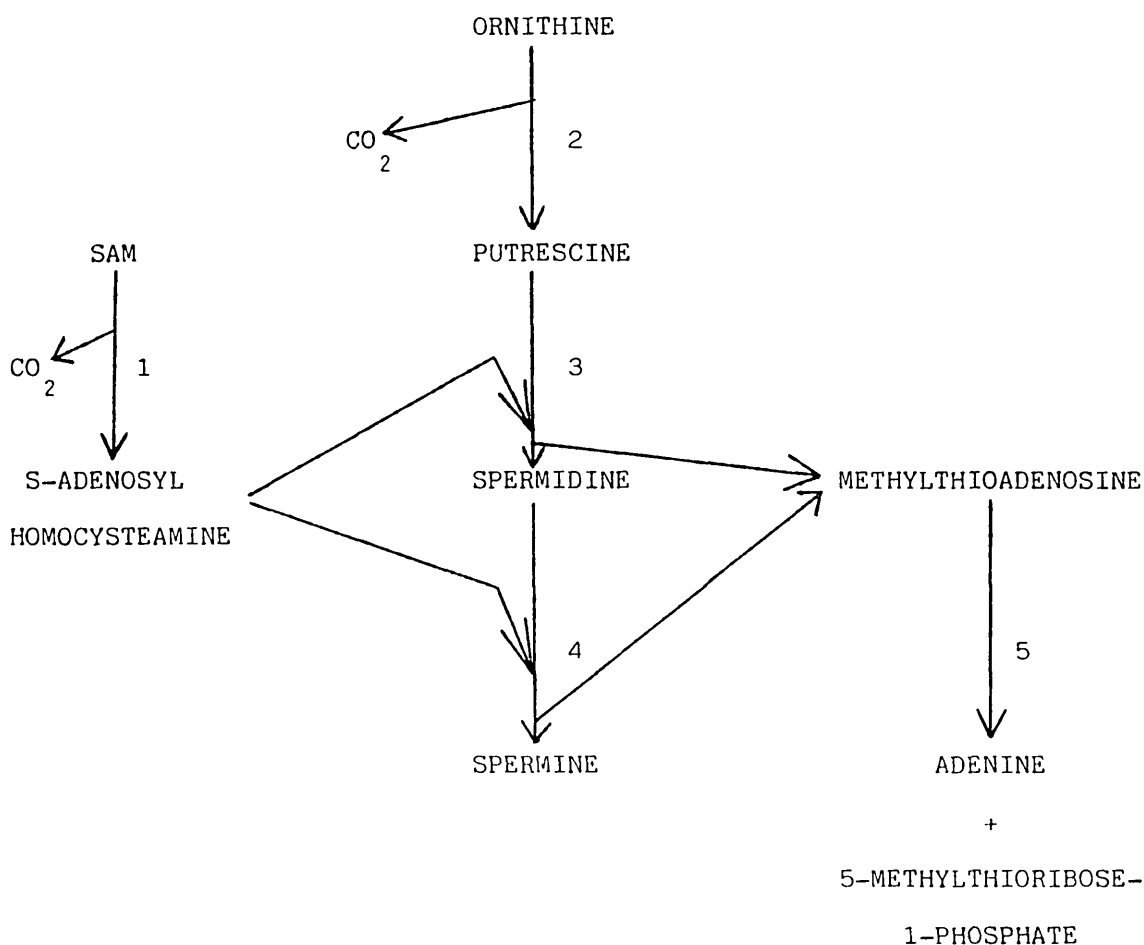


Fig.12 Biosynthesis of polyamines

- (1: SAM decarboxylase; 2: ornithine decarboxylase;
 3: spermidine synthase; 3: spermine synthase;
 4: methylthioadenosine phosphorylase;
 5: MTA phosphorylase).

The important roles of PLP as the cofactor of ODC and of methionine, as the precursor of SAM, are suggested by the observations that in the liver of rats fed on a pyridoxine deficient diet there was a decrease in the concentration of putrescine and in the activity of ODC (144), and that the feeding to rats of a methionine deficient diet produced an increase in the spermidine/spermine ratio, suggesting a reduced SAM availability for polyamine biosynthesis (154).

It has been reported that administration of L-dopa has a stimulatory role on polyamine biosynthesis; thus, in this condition, there is an increased requirement for polyamines, either to stimulate de novo protein synthesis or to stabilize existing macromolecules (155). Administration of carbidopa and iproniazid together with L-dopa, may interfere in the stimulation of polyamine synthesis, since ODC, like many PLP dependent enzymes, is inhibited by hydrazine derivatives (156); moreover its inhibition leads to accumulation of S-adenosylhomocysteine, an inhibitor of methyltransferases in vitro (157).

Because of their polybasic nature polyamines bind to nucleic acids; for example, two molecules of spermidine or spermine are bound to transfer-RNA (t-RNA) (158). It is believed that one of the most important biological effects of polyamines is to stimulate and optimize protein synthesis (159). Their roles include the stimulation of t-RNA methylation, which seems to be related to the structural and functional properties of the t-RNA molecules (160), whose major biological role is as a specific acceptor of amino acids in the initiation and elongation of the peptide chain. The in vitro methylation of t-RNA is enhanced by polyamines; in vivo a 50% decrease in polyamine concentration in bacteria causes incomplete methylation of t-RNA, whereas inhibition of polyamine synthesis in rat hepatoma cells caused a block in t-RNA methylation (158).

One of the aims of this work was then to study if chronic treatment with L-dopa and its catabolic inhibitors might interfere with the methylation of t-RNA via their effects on the metabolism of vitamin B6, methionine and SAM.

Perturbation of the normal methylated base content of t-RNA have been elicited experimentally and shown to greatly influence t-RNA function: undermethylated t-RNA, failed to support in vitro protein synthesis at the translational step (161). A change in the methylation pattern of t-RNA may also affect its role as a regulator in protein synthesis (162), or in the biosynthesis of some amino acid (163), by a mechanism similar to the one known to occur in lower eukaryotes (164).

MATERIALS AND METHODS

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CHAPTER 3) MATERIALS

3.1 CHEMICALS AND EQUIPMENTS

3.1.1 Commercially available

General laboratory reagents of the highest purity available, usually ANALAR grade or the equivalent, were used throughout these investigations. The sources of some chemicals and biochemicals were the following:

3.1.1.1 Radioactive compounds.

Tyrosine and glutamic acid both uniformly labelled with ^{14}C (Specific Activity 11-18.5 GBq/mmol).

S-Adenosyl-L-(methyl ^3H)methionine (Specific Activity 550 GBq/mmol).

S-Adenosyl-L-(methyl ^{14}C)methionine (Specific Activity 2.17 GBq/mmol).

Adenosine labelled with ^{14}C in the 8 position (Specific Activity 2.11 GBq/mmol).

Potassium Boro(^3H)-Hydride (Specific Activity 144 GBq/mmol).

All were purchased from the Radiochemical Centre, Amersham.

3.1.1.2 Enzymes.

Pancreatic ribonuclease from Sigma.

Alkaline phosphatase and snake venom phosphodiesterase from Worthington.

Adenosine deaminase and amyloglucosidase from Boehringer Mannheim.

3.1.1.3 Chromatographic materials.

Cellex-P (H^+ form) cation exchange cellulose from Bio-Rad laboratories
Dowex-50W (8% cross linked, 100-200 mesh) cation exchange resin from Sigma.

Spherical ion-exchange resins for amino acid analysis were either Type PA-35 (basic amino acid) on Type PA-28 (acidic and neutral amino acids); both resins were purchased from Beckman.

Sigma cell Type 100 fibrous cellulose from Sigma.

Plates coated with microcrystalline cellulose (Avicel) from Scientific Supplies. Their size was 20 x 20 cm and the thickness of the coating was 0.25 mm.

3.1.1.4 Chemicals

Aquasol and Aquasol-2 from New England Nuclear.

Standards for amino acid analysis were purchased from Hamilton and were either type P.B. (basic amino acids) or type P. A/N (acidic and neutral amino acids).

Ninhydrin from Pierce and methyl cellosolve from Union Carbide.

S-Adenosyl-L-methionine chloride salt (85% pure), S-adenosyl-L-homocysteine, Deoxyribonucleic acid of calf thymus, pyridoxal-5'-phosphate, L-(3,4)dihydroxyphenylalanine (L-dopa), 1-isonicotinoyl-2-isopropylhydrazine phosphate (iproniazid phosphate), amino acids and nucleosides were from Sigma.

Chemicals and standards for the determination of glucose were purchased as a kit from Boehringer Mannheim.

Carbidopa (L- α -(3,4-dihydroxybenzyl)- α -hydrazino propionic acid) was a generous gift from Merck Sharp & Dohme Laboratories.

3.1.1.5 Special equipments.

Filters for the preparation of amino acids samples were of the type GS (pore size 0.22 μ m) from Millipore.

Hamilton syringes from Scientific Supplies.

X-ray films, type X-OMAT RP (18 x 24 cm) from Kodak.

3.1.2 Preparations

3.1.2.1 L-Homocysteine.

This amino acid was prepared from homocysteine thiolactone by cleavage of the lactone ring with alkali (165).

0.2 mMole homocysteine thiolactone was incubated at room temperature with 0.30 ml 5M NaOH; after 5 minutes, 1.5 ml of 1.0M KH_2PO_4 and 0.20 ml of $\text{d.H}_2\text{O}$ were added. As the conversion is about 85%, homocysteine concentration was taken as 85 mM; this preparation was immediately used in the synthesis of S-(8- ^{14}C)adenosylhomocysteine.

3.1.2.2 S-(8- ^{14}C) Adenosylhomocysteine.

This compound was synthesized by the condensation of adenosine and L-homocysteine catalyzed by SAH hydrolase (see Fig.11) (144).

Incubations were carried out at 37°C for 12 minutes. The incubation mixture contained 3 mM L-homocysteine, 0.2 mM dithiothreitol, 0.1 M potassium phosphate buffer (pH 7.3), 550 KBq of (8- ^{14}C)adenosine (2.11 GBq/mmol), 2 mM 8-Azaguanine and about 4 mg protein of a fresh liver homogenate, in a total volume of either 0.50 or 1.0 ml. 8-Azaguanine was added to inhibit adenosine deaminase (166). The reaction was stopped by the addition of 25 μl of 30% (w/v) perchloric acid.

Further steps were performed at 6°C. The incubation mixture was centrifuged and the supernatant was applied to a cellex-P (H^+ form) column (1 x 5 cm). The column was washed with 1 mM HCl (about 100 ml) and 10 mM HCl (about 30 ml). Labelled S-(8- ^{14}C)adenosylhomocysteine was eluted in 2.5-5.0 ml fractions with 0.1 M HCl. The fractions of high specific activity were pooled, adjusted to pH 9.0 with 10 M NaOH and evaporated to dryness at 30°C in vacuo. The residue was dissolved in water and stored at -20°C.

A typical elution profile of the acid supernatant is show in Fig.13.

Generally, total recovery was around 90%; recovery in the (8- ^{14}C) S-adenosylhomocysteine fraction was around 70%. The specific activity of the labelled preparations was calculated to be around 1.97 GBq/mmol.

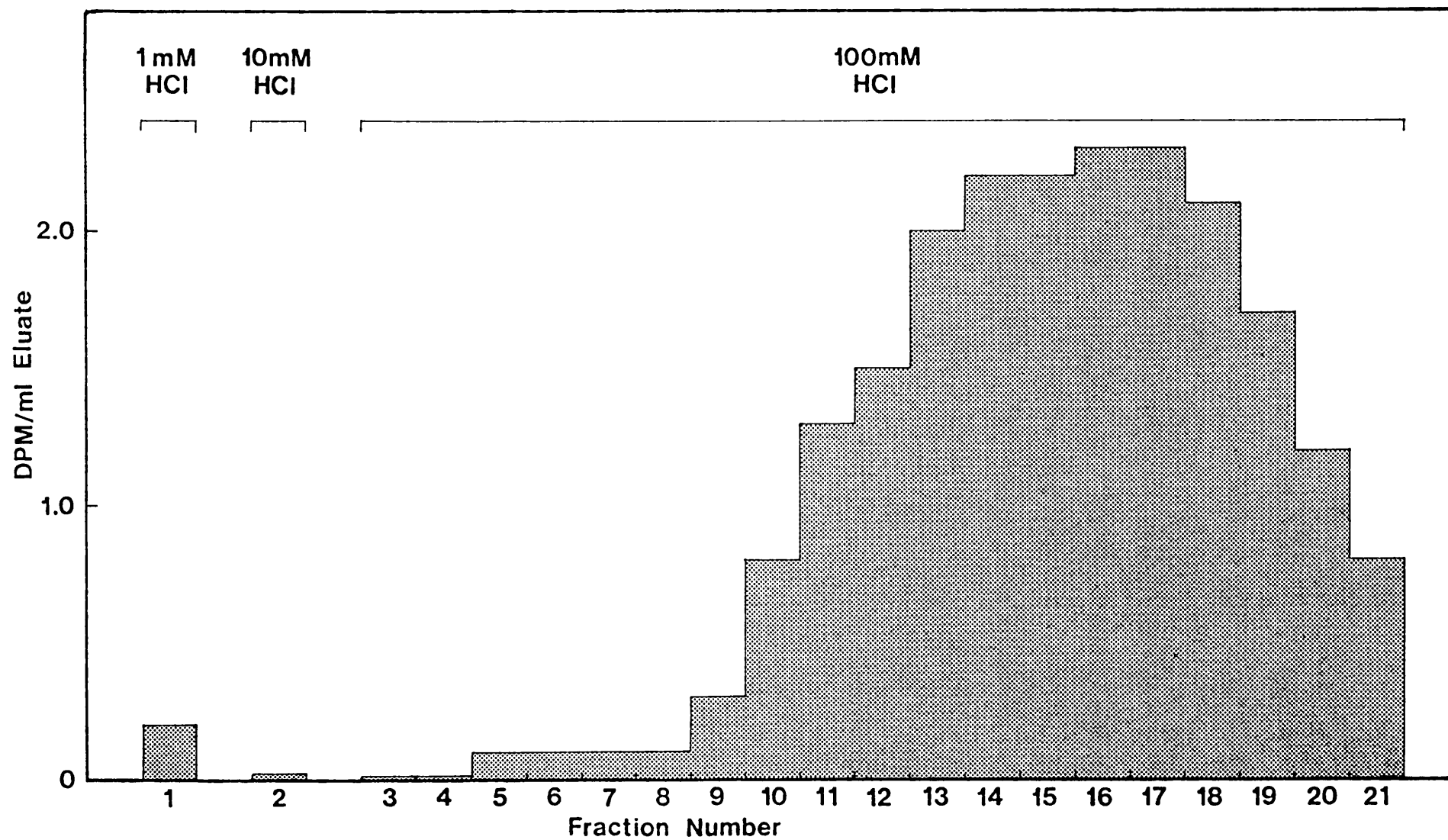


Fig. 13 Elution profile of labelled SAH and contaminants.

3.1.2.3 Plates for thin-layer chromatography.

A 15% aqueous suspension of fibrous cellulose was blended for 60 seconds in a Waring blender. The plates were coated with a thickness of 0.25 mm of cellulose.

3.2 INSTRUMENTS

- Amino acid analyzer: Beckman 120 C Model.
- Homogenizer: Braun Melsungen
- Spectrophotometers: Gilford Model 240
MSE Spectroplus
Corning 256
Unicam SP 800
- Liquid scintillation spectrometers: Packard Tri-Carb Model 3390
Searle Mark III Model 6880
- Centrifuges: MSE high speed 18
MSE super speed 100
- Automatic x-ray film processor: Kodak RP X-OMAT
- Automatic blood analysis: Coulter counter Model Ssr.

CHAPTER 4) METHODS.

4.1 ANIMAL MODELS.

The experimental animals used throughout these studies were adult male albino rats of Wistar strain, weighing 250 g ($\pm$ 20%).

During treatment animals were kept in individual cages and exposed to light from 8 a.m. to 8 p.m., at a constant temperature of 23°C, C.R.M. labware animal food and tap water were freely available; the daily intake of food and water were measured by weighing the solid pellets and the water container.

All animals were treated every morning between 10 a.m. and 1 p.m. for 15 days. Prior to treatments they were lightly anaesthetized with Nitrous Oxide: Oxygen (30:70) admixed with fluothane.

Observations were made on five groups of animals divided according to treatment:

a) Control rats.

Daily intraperitoneal (i.p.) and/or intravenous (i.v.) injection of physiological saline containing 0.2% (v/v) of the non-ionic detergent Tween 80 (vehicle). The i.p. or i.v. route were chosen to match control rats to different groups of experimental rats (see below). The volumes injected either i.p. or i.v. were equivalent to the volumes injected in the drug treated groups.

b) Carbidopa treated rats.

Daily i.p. injection of 20 mg/kg of a carbidopa suspension in vehicle (0.1 g/10 ml) for the first 12 days; on the last three days the dose was doubled.

c) L-Dopa and carbidopa (Sinemet) treated rats.

Daily i.p. injection of 200 mg/kg and 20 mg/kg of L-dopa and carbidopa, respectively, suspended in vehicle (1 g and 0.1 g in 10 ml, respectively) for the first 12 days; on the last three days the dose was doubled.

d) Iproniazid treated rats.

Daily i.v. injection into the tail vein of 10 mg/kg of iproniazid solution in vehicle (0.1 g/10 ml). The iproniazid was administered by the intravenous route to maximize the proportion of the drug entering the CNS.

Chronic injections of the drug via the tail vein became more problematic as the treatment progressed, because of increasing damage to the blood vessels; however with increasing experience this problem was largely overcome.

e) L-Dopa and iproniazid treated rats.

Daily i.v. injection into the tail vein of 10 mg/kg of iproniazid solution in vehicle (0.1 g/10 ml); this was followed by daily i.p. injection of 100 mg/kg of L-dopa suspended in vehicle (1 g/10 ml).

The suspensions were freshly prepared and homogenized every three days, and stored for this period at 4°C.

On the 15th day the rats were anaesthetized with ether 90 minutes after the last injection. When blood was required, the chest was opened and the blood removed by heart puncture; the liver was briefly perfused with ice-cold saline and the brain was washed with ice-cold saline.

Tissues were frozen in liquid nitrogen and stored at -20°C until used for analysis. For some experiments (see 4.5) the animals were decapitated, whole heads were plunged in liquid nitrogen, and stored at -20°C until analysis.

4.2 DETERMINATION OF BIOCHEMICAL AND HAEMATOLOGICAL PARAMETERS

4.2.1 Estimation of tissue protein concentrations.

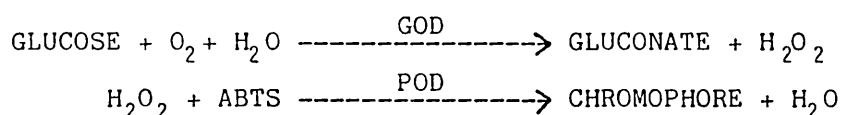
Protein determinations were carried out by a modified Lowry method (167), using bovine serum albumin as standard. This procedure gives a more linear relationship between protein concentrations and absorbance than the original method (168) from which it is derived.

4.2.2 Estimation of haematological parameters.

These determinations were carried out automatically by a Coulter counter on fresh blood samples treated with EDTA.

4.2.3 Estimation of blood glucose.

The method is based on measurement of the glucose present in the protein free supernatant by treatment with glucose oxidase. The hydrogen peroxide thereby formed is assayed spectrophotometrically by following the formation of the chromophore produced from its interaction with a chromogen (ABTS) in the presence of a peroxidase (169). The reagents and the standard are commercially available as a kit (see 3.1.1.4).



where:

GOD = Glucose oxidase

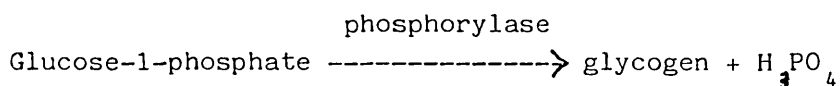
POD = Peroxidase

ABTS = di-ammonium 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate).

0.1 ml of blood was added to 0.9 ml of deionized water. Following protein precipitation by 0.5 ml of 0.15 M Ba(OH)₂ and 0.5 ml of 0.15 M ZnSO₄, the samples were centrifuged; 0.1 ml of the clear supernatants were added to

4.2.5 Estimation of liver phosphorylase activity.

The method is based on the determination of the orthophosphate liberated from glucose-1-phosphate in the formation of glycogen; this reaction is catalyzed by glycogen phosphorylase (172):



The orthophosphate is reacted with molybdic acid to form phosphomolybdate which is assayed spectrophotometrically after reduction to molybdenum blue (173).

4.2.5.1 Preparation and analysis of samples.

The following reagents, all prepared in deionized distilled water, were utilized:

substrate (pH 6.1): 0.15 M glucose-1-phosphate
 2 % glycogen
 0.2 mM sodium fluoride
 3 mM AMP (when present)

ammonium molybdate: 2.5 % solution in 2.5 M H_2SO_4

aminonaphtholsulphonic

acid reagent (ANSA): 79 ml of a 45 % sodium bisulphite solution were diluted to 200 ml, and to 195 ml of this dilution, 500 mg 1-amino-2-naphtol-4-sulphonic acid were added with mixing.

Then 5 ml and further 1 ml aliquots of a 20% solution of Na_2SO_3 were added with mixing, until all the powder had dissolved and a clear solution was obtained. The solution (stable for 4 weeks) was stored in the dark.

Weighed amount of tissues (1-3 g) were homogenized in 5 volumes of distilled water at 4°C. Incubation mixtures contained 50 µl of substrate (with or without AMP) and 50 µl of the liver homogenate. Incubations were carried out for 10 minutes at 37°C, and stopped with 200 µl of 10% trichloroacetic acid. After centrifugation, 200 µl of the supernatants were transferred into clean tubes and diluted to 4 ml with distilled water; 0.5 ml of the ammonium molybdate solution and, after mixing, 200 µl of ANSA solution were added to each tube. The colour developed after 10 minutes and was read at 680 nm. Blanks (where the acid was added before the enzyme preparation, or where the latter was replaced by an equivalent amount of water) and standards were prepared, and treated in the same way as the samples.

4.2.5.2 Analysis of data

Phosphorylase activity (A), expressed as µmole orthophosphate liberated/hr/100 mg of protein, was calculated according to the following formula:

$$\text{Activity} = A \times S \times \frac{1}{31} \times \frac{60}{t} \times \frac{0.3}{0.2} \times \frac{1}{V} \times \frac{100}{P}$$

where:

- A = absorbance of samples at 680 nm
- S = slope of standard curve (µgPi/ O.D.₆₈₀)
- t = incubation time in minutes
- 0.3/0.2 = correction for volume used in the colorimetric assay
- V = volume of homogenate incubated with substrate in ml
- P = protein concentration of homogenate in mg/ml
- 31 = atomic weight of inorganic phosphate.

4.3 DETERMINATION OF FREE AMINO ACID POOLS

The analysis of acid soluble amino acids was carried out by an automatic amino acid analyzer. The method is based on the separation of the amino acids by adjustment of temperature, pH and ionic gradients in an ion exchange column. After reacting with ninhydrin each compound is spectrophotometrically quantified (174). Similar methods have been described (175,176,177).

4.3.1 Preparation of tissue extracts

The preparations of both plasma and tissue samples were carried out at 0-4°C. Fresh heparinized blood was centrifuged (3,000 rpm) for 5 minutes, the plasma was removed and any haemolysis was noted. An equal volume of 5% sulphosalicylic acid was added to the plasma with mixing, and after centrifugation the supernatant was filtered (pore size of filters 0.22 μ m, see 3.1.1.5) frozen in liquid nitrogen and stored at -25°C until analysis. Weighed amounts of liver (about 2 g) or whole brains were homogenized in 3% sulphosalicylic acid (1:3 wt/vol). The suspensions were centrifuged at 20,000 g for 15 minutes; the supernatants decanted and the pellets resuspended, rehomogenized (in 1 vol of 3% sulphosalicylic acid) and recentrifuged. If necessary, the combined supernatant fluids from each tissue preparation were adjusted to pH 2.2, by the addition of 1.0M NaOH. The acid extracts were then adjusted to a final concentration of 0.2 g tissue/ml, frozen in liquid nitrogen and stored at -25°C until analysis. For all the liver samples and for most of the brain samples, the following procedure was used to remove glutathione. A portion of the acid extract was adjusted to pH 7.2-7.5 with 1.0 M NaOH; then, 0.80 ml of a freshly prepared solution of 0.5 M sodium sulphite were added per gram of tissue extracts and the solution was allowed to stand, open to the air, for 4 hours. The pH was then adjusted to 2.0-2.2 with 1.0 M HCl and the solution was diluted to give a final concentration of extract of 0.1 g tissue in 1.0 ml. This fraction was stored as described above. Before analysis all the samples were filtered through a Millipore filter (see 3.1.1.5).

4.3.2 Analysis of samples

Tissue extracts were analyzed on two different columns; one column for acidic and neutral amino acids and the other for basic amino acids.

The conditions for analysis are summarized in Table 1.

Slight changes in the timing of buffers and temperature or in the pH of buffers were often introduced to optimize the separation. For example, in some cases it was possible to elute methionine with the first buffer (and thus before cystathionine, see Fig.14), by lowering the pH of the first buffer to below 3.0 and by introducing the temperature change at 100 instead of 115 minutes.

4.3.3 Analysis of data

Amino acids concentrations in the samples were calculated by comparison to standard amino acids mixtures of known concentrations. A standard was run every 3 to 6 days depending on the age of the ninhydrin solution.

The percentage differences (%D) in the concentrations of ninhydrin positive compounds between control and treated rats were calculated according to the formula:

$$\%D = \frac{A - Ac}{Ac} \times 100$$

where:

Ac = amino acid concentrations in the tissues of control rats

A = amino acid concentrations in the tissues of treated rats.

TABLE 1 -CONDITIONS FOR AMINO ACID ANALYSIS-

		AMINO ACID ANALYSIS	
		ACIDIC & NEUTRAL	BASIC
COLUMN SIZE	length (cm)	70.0	23.0
	width (cm)	0.9	0.9
FIRST BUFFER	pH	3.25	4.25
	Sodium citrate conc. (M)	0.20	0.38
	Running time (min)	0 → 185	0 → 185
SECOND BUFFER	pH	4.25	5.25
	Sodium citrate conc. (M)	0.20	0.38
	Running time (min)	185 → 310	185 → 230
FIRST TEMPERATURE (30°C)			
	Running time (min)	0 → 115	0 → 185
SECOND TEMPERATURE (62.5°C)			
	Running time (min)	115 → 325	185 → 240
2 M NaOH "WASH" (min)		15	10
RE-EQUILIBRATION TIME (min)			
	(First buffer)	60	60
TOTAL RUNNING TIME/SAMPLE (min)		385	300

4.3.4 Comments on experimental procedure

Treatment with sodium sulfite removed most of the oxidized glutathione (GSSG), but not all of the reduced glutathione (GSH). GSSG was eluted as a broad peak between proline and alanine; GSH was eluted as a sharp peak before aspartate (Fig.14). If the amino acid analysis was carried out on freshly prepared samples, the interference by GSSG was not significant. Determinations were not carried out on plasma samples that showed significant haemolysis because of the different concentrations of amino acids between plasma and RBC (178). Slight haemolysis did not interfere in the analysis.

The elution of some amino acid relevant to this work was studied (Fig. 14) and the details are given below:

- (i) L-dopa is eluted between leucine and tyrosine;
- (ii) 3-O-methyldopa is eluted between tyrosine and phenylalanine; in Sinemet treated rats, the interference by the O-methylated compound was marked, especially in relation to the phenylalanine peak. Therefore, this amino acid was not quantified in these animals (Tables 7,10 and 13).

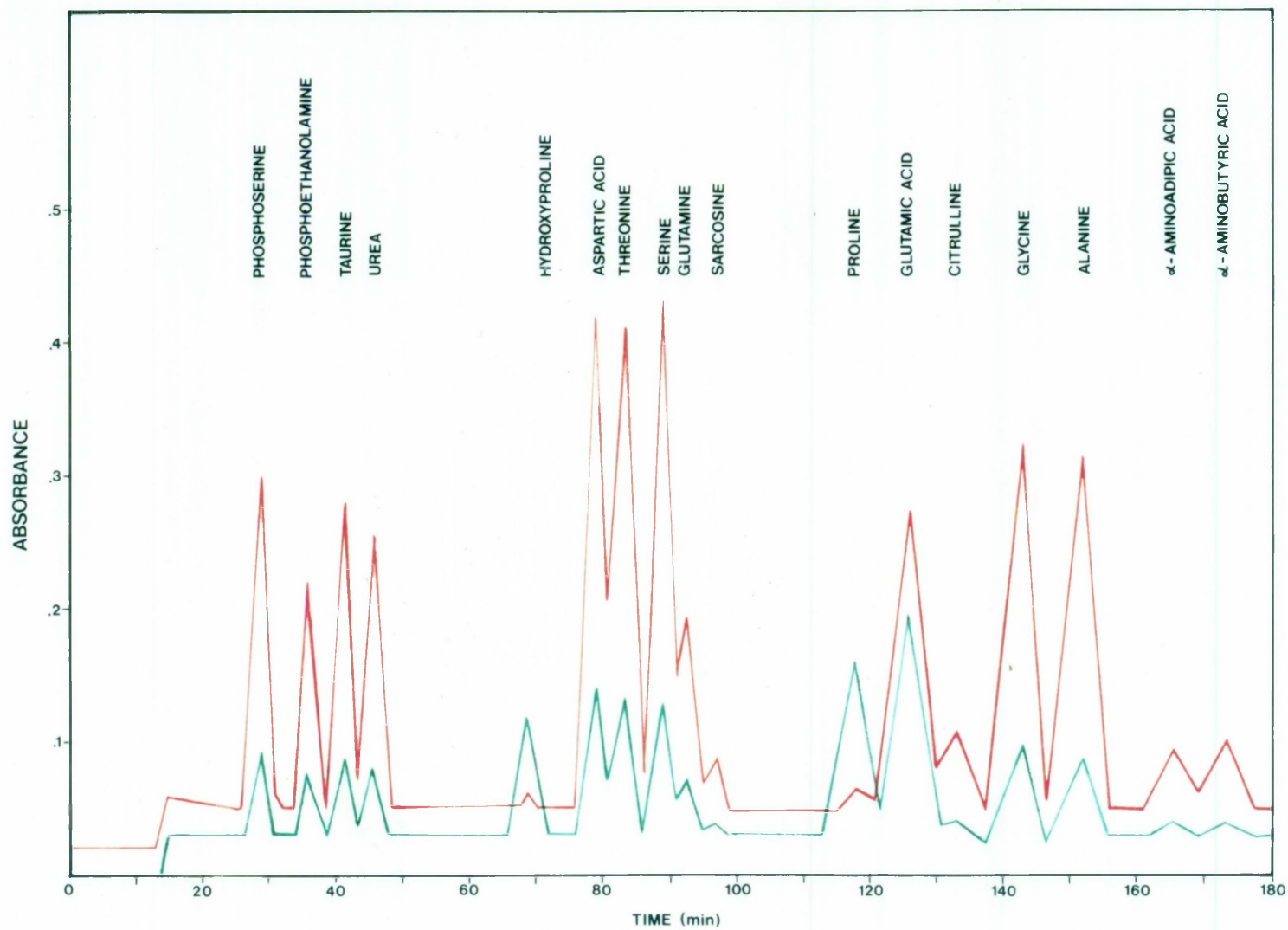


Fig. 14 Elution profile of a standard mixture of acidic and neutral amino acids.

The red and green lines represent the absorbance at 570 and 440 nm. respectively

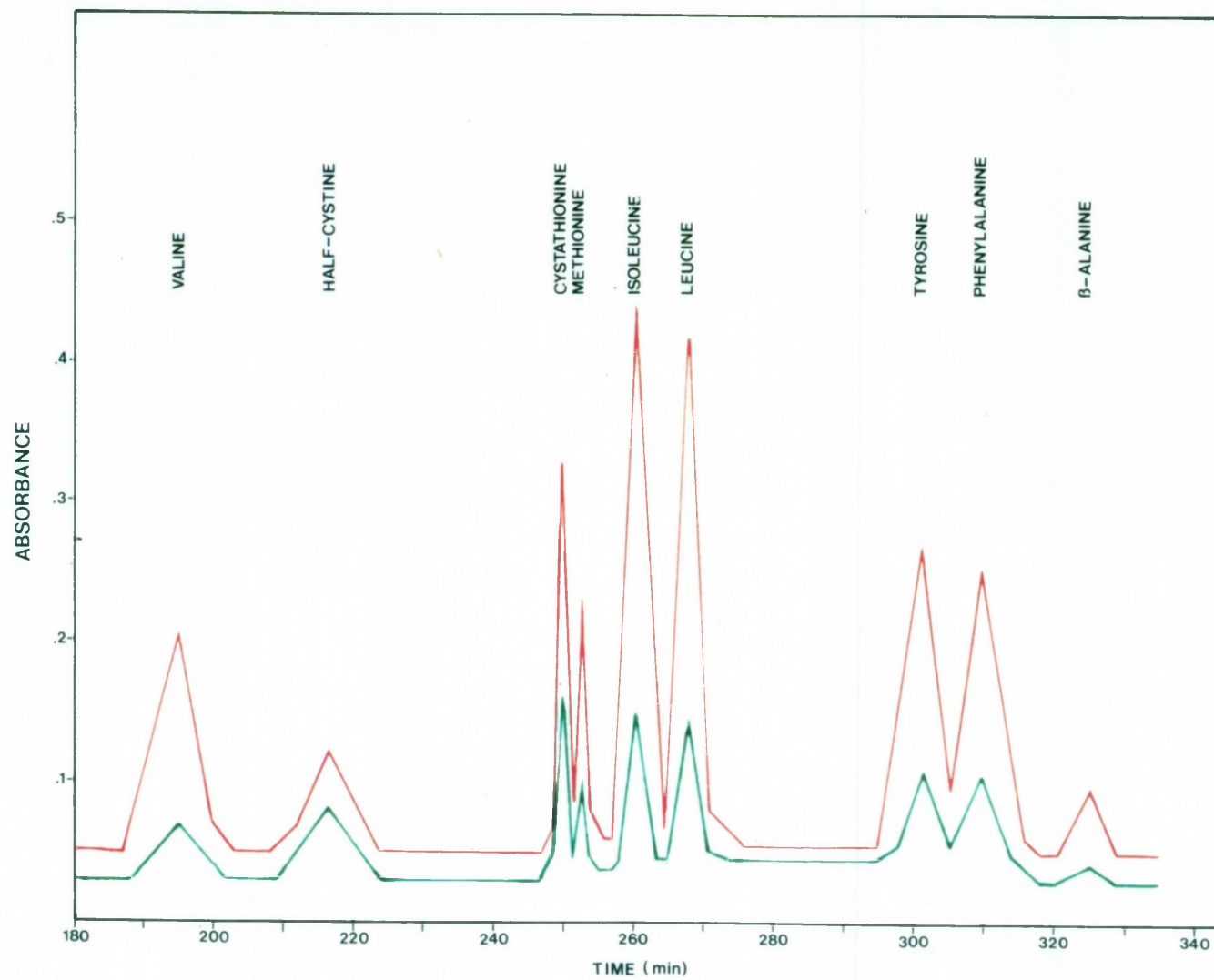


Fig.14 Continued

4.4 DETERMINATION OF CATECHOL-O-METHYLTRANSFERASE ACTIVITIES

COMT activities were measured by a radiochemical method based on the solvent extraction of the labelled homovanillic acid formed enzymatically from 3,4 dihydroxyphenylacetic acid and (³H) methyl labelled S-adenosyl-L-methionine (179).

4.4.1 Enzyme preparations

Deionized distilled water was used throughout the procedure. Fresh heparinized blood was centrifuged (3,000 rpm) for 5 minutes and the plasma and buffy coats were removed. The erythrocytes were washed three times with ice-cold saline. Generally enzyme activities were assayed immediately; alternatively packed cells were stored at -25°C for no longer than 2-3 days. Erythrocytes were haemolyzed by adding approximately 8.0 volumes of ice-cold water; after 30 minutes at 4-6°C with stirring, cell debris were removed by centrifugation and haemoglobin was determined by the cyanomethaemoglobin method (180).

Whole brains were homogenized in a Potter homogenizer at 4°C in 5 volumes of a medium of the following composition:

0.01 mM triethanolamine pH 7.6
0.5% (w/v) Triton X-100

Homogenates were centrifuged at 15,000 g for 10 minutes and the supernatants taken for COMT assay.

4.4.2 Preparation and analysis of samples

Incubations were carried out in a shaking water bath at 37°C.

The substrate mixture was of the following composition:

80 mM triethanolamine HCl pH 8.0
1.6 mM MgCl₂
3.0 mM 3,4-dihydroxyphenylacetic acid
0.9 mM (³H)-labelled S-adenosyl-L-methionine (50 GBq/mol).

The enzyme preparation (either 200 μ l of the brain supernatant or 300 μ l of the erythrocytes extract) and 2.0 units of adenosine deaminase (to hydrolyze the highly inhibitory S-adenosyl-L-homocysteine formed during the assay) were preincubated for 15 minutes; 500 μ l volumes of the substrate mixture, also preincubated, were added at zero time.

The reaction was studied within the limit of linearity with respect to both amount of protein and time. To satisfy these requirements, the amount of protein in the incubation mixtures varied from about 2 mg to about 4 mg protein (for brain and erythrocyte preparations, respectively) and the time of incubation was 60 minutes.

The reaction was stopped by the addition of 1.0 ml of 3 M HCl. Then 10 ml of a toluene/isoamyl alcohol mixture (3:2 v/v containing 0.4% 2,5-di-phenyloxazole and 0.01% 1,4-di-(2-(5-phenyloxazolyl)-benzene), w/v in both cases) were added to each vial. The mixture was subjected to vortex mixing for 15 seconds to extract the (3 H)-labelled homovanillic acid formed in the reaction. The mixture rapidly separated into two phases upon standing and was counted in the Searle scintillation counter without further treatment.

Most of the denatured protein separated in the aqueous layer, but some formed a layer at the interface between the aqueous and the organic phases. In all samples, the organic phase was clear and colourless; however for the erythrocyte COMT assay, the organic phase acquired a faint brown colour upon standing for 1 or 2 days at room temperature, which slightly decreased the counting efficiency.

Denatured proteins did not significantly interfere with the counting of the samples. This was checked in two ways; firstly by the addition of external standards to samples and blanks; secondly, by counting aliquots of the organic phase and comparing them to the radioactivity of the biphasic samples. The efficiency of the samples with both phases was above 10%, while the efficiency of the organic phase alone was around 20%.

In the blanks, hydrochloric acid was added before the enzyme; the vials were either treated as above and counted, or were incubated for 60 minutes at 37°C, treated as above and counted. Preliminary experiments showed that addition of L-dopa, in concentrations equivalent to those found in tissues of Sinemet treated rats (20-50 nmole; see Tables 7 and 10), did not interfere with the enzyme assays in vitro.

In the RBC preparations, the relatively high SAM hydrolase activity may interfere with the COMT assay. To discriminate between the two enzymic activities the following procedure was used (181). The samples were prepared and assayed as described above; then 5 ml aliquots of the organic solvent were transferred to clean vials; 5 ml of the scintillation mixture were added and the samples were recounted. These fractions were then evaporated to dryness to remove any (³H)-methanol formed enzymatically; 10 ml of the scintillation mixture were added and the samples were counted.

4.4.3 Analysis of data

Methyl transferase activities, expressed in nmole homovanillic acid formed/hr/mg protein or haemoglobin, were calculated according to the following formula:

$$\text{Activity} = \frac{\text{DPM}_s - \text{DPM}_b}{\text{DPM}_t} \times A \times \frac{1}{P} \times \frac{60}{t}$$

where:

DPM_s = dpm of sample

DPM_b = dpm of blank

DPM_t = total dpm in incubation mixture

A = nmole of SAM in incubation mixture

P = mg protein (or haemoglobin) in incubation mixture

t = incubation time in minutes.

4.5 DETERMINATION OF S-ADENOSYL-L-METHIONINE AND S-ADENOSYLHOMO-CYSTEINE CONCENTRATIONS.

The procedure is based on the acid extractions of S-adenosyl-L-methionine (SAM) and S-adenosylhomocysteine (SAH) from tissues, with the addition of labelled internal standards. The two compounds are separated from contaminants by ion-exchange chromatography and quantified by spectrophotometric assays (144).

4.5.1 Preparation of samples

Weighed amounts of frozen liver (2-3 g) were used. Whole brains were removed from the frozen head. The procedure, carried out at 4°C, is summarized in Fig.15.

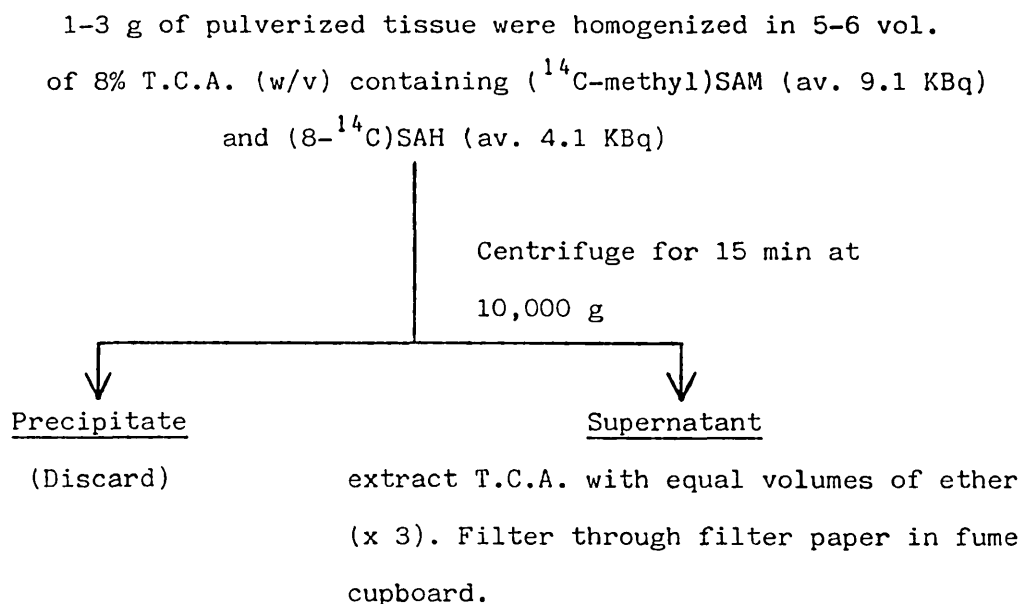


Fig.15 Preparation of SAM and SAH samples for chromatographic analysis.

4.5.2 Analysis of samples

Columns (1 x 4 or 1 x 5 cm) of Cellex-P (H⁺ form) were equilibrated with 1 mM HCl (about 100 ml); elutions were carried out in the cold room (4-6°C). The supernatants were applied to the columns and were eluted as follows:

100	ml	1 mM	HCl	
100	ml	10 mM	HCl	
2-3	ml	150 mM	HCl	-void volume-
15-20	ml	150 mM	HCl	-SAH fraction(s)-
50-60	ml	50 mM	HCl	
2-3	ml	500 mM	HCl	-void volume-
15-25	ml	500 mM	HCl	-SAM fraction(s)-

Before elution the temperature of the acid solutions were brought to 4°C. Generally the SAH and SAM fractions were collected in two subfractions. The amounts of SAH and SAM were determined by measuring the absorbance at 257 nm and using a molar extinction coefficient of 15,000 litre mol⁻¹ cm⁻¹ for both substances. The absorbance at 257 nm of the void volume fractions, was taken as the background absorbance.

The volumes of the SAM and SAH fractions were recorded. The radioactivity of each was determined by adding aliquots to 15 ml Aquasol and counting the samples in a liquid scintillation counter with automatic quench correction.

4.5.3 Analysis of data

The following formula was used:

$$\frac{\text{nmole SAH (or SAM)}}{\text{g of tissue}} = \frac{1}{W} \left(\frac{\text{DPM}_S}{\text{DPM}_f} \times \frac{A}{E} \times V - \frac{\text{DPM}_f}{SA} \right)$$

where:

W = weight of tissue used in grams
DPM_s = dpm of standard labelled isotope added to each sample
DPM_f = dpm recovered in fraction
A = absorbance of fraction at 257 nm
E = molar extinction coefficient at 257 nm (0.015 L/ μ Mole/cm)
V = volume of fraction in ml
SA = specific activity of standard labelled isotope.

4.5.4 Comments on experimental procedure

Fig.13 shows the elution profile of labelled SAH following its synthesis from adenosine and homocysteine. The volume required to elute SAH was consistently higher than reported; furthermore elution was extremely slow. These difficulties were partly overcome by repeated removal of fine cellulose particles, by soaking the cellulose for a short time and by decreasing the bed volume to a minimum (182). Even after these precautions elution was very slow and SAH was eluted as a broad peak (see Fig.13).

Thus, for analysis of samples (but not for preparation of SAH) 150 mM HCl was used to elute the sulfonium compound. Although this had no effect on elution time, it considerably diminished the volume required to elute SAH.

Purity was checked by thin-layer chromatography on cellulose using n.butanol : acetic acid : water (12:3:5) as mobile phase (183). Two spots were identified under u.v. light: one migrating like SAH and the other like adenine. The SAH spot accounted for 85-90% of the total radioactivity eluted in the SAH sample; the adenine spot accounted for about 10%. No spot for SAM was revealed by spraying the plate with ninhydrin.

Under these conditions, the recoveries of SAH were between 70% and 85%. Following elution of SAH, the columns were washed and SAM was eluted as suggested in the original procedure. Under these conditions the recoveries of SAM were around 90%.

4.6 DETERMINATION OF NUCLEIC ACID CONCENTRATIONS

4.6.1 Total DNA and total RNA

Perchloric acid extracts of tissue homogenates were treated with alkali which hydrolyses RNA but not DNA. Following acidification the nucleic acids were separated by centrifugation and quantified either by ultraviolet or by colorimetric assays (184).

4.6.1.1 Preparation of samples

The procedure was carried out at 4°C.

Weighed amounts of liver (about 1 g) and whole brains were homogenized in 20 and 10 volumes of distilled water, respectively.

The method is summarized in Fig.16.

4.6.1.2 Analysis of samples

Total RNA was estimated by measuring the extinction of the RNA solution at 260 nm, assuming that an absorbance of 1.00 is equivalent to 32 µg RNA/ml. To check the purity of the RNA solutions, the absorbance at 232 and 280 nm was also measured.

Total DNA was estimated by the indole reaction. 1.0 ml of 0.04% indole reagent and 1.0 ml of concentrated HCl were added to 2.0 ml of the DNA preparations. The samples were placed in a boiling water bath for 10 minutes; after cooling to room temperature, the samples were extracted three times with 4 ml of CHCl_3 . Standards and blanks were prepared in 0.1 M KOH and treated in the same way. The absorbance was measured at 490 nm.

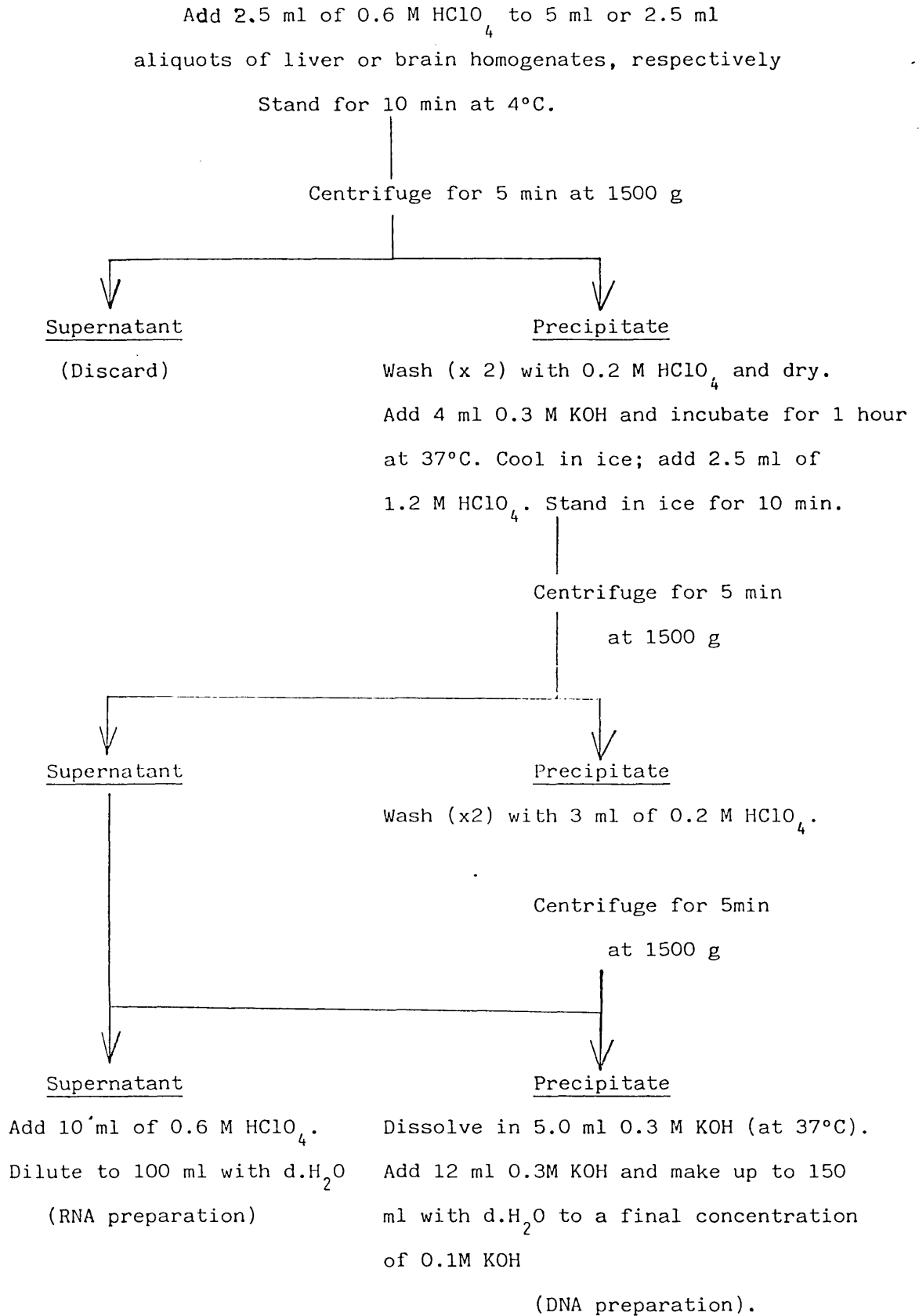


Fig.16 Separation of DNA and RNA from tissue homogenates.

4.6.2 Total transfer RNA

The method is based on the separation of the post microsomal supernatant by high speed centrifugation followed by the precipitation of t-RNA, and associated protein, by adjustment of the pH to 4.9. The precipitate is resuspended in saline and the protein removed by phenol extraction. The t-RNA is precipitated by acetonitrile-ethanol after readjustment to pH 5.0 (185).

4.6.2.1 Preparation of samples

The following buffers were used:

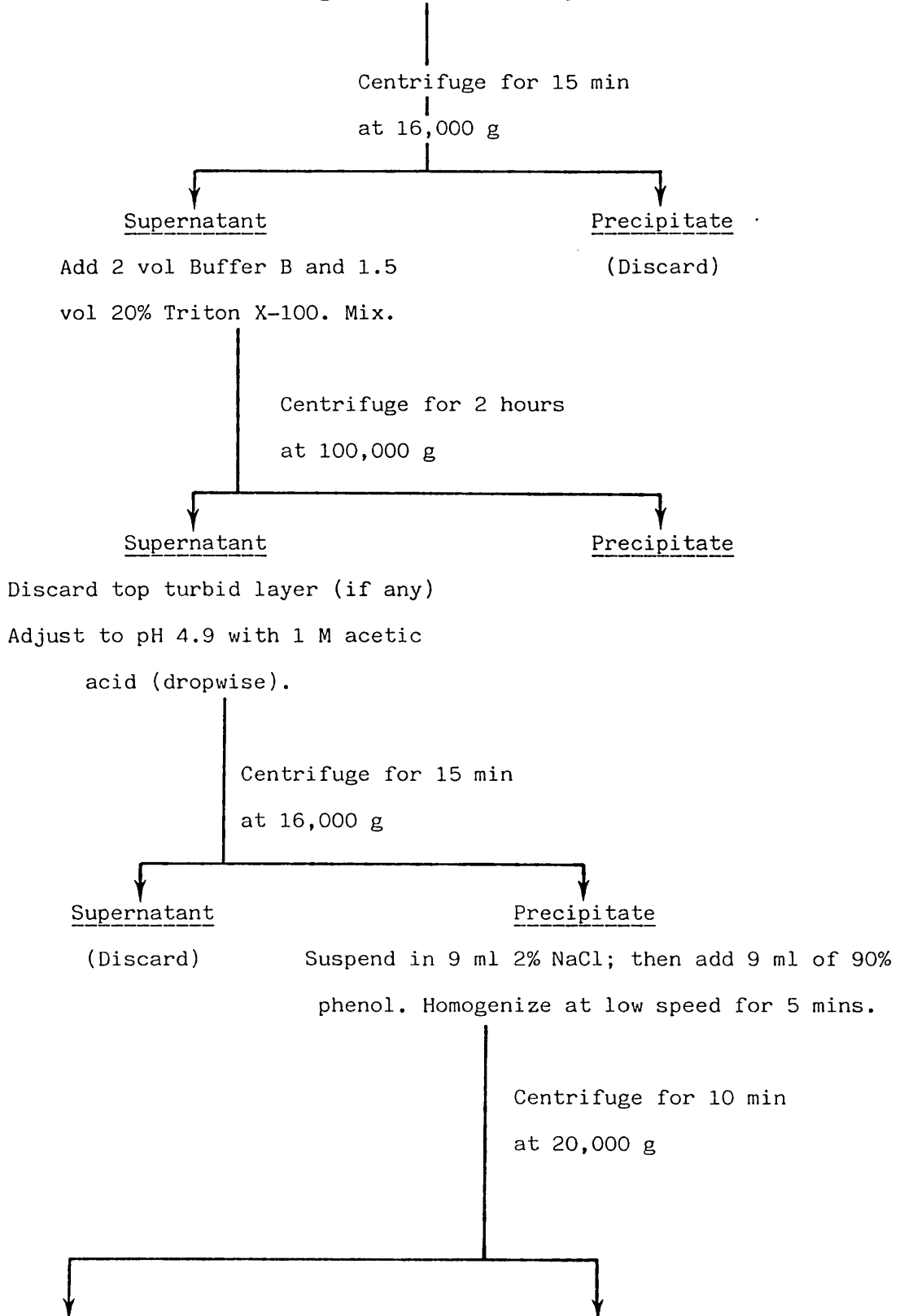
Buffer A: 0.25 M sucrose (Ribonuclease free)
 5 mM MgCl_2
 25 mM KCl
 25 mM KH_2PO_4
 Adjusted to pH 6.6 with KOH.

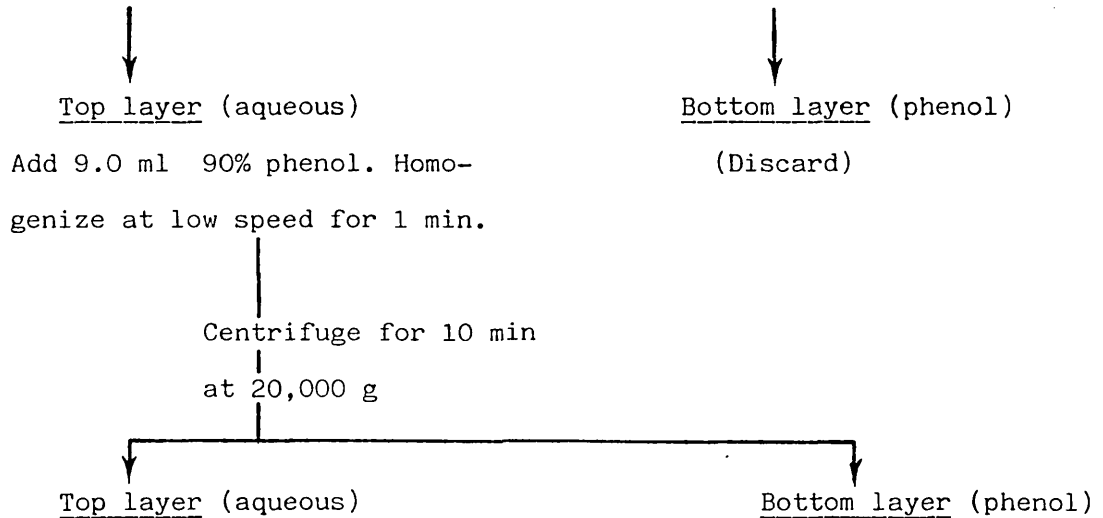
Buffer B: 5 mM MgCl_2
 25 mM KCl
 25 mM KH_2PO_4
 Adjusted to pH 6.6 with KOH.

The solution of 90% phenol was prepared one day before use and was stored in the dark at room temperature.

The procedure, summarized in Fig.17, was carried out at 4°C.

Weighed amounts (about 2.0 g) of tissue were suspended in 5 ml Buffer A and homogenized at lowest speed for 3-4 min.

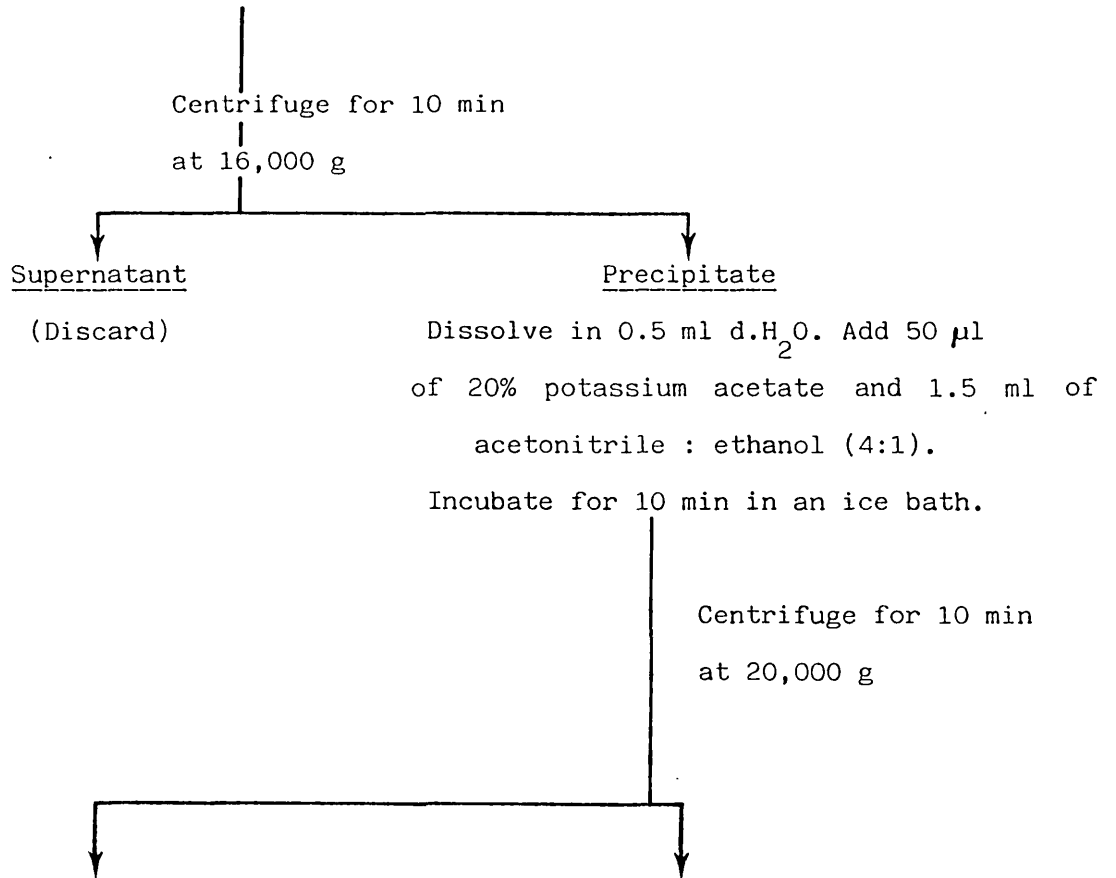




Remove about 8.0 ml taking care not to remove any protein (white interface between two layers). Record volume of removed solution.

Add 0.1 vol of 20% potassium acetate pH 5.0 and 3.0 vol of acetonitrile : ethanol (4:1).

Incubate for 10 min. in an ice bath.



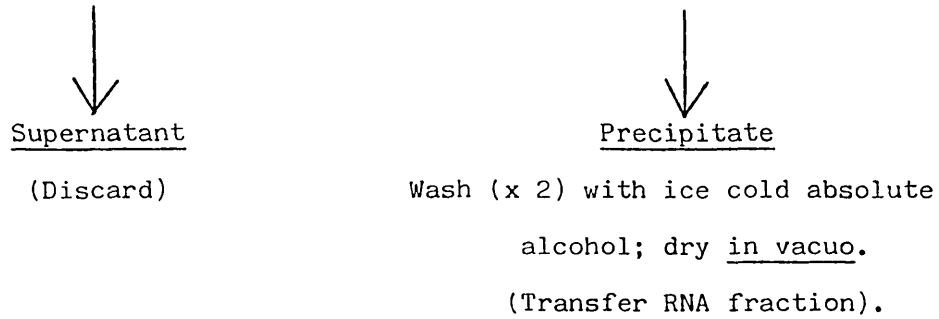


Fig. 17 - Isolation of transfer RNA from rat tissues.

4.6.2.2 Analysis of samples

The dry residues were dissolved in 200 μ l of d.H₂O. The absorbance of diluted aliquots (1:200 and 1:400) was measured at 260 nm assuming that 1.00 O.D. is equivalent to 40 μ g or 50 μ g of t-RNA for liver and brain respectively (185,186). Absorbance was also measured at 280 and 250 nm and the values were used to calculate the level of impurities.

4.7 DETERMINATION OF TRANSFER RNA BASE COMPOSITION

The method involves the enzymatic degradation of the t-RNA molecule to its constituent nucleosides. The pentose rings of each nucleoside is then opened by periodate oxidation to the corresponding hydroxydialdehyde, which is then reduced by tritiated potassium borohydride to the triol with stoichiometric incorporation of tritium.

The various triols are separated by two dimensional thin-layer chromatography on cellulose. The labelled triols are located by fluorography, the corresponding areas are scraped out and assayed by liquid scintillation counting. The method and some of its applications have been extensively studied (185,187,188,189).

4.7.1 Preparation of samples

4.7.1.1 Tissue extract of t-RNA

As described in 4.6.2.1

4.7.1.2 Enzymatic digestion of t-RNA

Acid washed tubes and deionized-distilled water were used throughout the procedure.

Hydrolysis of t-RNA was carried out at 37°C for 12 hours. The incubation mixture contained per "μl":

0.535	μg	of t-RNA preparation
30	nmole	bicine-Na (pH 8.0)
10	nmole	MgCl ₂
0.2	μg	venom phosphodiesterase
0.2	μg	ribonuclease
0.165	μg	alkaline phosphatase.

The total volume of the incubation mixture was either 25 or 50 μl, made up with d.H₂O, if necessary.

Incubations were stopped by storing the test-tubes at -25°C; enzymes or electrolytes were not removed.

4.7.1.3 Tritium labelling of nucleosides.

The procedure was carried out with acid-washed tubes, in a fume cupboard and at room temperature (23°C), unless otherwise stated.

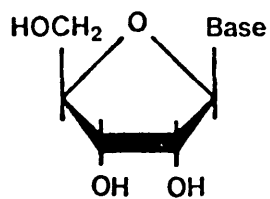
The (^3H)labelled- KBH_4 solution was prepared in the following way; 3.66 GBq of (^3H)- KBH_4 were dissolved in 0.1 M KOH (carbonate free) to give a final concentration of 0.1 M (^3H)- KBH_4 . The solution was divided into aliquots of 50 μl and stored at -25°C. The preparation was used within 4 months.

Nucleosides solutions were incubated at 37°C for 5 minutes and mixed at high speed.

The procedure is summarized in Fig.18

Finally, 75 μl of 1.0M acetic acid (75,000 nmol) were added to each incubation mixture; the solutions were allowed to stand at room temperature for 30 min in unstoppered test tubes and were then freeze dried. The dry residues were dissolved in 50 or 25 μl of 0.1 M formic acid. Preparations were assayed for radioactivity; if this was equal or higher than about 1×10^6 dpm/ μl they were stored at -25°C and analyzed within 4 weeks.

When the specific activity of the solutions was markedly less than 1×10^6 dpm/ μl , the chromatographic resolution of the compounds was poor and unidentified spots appeared on the X-ray film, suggesting an incomplete hydrolysis of t-RNA or an unsuccessful oxidation of the dialdehydes.



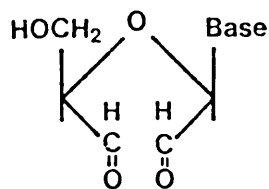
Nucleosides

10 μ l enzymatic digest of t-RNA (16.1 nmol nucleosides)

Add: 1 μ l 42 mM NaIO_4 (42 nmol)

13 μ l $\text{d.H}_2\text{O}$ (carbonate free)

Incubate for 2 hours in the dark.



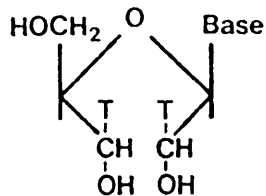
Nucleoside dialdehydes

Cool for 5 min in an ice-bath.

Add: 6.0 μ l 75 mM phosphate buffer pH 6.8 (450 nmol)

2.5 μ l 0.1 M (^3H)- KBH_4 (250 nmol)

Incubate for 2 hours in the dark.



Tritium labelled triols.

Fig.18 Oxidation-reduction of nucleosides.

4.7.2 Analysis of samples

The labelled nucleoside derivatives were quantitatively and qualitatively analysed by two-dimensional thin-layer chromatography.

All plates were oven dried for 30 min at 70-80°C and then allowed to cool to room temperature before use.

The solvent used were of the following composition:

Solvent A : acetonitrile : 4M NH_4OH (3.4:1 by volume)

Solvent B : t-amyl alcohol : methyl ethyl ketone : acetonitrile :
ethyl acetate : water : formic acid
(4:2:1.5:2:1.5:0.18 by vol).

The solvent mixtures were stored at 6°C (but allowed to come to room temperature before use) and used within three days.

The molarity of NH_4OH was checked by titration against HCl.

Plates were spotted in a corner (2 cm from each side) with 150/220 KBq of sample applied in aliquots of 1 μl with intermediate drying.

After chromatography with solvent A, the plates were dried, developed in the second dimension with solvent B, and again they were thoroughly dried. They were then briefly immersed in a 7% solution of 2,5-diphenyl oxazole in diethyl ether and dried. The plate were then spotted at positions close to their edge with a mixture of methyl blue and $(^3\text{H})\text{-KBH}_4$. These reference spots defined the position of the X-ray plate relative to the thin-layer chromatogen.

Fluorography was carried out in the dark; X-ray films were sandwiched between one chromatogram and glass plate and fixed in position by clamps. Exposure varied between one and two weeks, depending on the temperature at which it was carried out (either -80°C or -25°C) and the amount of radioactivity on each plate.

It was noted during the course of these experiments that the derivatives were more readily visible on a photocopy of the X-ray film, rather than on the original.

Following development of the X-ray film, the nucleoside derivatives were located on the chromatogram by using the reference spots to line up the plate and the film. Each compound was scraped out, transferred into a vial containing 1.9 ml of $\text{d.H}_2\text{O}$, the vials were vigorously shaken and 8.0 ml of Aquasol-2 were added. In this proportion water and Aquasol form a stable gel on mixing. The opalescent gels were counted in a β -scintillation spectrometer at a counting efficiency of over 20%.

The reproducibility of counting was improved by the use of a gel suspension of the cellulose; there was no loss in sensitivity, and only a slight decrease in counting efficiency when compared to samples where the cellulose was added to a clear solution of Aquasol (190). The size of the spots (and thus the amount of cellulose added to each vial) had a negligible influence on counting efficiencies, and did not affect the dpm.

4.7.3 Analysis of data

Four t-RNA preparations from both liver and brain were used and the base composition of each t-RNA preparation is the average of three chromatographic analysis.

A two-dimensional chromatogram of tritium labelled, partially purified digest of rat liver t-RNA is shown in Fig.19.

The following nomenclature is used:

U' Nucleoside triols derived from					Uridine
A'	"	"	"	"	Adenosine
C'	"	"	"	"	Cytidine
G'	"	"	"	"	Guanosine
I'	"	"	"	"	Inosine
Y'	"	"	"	"	Pseudouridine
hU'	"	"	"	"	Dihydrouridine
m ₁ A'	"	"	"	"	1-methyladenosine
m ₁ G'	"	"	"	"	1-methylguanosine
m ₂ G'	"	"	"	"	N ² -methylguanosine
m ₂ ² G'	"	"	"	"	N ² ,N ² -dimethylguanosine
m ₃ C'	"	"	"	"	3-methylcytidine
m ₅ C'	"	"	"	"	5-methylcytidine
m ₅ U'	"	"	"	"	5-methyluridine
m ₇ G'	"	"	"	"	7-methylguanosine

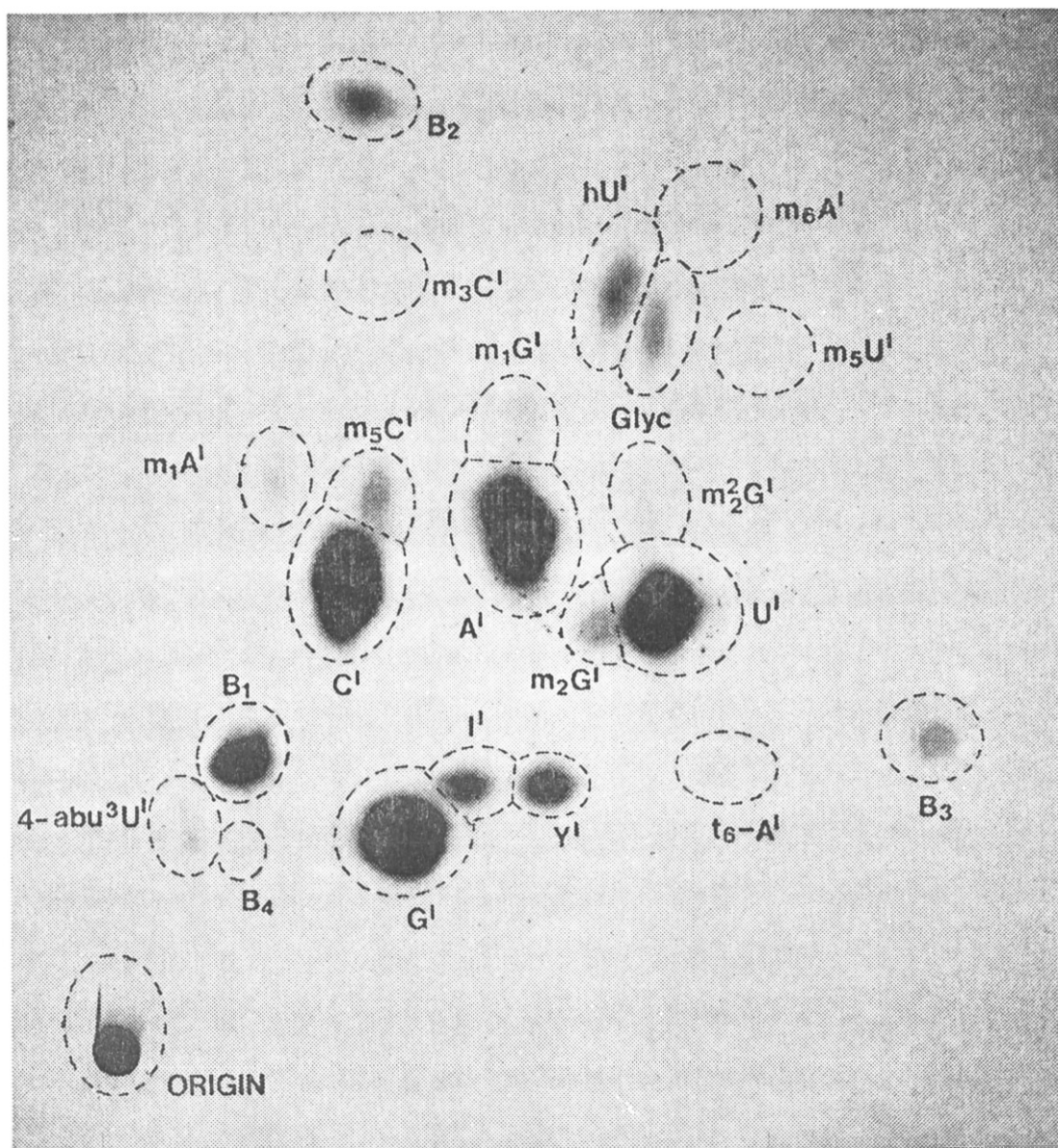


Fig.19 Two dimensional chromatogram of nucleoside trialcohols derived from rat liver t-RNA. The nomenclature is as described above; additional spots refer to triols derivatives of the following compounds: Glyc =glycogen; $t_6-A' = N-[9(\beta\text{-D ribofuranosyl})\text{purin-6-yl}] \text{ carbamoyl threonine}$; $4\text{-abu}^3U' = 3\text{-(3-amino-3 carboxypropyl) uridine}$; $m_6A' = 6\text{-methyl adenosine}$; $B_1, B_2, B_3, B_4 = \text{background}$. The total radioactivity recovered in these spots and in the origin is consistently less than 10% (with B_1, B_3 and the origin accounting for well over half of this). Within this group the recoveries are $B_1 \approx B_3 \approx OR \approx B_2 \approx \text{Glyc} \approx t_6-A' \approx B_4 \approx 4\text{-abu}^3U'$; the last compound is not always detected.

The proportion of each base in the transfer RNA preparation is expressed as a percentage of the total (f_1) according to the formula

$$f_1 = \frac{\sum_{i=1}^N \text{DPM}_i}{\text{DPM}_i} \times 100$$

where:

N = number of radioactive nucleoside derivatives separated

DPM_i = DPM of individual compounds.

The value of N in the formula above refers only to the following derivatives: U', A', C', G', I', Y', hU, m₁A', m₁G', m₂G', m₂²G', m₃C', m₅C', m₅U', and m₇G'.

The derivative m₆A' is formed by the breakdown of m₁A' (185), so that the corrected recovery of m₁A' is equal to the sum of m₁A' and m₆A'.

The resolution of G' and I' is not always satisfactory; for this reason, the % base composition for these two compounds is expressed as the sum of the radioactivity recovered in the two spots.

4.8 DETERMINATION OF AMINOTRANSFERASE ACTIVITIES AND THEIR "IN VITRO" STIMULATION BY PYRIDOXAL PHOSPHATE.

The method is based on the ion-exchange separation of labelled amino acid substrates and of labelled keto acid products formed during the in vitro incubation with enzyme preparations. Enzyme activities are calculated by measuring the radioactivity in the amino acids and in the keto acid eluates. Similar methods have been described (191,192).

4.8.1 "In vitro" studies

4.8.1.1 Condensation studies

The condensation between pyridoxal phosphate and L-dopa was studied at pH 6.8, 7.4 and 8.0 at 37°C. The incubation mixtures were of the following composition:

- 200 μ M L-dopa
- 200 μ M pyridoxal phosphate
- 800 μ M dithiothreitol (DTT)
- 0.1 M potassium phosphate buffer at varying pH.

DTT is added to the incubation mixture, as at pH 8.0 L-dopa oxidation interferes with the ultraviolet spectrum. Oxidation of DTT at pH 8.0 produces a broad peak with maximum absorption at 280 nm; at pH 8.0 the absorption around this wavelength was corrected for DTT oxidation. The ultraviolet spectrum of L-dopa shows an absorption peak at 280 nm at all three pH(s); the absorption peak of pyridoxal phosphate shifts from 384 at pH 6.8 to 388 and 412 nm at pH 7.4 and 8.0, respectively.

The condensation between pyridoxal phosphate and carbidopa is studied at pH 7.4 at 37°C. The following concentrations were used:

- 150 μ M carbidopa
- 150 μ M pyridoxal phosphate
- 0.1 M potassium phosphate pH 7.4

At this pH, carbidopa has an absorption peak at 280 nm.

Condensation was followed in the Unicam ultraviolet spectrophotometer; suitable wavelengths were chosen to measure the appearance of the condensation products vs. time and the disappearance of the absorption maximum of pyridoxal phosphate vs. time.

4.8.1.2 Glutamate aminotransferase activity and the effect of pyridoxal-5'-phosphate, L-dopa, carbidopa and iproniazid addition in vitro.

The drugs used in the treatment of the animals (see 4.1) were used to study their in vitro effect on glutamate aminotransferase activity. When added to the incubation mixture their final concentrations were 500 μ M L-dopa, 50 μ M carbidopa and 50 μ M iproniazid phosphate. When pyridoxal phosphate was added to the incubation mixture its final concentration was 25 μ M.

Incubation, preparation and analysis of samples were carried out as described in section 4.8.2.2, the only difference being that, in the incubation mixture 0.1 M potassium phosphate buffer pH 7.4 was used in place of the triethanolamine buffer. A crude homogenate of rat liver was used as the enzyme source.

4.8.2 Studies of the "in vivo" influence of carbidopa, iproniazid and L-dopa on the activity of aminotransferases

4.8.2.1 Enzyme preparations.

Deionized-distilled water was used throughout the procedure. The enzyme was prepared from the erythrocytes as already described (see 4.4.1). Weighed amounts of liver (about 2-3 g) and whole brains were homogenized at 4°C in 5 volumes of a medium of the following composition:

0.01 M triethanolamine buffer pH 7.6
0.10 mM dithiothreitol
0.2% w/v Triton X-100.

Triethanolamine was the buffer used in this study, as phosphate buffers interfere in the reactivation of some transaminase apoenzymes by pyridoxal phosphate (193,194).

Homogenates were filtered through a gauze and the protein concentration was determined by a modified Lowry method (167). Dialysis of homogenates was not carried out in this study for the following reasons:

- a) not to alter the apoenzyme/holoenzyme ratio
- b) the endogenous amino acids do not affect enzyme activity (191).

This was checked also in relation to the relatively high levels of L-dopa and 3-O-methyldopa in the tissues of Sinemet treated rats (Tables 7,10 & 13).

4.8.2.2 Preparation of samples; incubations.

Deionized-distilled water and acid washed glassware were used throughout the procedure.

Incubations were carried out at 37°C in a shaking water bath. The incubation mixture contained:

0.1 M triethanolamine buffer pH 8.0
0.2 mM dithiothreitol
10 mM α -ketoglutaric acid
6-10 KBq (^{14}C)labelled amino acid

and the corresponding amino acid depending on the enzyme assay; thus for glutamate aminotransferase 33 mM of glutamate and for tyrosine aminotransferase 3.3 mM of tyrosine were used.

If necessary the pH was adjusted with NaOH or HNO_3 to pH 8.0; HCl was not used as chloride ions are known to affect the activity of certain aminotransferases (193).

Each incubation was carried out with or without addition of pyridoxal phosphate (final concentration 150 μ M). The total volume of each incubation mixture was adjusted to 1.0 ml with d.H₂O. The enzyme preparations were equilibrated with pyridoxal phosphate (or an equivalent volume of d.H₂O) at 37°C for 5 minutes; reactions were started by the addition of the incubation mixture pre-equilibrated at 37°C. The reactions were studied within the limits of linearity with respect to both amount of protein and time. To satisfy these requirements the amount of protein added to the incubation mixtures varied from 0.01 to 5.5 mg protein, and the time of incubation varied from 10 to 60 mins. Incubations were stopped by the addition of 0.38 ml of 3.5% perchloric acid. The samples were centrifuged and the supernatants were frozen and stored at -25°C until analysis.

Blanks (in which the perchloric acid was added to the incubation mixture before the enzyme preparation, or, alternatively, the enzyme preparations were replaced by an equal volume of water) were prepared and treated in the same way.

4.8.2.3 Analysis of samples.

The procedure was carried out at room temperature. 12 columns (1 x 6 cm) of Dowex 50 (H⁺ form) were washed with 3 M HCl (about 100 ml) and then with d.H₂O (about 120 ml). The same column was used for all the samples obtained from one enzyme preparation. The perchloric acid supernatant fluids were applied to the columns. The keto acids were eluted in a first fraction with d.H₂O; the amino acids were eluted in a second fraction with 1.5 M HCl. The exact volume of each fraction was recorded (40-50 ml and 100-110 ml of d.H₂O were required to elute labelled α -ketoglutarate and labelled p-hydroxyphenylpyruvic acid respectively; 100 ml and 180 ml of 1.5 M HCl were required to elute labelled glutamic acid and labelled tyrosine, respectively).

Since small amounts of labelled amino acids were eluted with water, a blank was applied to each column and the amount of labelled amino acid eluted by water was determined (about 1% of (^{14}C)-glutamate and 2% of (^{14}C)-tyrosine). The radioactivity of each fraction was measured by adding 1.0 ml aliquots to 15 ml of Aquasol-2 and counting was carried out in a β -scintillation counter.

After each chromatographic analysis the resin was recycled by washing with 1 M NH_4OH (about 30 ml), 3 M HCl (about 100 ml) and 120 ml $\text{d.H}_2\text{O}$. The resin was discarded after 3-4 chromatographic analysis and the column was re-standardized.

4.8.2.4 Analysis of data.

The following formula was used to calculate enzyme activities:

$$\mu\text{mol amino acid converted/hr/100 mg protein} = \frac{(E-C)}{100} \cdot A \cdot \frac{60}{t} \cdot \frac{100}{P}$$

where:

E = % label eluted in first fraction of experimental run

C = % labelled amino acid eluted in first fraction of blank run

A = μmole of amino acid in 1 ml of incubation mixture

t = incubation time in minutes

P = mg protein added to 1 ml incubation mixture.

The percentage activation (% D) resulting from in vitro addition of pyridoxal phosphate to the enzyme preparation was calculated according to the following formula:

$$\% D = \frac{E_c - E}{E} \times 100$$

where:

E_c = enzyme activity with pyridoxal phosphate

E = enzyme activity without pyridoxal phosphate.

4.9 CALCULATIONS

Throughout this study, results are expressed as means $\pm$ standard deviations and the number of determinations in any given experiment is indicated in parenthesis.

Student t-test was used to determine the statistical significance of differences among the results, only when four or more determinations were carried out. In the determinations of the free amino acid pools, differences in results are reported as significant only when the "p" values are equal or less than 0.01; in the other experiments, differences are reported as significant only when the "p" values are equal or less than 0.05.

RESULTS AND DISCUSSION

CHAPTER 5) RESULTS

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CHAPTER 5) RESULTS

5.1 WHOLE ANIMALS

5.1.1 Behaviour and general appearance

Carbidopa and iproniazid treatments did not have clear effects on the behaviour and appearance of the animals, at the dosage utilized in these experiments.

Sinemet treatment induced well established behavioural patterns: motor hyperactivity, muscle jerks, jumping, convulsions and salivation (195). These were observed for 3-4 hours after the injection. The daily response to the drug appeared to slowly subside, so that the animals gradually reverted to a normal appearance and behaviour.

After 2 to 5 days, a pink/orange tint appeared on the hair of the back, near the base of the skull, which generally persisted until the end of the treatment. Responses of the animals to drug treatment (as described above) changed with time: in the first 1 to 3 days of treatment, they were not as strong as observed after about one week. However, after 8 to 10 days the animals reverted to weaker responses. For this reason, the dose was doubled on the 13th day in order to reinduce strong behavioural responses.

Following dissection, the appearance of tissues of control and experimental animals was similar. There were no cases of mortality.

Joint treatment with iproniazid and L-dopa also induced well recognized behavioural symptoms: muscle jerks, impaired movement, gnawing, corkscrew tails, catatonic state posturing, biting the wires of the cage, elevation of the hair and protuberance of the eyes (195,196). These drug-induced responses were observed for longer than three hours after each injection. The animals did not gradually revert to a normal behaviour; once the most visible effects of the treatment were no longer seen, they appeared exhausted and less active than normal.

Although the daily response to iproniazid and L-dopa was similar in intensity throughout the treatment, the general appearance of the animal deteriorated with time. Loss of hair was apparent throughout the body; 7 to 9 days after the start of the treatment about 50% of the animals developed a bald patch in a well defined area under the chin, which, however, was reversible.

About 30% of the animals died during this treatment and the first deaths occurred after 6 to 8 days.

When the tissues were removed, damage to the kidneys was often observed. The amount of fatty tissues appeared to be less than in rats subjected to the other treatments.

5.1.2 Physical parameters; food and water intake

As described above, the behavioural response and the appearance of the animals changed with time and with the different treatments. Differences in body weights were observed between experimental and control rats at the end of each treatment. Therefore, day to day changes in body weight and in food and water consumption were measured, to determine whether these parameters were related to the varying behavioural responses and appearances of the animals.

The following tables and figures summarize the results:

Table 2 Effect of treatments on body and organ weights

Table 3 Effect of treatments on food and water intake.

Fig.20 Effect of treatments on:

- A) normalized weight
- B) cumulative consumption of water
- C) cumulative consumption of food.

Fig.21 Effect of treatments on:

- A) day to day changes in weight
- B) daily consumption of water
- C) daily consumption of food.

In these figures the arrows indicate the days from which the doses of Sinemet and carbidopa were doubled.

The tables below the figures summarize the significant differences. The meaning of the various symbols and colours is as follows:

red symbols : differences which are highly significant ($p < 0.01$)
blue symbols: differences which are significant ($0.05 > p > 0.01$)
dots : significantly different from control
circles : significantly different from treatment with the inhibitor alone.

TABLE 2 -EFFECT OF TREATMENTS ON BODY AND ORGAN WEIGHTS-

TREATMENT	BODY WEIGHT (g)			ORGAN WEIGHTS** (g)			
	INITIAL	FINAL	%D*	BRAIN	% of whole body	LIVER	% of whole body
CONTROL	256 $\pm$ 24(12)	267 $\pm$ 20(12)	+4.5 $\pm$ 3.3	1.66 $\pm$ 0.15(12)	0.653 $\pm$ 0.038	8.63 $\pm$ 0.66(9)	3.241 $\pm$ 0.346
CARBIDOPA	269 $\pm$ 24(12)	283 $\pm$ 19(12)	+5.6 $\pm$ 2.9	1.81 $\pm$ 0.11(12)	0.641 $\pm$ 0.066	9.22 $\pm$ 0.70(8)	3.223 $\pm$ 0.270
SINEMET	261 $\pm$ 21(12)	246 $\pm$ 15(12) ^{b, d}	-5.4 $\pm$ 3.3 ^{a, b}	1.68 $\pm$ 0.14(12)	0.713 $\pm$ 0.066 ^f	8.33 $\pm$ 0.54(9)	3.485 $\pm$ 0.301 ^g
IPRONIAZID	261 $\pm$ 21(12)	255 $\pm$ 16(12)	-2.3 $\pm$ 4.0 ^d	1.75 $\pm$ 0.09(12)	0.690 $\pm$ 0.071	8.81 $\pm$ 0.43(8)	3.486 $\pm$ 0.439
L-DOPA+IPRONIAZID	256 $\pm$ 17(12)	237 $\pm$ 13(12) ^{a, c}	-7.4 $\pm$ 4.0 ^{a, c}	1.72 $\pm$ 0.13(12)	0.775 $\pm$ 0.070 ^{d, e}	9.11 $\pm$ 1.54(7)	3.858 $\pm$ 0.647

a) p<0.001 from CONTROL

e) p<0.01 from IPRONIAZID

b) p<0.001 from CARBIDOPA

f) p<0.02 from CONTROL

c) p<0.001 from IPRONIAZID

g) p<0.05 from CONTROL

d) p<0.01 from CONTROL

* Percentage difference in weight from start of experiment.

** The organs were usually from the same groups of animals reported under "body weight". Otherwise, the organs were obtained from animals matched for their initial body weight.

TABLE 3 -EFFECT OF TREATMENTS ON FOOD AND WATER INTAKE-

TREATMENT	FOOD CONSUMPTION (g)	%D*	WATER CONSUMPTION (ml)	%D*
CONTROL	266 $\pm$ 29 (12)	-	357 $\pm$ 33 (4)	-
CARBIDOPA	291 $\pm$ 9 (6)	+ 9.4	379 $\pm$ 31 (4)	+ 6.2
SINEMET	220 $\pm$ 12 (10) ^{a, b}	-17.3	405 $\pm$ 6 (4) ^d	+13.4
IPRONIAZID	251 $\pm$ 6 (6)	- 5.6	349 $\pm$ 16 (4)	- 2.2
L-DOPA+IPRONIAZID	205 $\pm$ 28 (16) ^{a, c}	-22.9	458 $\pm$ 27 (8) ^{a, e}	+28.3

a) $p < 0.001$ from CONTROLb) $p < 0.001$ from CARBIDOPAc) $p < 0.01$ from IPRONIAZIDd) $p < 0.05$ from CONTROLe) $p < 0.001$ from IPRONIAZID

* Percentage difference from control.

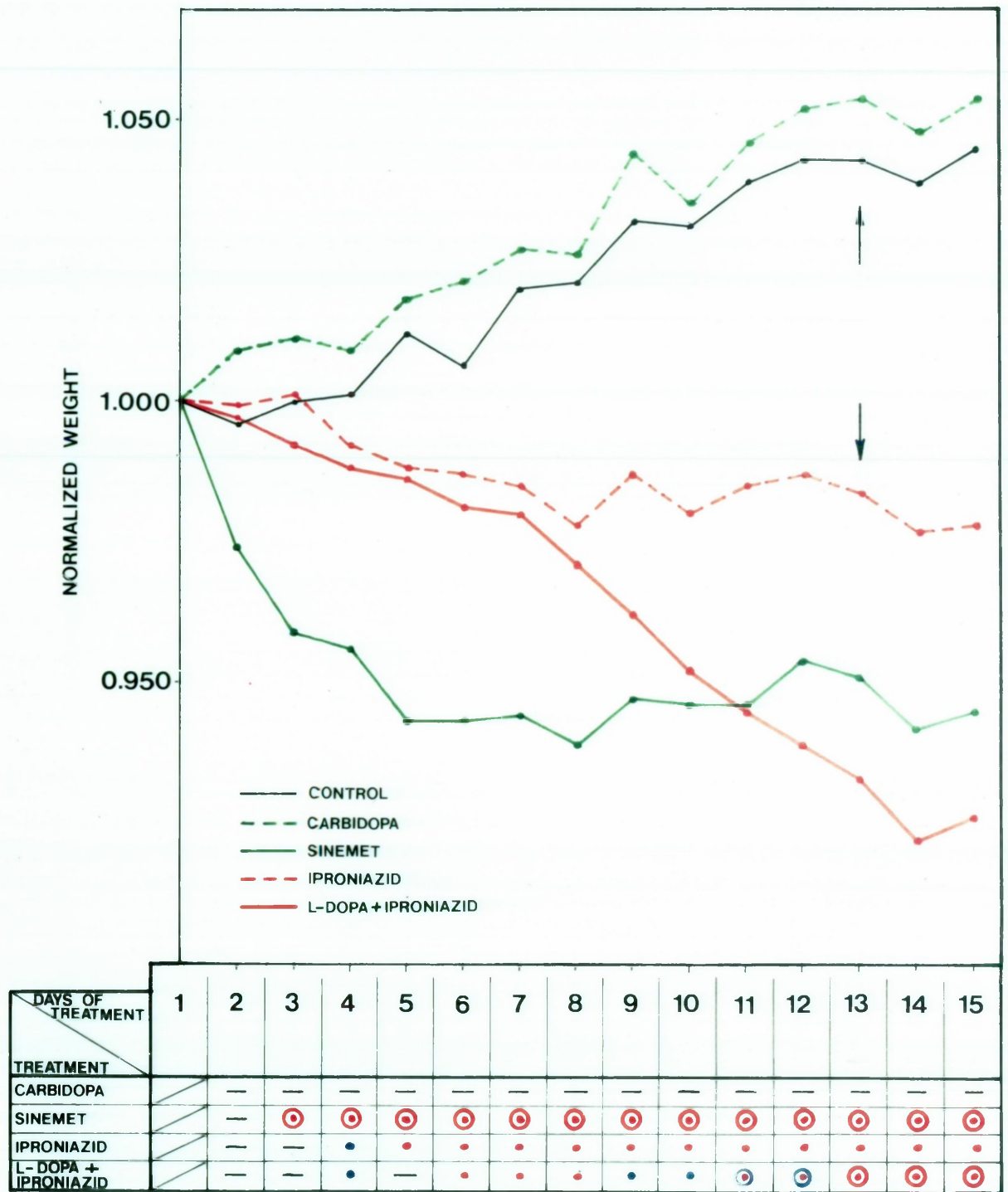


Fig.20A Effect of treatments on daily growth rate.

Each point is the mean of 12 determinations.

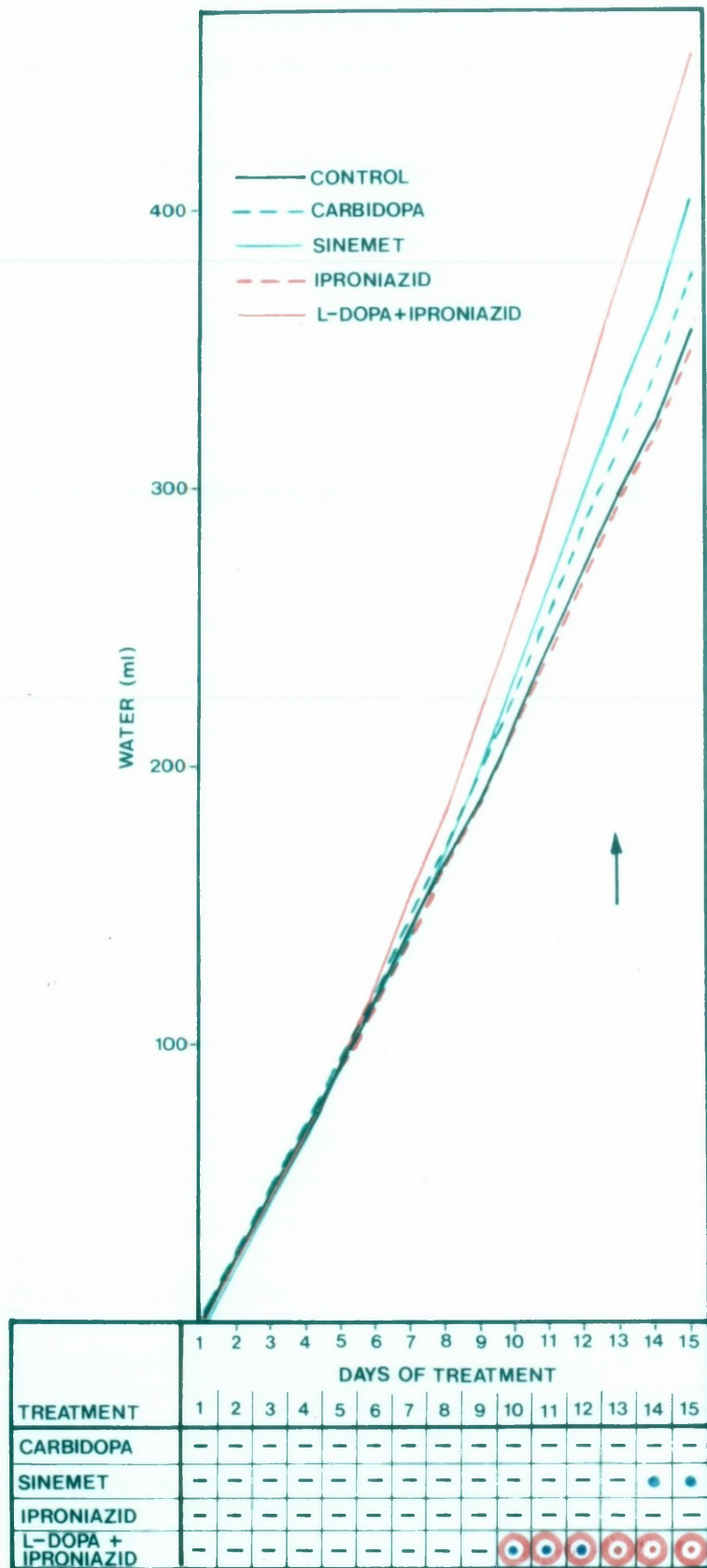


Fig.20B Effect of treatments on the cumulative consumption of water.

Each point is the mean of four to eight determinations.

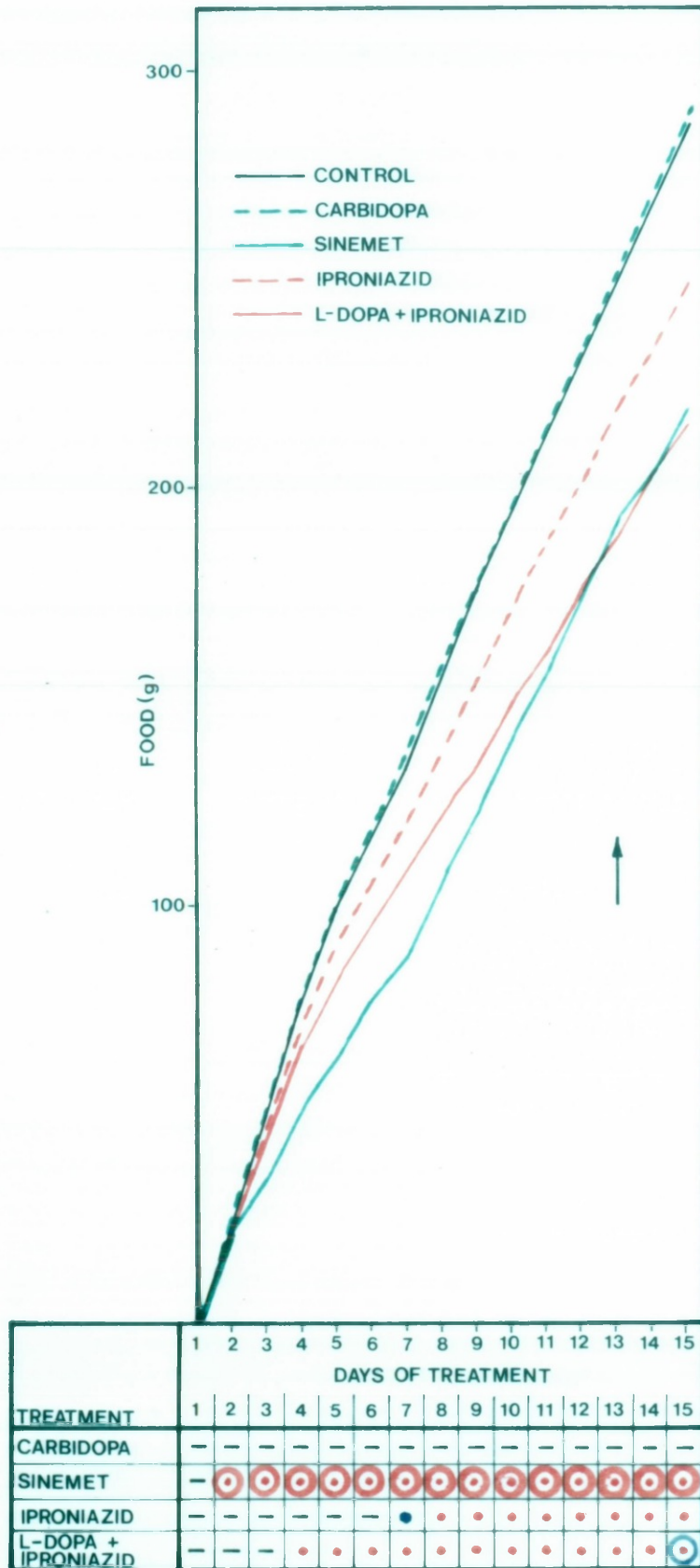


Fig.20C Effect of treatments on the cumulative consumption of food.

Each point is the mean of four to eight determinations.

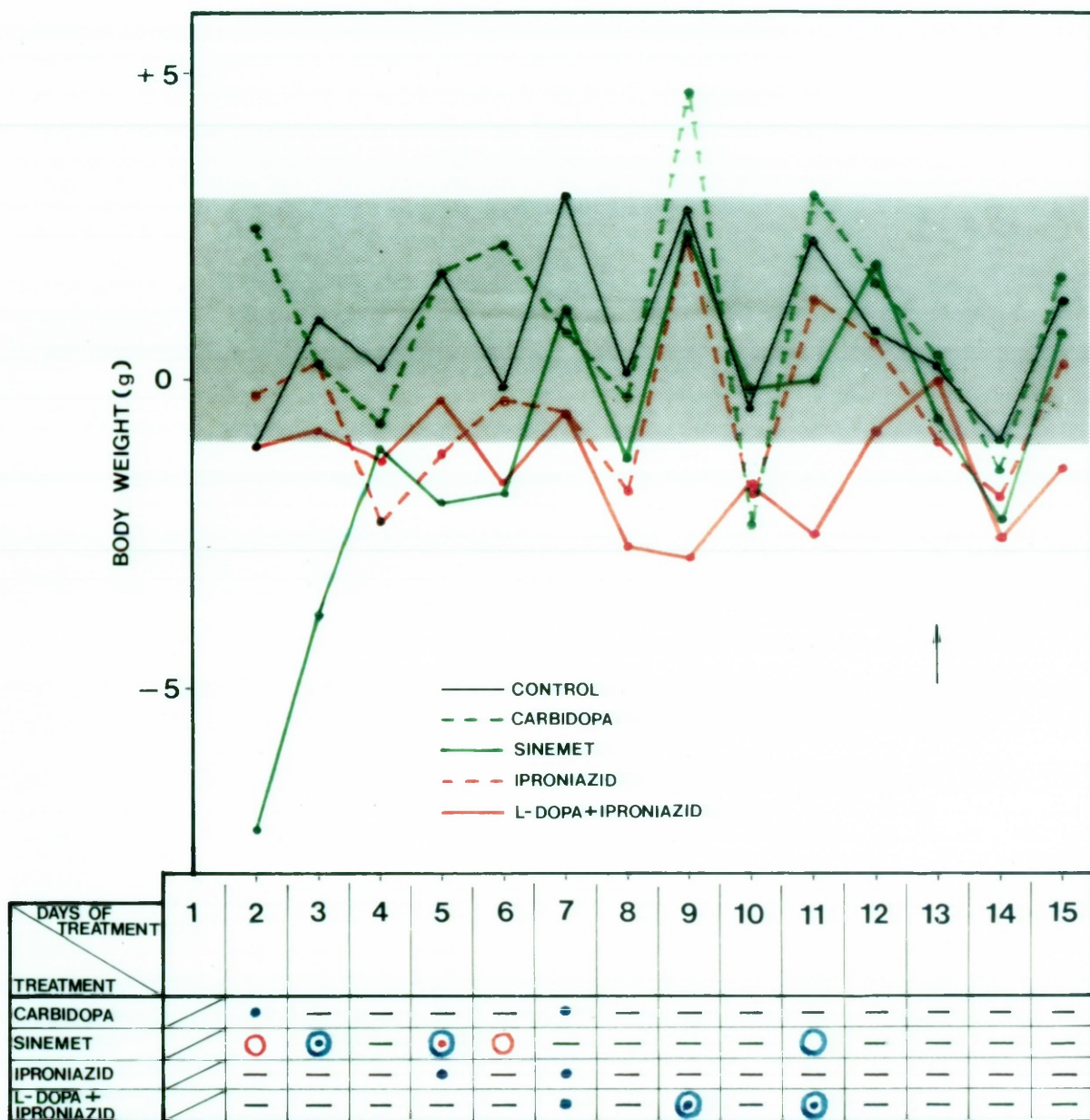


Fig.21A Effect of treatments on day to day changes in body weight.

Each point is the mean of 12 to 18 determinations. For the purpose of clarity, the shaded area represents variations in day to day weight which are assumed to be within normal limits.

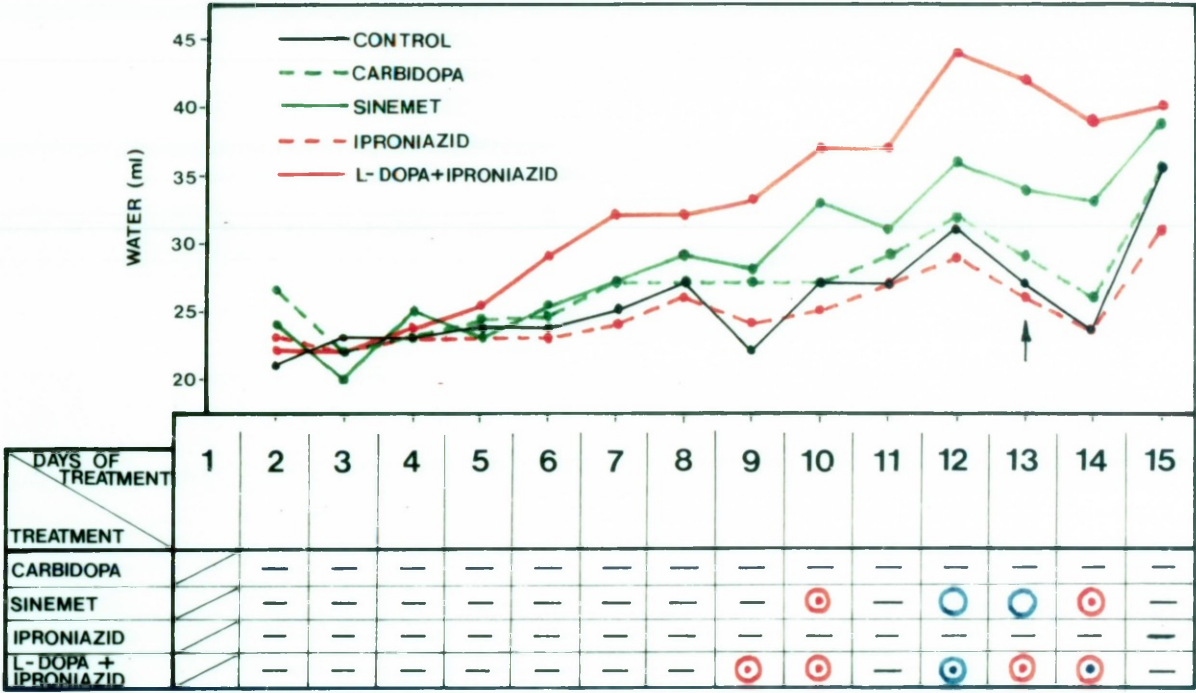


Fig.21B Effect of treatments on daily water consumption.

Each point in the mean of six to eight determinations.

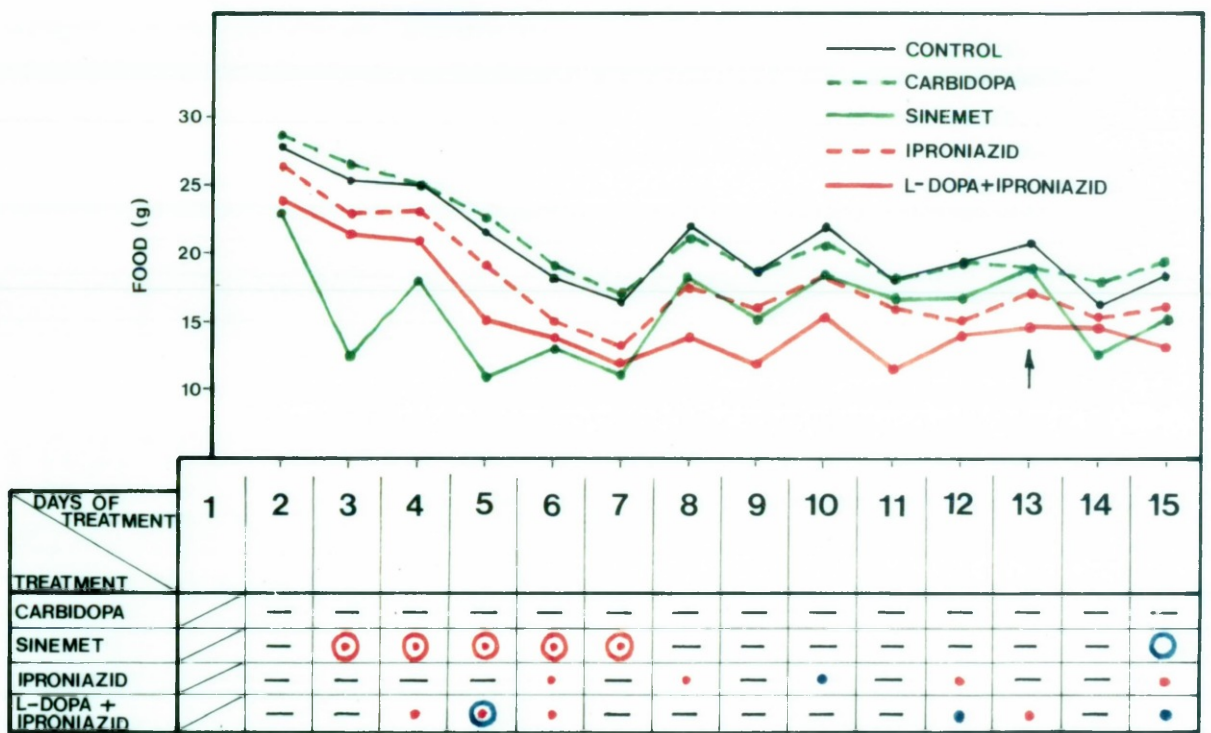


Fig.21C Effect of treatments on daily food consumption.

Each point in the mean of six to eight determinations.

5.1.3 Comments

All the animals appeared to change their drinking and eating habits during the course of the treatments. The amount of food consumed declined each day in the first week (Fig.21 C), whereas the amount of water drunk daily was constant in the first week, after which it consistently increased (Fig.21 B). These changes, which were superimposed on the effects of the different drugs, were reflected in the cumulative consumption of food and water; after about one week there was an overall increase in water consumption and an overall decrease in food consumption in all groups of animals (Fig.20 B and C). These observations may be partly interpreted as an adaptation of the animals to their new environment; for example, it was observed that animals caged individually ate more than animals sharing the same cage. Moreover the effects of daily handling of the rats cannot be discounted.

The effect of carbidopa treatment on behaviour and appearance was negligible; however, the final weight of these rats was very often slightly higher than that of control rats.

Sinemet treatment significantly reduced the weight of the animals, although the weight of liver and brain were unaffected (Table 2). The ratios of organ to body weight significantly increased (Table 2), suggesting that the weight loss was not due to a generalized phenomenon affecting the whole body, but that it was more specific for some particular tissue. This is supported by two observations:

- i) The normalized weight sharply dropped in the first few days, so that after about four days the weight loss was equivalent to that observed at the end of the treatment (Fig.20 A).
- ii) On a daily basis the animals lost weight only on the 2nd, 3rd, 5th and 6th day but not later on (Fig.21 A). The doubling of the Sinemet dose did not cause a sharp decrease in body weight as seen at the beginning of the treatment.

These weight changes were concomitant with a diminished food consumption, and an increased water intake (Table 3, Fig.20 B & C). On a day to day basis, variations in food consumption reflected changes in body weight (Fig.21 A & C), whereas water intake seemed to increase only in the second week of treatment (Fig.21 B).

The response of rats to Sinemet treatment can be divided into different phases, as summarized in Fig.22.

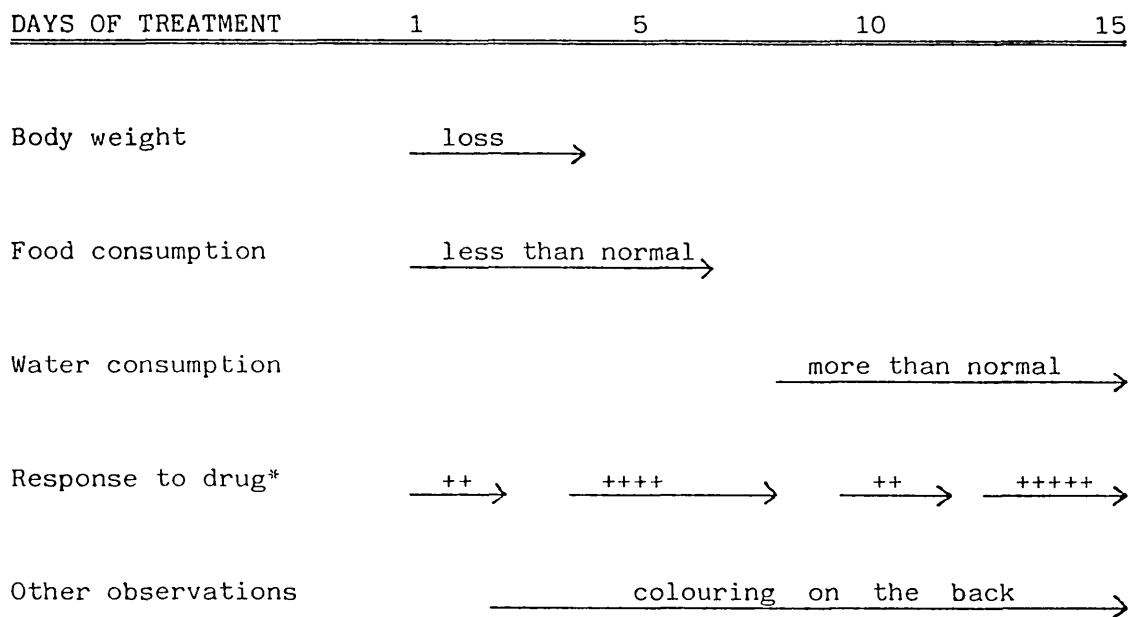


Fig.22 Summary of the changes observed in Sinemet treated rats over a period of 15 days.

* 0 = no response; +++++ = maximum response.

The loss of weight observed in the first few days can be attributed to the decreased ingestion of food. The effect of L-dopa on appetite has been reported (195,197), and it is in keeping with the observation that the Sinemet treated animals consistently ate less than either control or carbidopa treated rats throughout the treatment (Fig.21C). The hyperthermic effect of L-dopa (198), may account for the increased consumption of water.

The changes in response, observed during the 15 days of treatment, can be attributed to stimulation of different peripheral mechanisms which decrease the availability of L-dopa to the CNS (100,115,199), to metabolic adjustments within the CNS (200), and to changes in dopamine receptors, which have been reported after 11 days of Sinemet treatment (201).

Treatment with iproniazid alone induced a progressive loss in body weight (Fig.20 A). These animals consistently ate less than normal throughout the treatment, with significant differences in the daily food intake starting to occur after about one week (Fig.21 C). At the end of the treatment, their total food intake was not different from normal, if animals from different batches are compared (Tab. 3). However, if controls and iproniazid treated rats from the same batch are compared, the difference is highly significant (Fig.20 C). This discrepancy is presumably a reflection of the initial difficulties encountered in the treatment of these animals, as described above (see 4.1).

Joint treatment with iproniazid and L-dopa significantly reduced the body weight while liver and brain weights were unaffected (Table 2). The ratio of brain to body weight is higher than normal, suggesting that the weight of this organ was not affected as other tissues. These animals ate less than normal, but they drank significantly larger quantities of water (Tab. 3). The loss in body weight, as well as the changes in food and water consumption, appear to be biphasic:

- i) in the first week the pattern was the same as for iproniazid treated rats (Fig.20 A, B & C); body weight and water intake were, on a daily basis, similar to control or iproniazid treated rats (Fig.21 A & B), but food consumption was significantly decreased;

ii) after about one week the normalized weight steadily declined (Fig.20 A) and the consumption of food and water became increasingly different from those of control rats or of rats treated with iproniazid alone (Fig.20 B & C). On a daily basis, the changes in body weight were significantly lower only on the 9th and 11th day (Fig.21 A), whereas the water consumption appeared to steadily increase around the end of the treatment (Fig.21B).

The above observations are summarized in Fig.23.

DAYS OF TREATMENT	1	5	10	15
Body weight	slight loss		marked loss	
Food consumption	markedly less than normal		less than normal	
Water consumption	progressively more than normal			
Response to drug	constant			
Other observations	occurrence of death			
	loss of hair			

Fig.23 Summary of the changes observed in L-dopa and iproniazid treated rats over a period of 15 days.

The decrease in body weight observed throughout this treatment can be attributed to a lower food consumption. The changes in normalized weight (Fig.20 A) and in the daily intake of food (Fig.21 C) observed in the first week are not different among animals treated with iproniazid alone or with iproniazid plus L-dopa. Thus, it seems that in the first few days, the additional injection of L-dopa had no effect on these parameters. However, after about one week marked changes appeared between the two groups of animals: the general appearance deteriorated and the first deaths occurred in the iproniazid plus L-dopa treated rats.

As iproniazid is an irreversible inhibitor of monoamine oxidase and this enzyme is not induced in the brain by either chronic inhibition (119) nor chronic L-dopa treatment (120), it is likely that the consistently high levels of monoamines in the brain (and also in the periphery) may be responsible for the toxic effects of this treatment. In this context, it is of interest that the first deaths occur after about one week, and that the half life of monoamine oxidase in the brain is about 7 to 10 days (119).

The intensity of response to the drugs appeared to be constant throughout the 15 days of treatment. As for Sinemet treated rats, the increased drinking habit of these animals can be attributed to the hyperthermic effect of L-dopa (119).

5.2 BIOCHEMICALS AND HAEMATOLOGICAL PARAMETERS

Some preliminary experiments were carried out on the tissues used in this study. Tissue protein concentrations and haematological parameters are shown because some of these data are used in later calculations. Blood glucose, which is the main source of energy for the brain, was also measured.

5.2.1 Results

The results are summarized in the following tables:

Table 4 Effect of treatments on tissue protein concentrations

Table 5 Effect of treatments on haematological parameters

Table 6 Effect of treatments on blood glucose and liver glycogen
 concentrations, and liver phosphorylase activity.

TABLE 4 -EFFECT OF TREATMENTS ON TISSUE PROTEIN CONCENTRATIONS-

TREATMENT	PLASMA (mg/ml)	LIVER (mg/g)	BRAIN (mg/g)
CONTROL	68.2 $\pm$ 1.8 (12)	201 $\pm$ 16 (8)	98.3 $\pm$ 2.4 (4)
CARBIDOPA	66.9 $\pm$ 3.3 (8)	199 $\pm$ 17 (8)	95.4 $\pm$ 3.5 (4)
SINEMET	69.7 $\pm$ 4.6 (7)	194 $\pm$ 12 (7)	99.4 $\pm$ 1.4 (4)
IPRONIAZID	65.6 $\pm$ 4.7 (8)	200 $\pm$ 20 (8)	96.0 $\pm$ 1.8 (4)
L-DOPA + IPRONIAZID	64.6 $\pm$ 4.0 (10) ^a	183 $\pm$ 15 (9) ^b	95.4 $\pm$ 5.8 (4)

a) $p < 0.02$ from CONTROL

b) $p < 0.05$ from CONTROL

TABLE 5 -EFFECT OF TREATMENTS ON HAEMATOLOGICAL PARAMETERS-

TREATMENT	WHITE BLOOD CELLS ($\times 10^9/L$)	RED BLOOD CELLS ($\times 10^{12}/L$)	HAEMOGLOBIN (g/dl)	HAEMATOCRIT	MEAN CELL VOLUME (fl)	MEAN CELL HAEMOGLOBIN (pg)	MEAN CELL HAEMOGLOBIN CONC. (g/dl)
CONTROL	4.40 $\pm$ 0.70 (9)	8.01 $\pm$ 0.23(10)	15.80 $\pm$ 0.50(10)	0.418 $\pm$ 0.014(10)	52.3 $\pm$ 1.6(10)	19.7 $\pm$ 0.4(10)	37.9 $\pm$ 0.6(10)
CARBIDOPA	4.20 $\pm$ 0.42 (4)	7.84 $\pm$ 0.08 (4)	15.78 $\pm$ 0.31 (4)	0.412 $\pm$ 0.007 (4)	52.5 $\pm$ 0.6 (4)	20.0 $\pm$ 0.3 (4)	38.3 $\pm$ 0.4 (4)
SINEMET	3.70 $\pm$ 1.20(11)	7.38 $\pm$ 0.47(12) ^b	14.46 $\pm$ 0.56(12) ^{d, f}	0.382 $\pm$ 0.014(12) ^{d, g}	52.3 $\pm$ 3.4(12)	19.7 $\pm$ 1.0(12)	38.0 $\pm$ 0.7(12)
IPRONIAZID	5.53 $\pm$ 0.95 (4) ^a	7.52 $\pm$ 0.26 (4) ^b	14.63 $\pm$ 0.38 (4) ^b	0.392 $\pm$ 0.017 (4) ^e	51.8 $\pm$ 0.5 (4)	19.4 $\pm$ 0.4 (4)	37.5 $\pm$ 1.1 (4)
L-DOPA + IPRONIAZID	4.00 $\pm$ 2.00(11)	7.45 $\pm$ 0.57(12) ^c	14.36 $\pm$ 1.15(12) ^b	0.383 $\pm$ 0.028(12) ^b	51.3 $\pm$ 1.0(12)	19.2 $\pm$ 0.2(12) ^a	37.6 $\pm$ 0.5(12)

a) $p < 0.05$ from CONTROLb) $p < 0.005$ from CONTROLc) $p < 0.01$ from CONTROLd) $p < 0.001$ from CONTROLe) $p < 0.02$ from CONTROLf) $p < 0.001$ from CARBIDOPAg) $p < 0.005$ from CARBIDOPA

TABLE 6 -EFFECT OF TREATMENTS ON BLOOD GLUCOSE AND LIVER GLYCOGEN CONCENTRATIONS,
AND LIVER PHOSPHORYLASE ACTIVITY-

TREATMENT	BLOOD GLUCOSE (mM)	LIVER GLYCOGEN (mM glucose kg ⁻¹)	LIVER PHOSPHORYLASE ACTIVITY *		%D**
			-AMP	+AMP	
CONTROL	6.26±0.95 (7)	203±41(10)	314±16 (4)	427±93(4)	36
CARBIDOPA	7.01±0.35 (4)	211±25 (4)	291±62 (4)	385±77(4)	32
SINEMET	6.70±0.55(11)	294±96 (6) ^a	343±49 (4)	505±80(4)	47
IPRONIAZID	6.79±0.38 (4)	250±27 (4)	263±101(4)	330±95(4)	25
L-DOPA + IPRONIAZID	8.83±2.26(10) ^a	101±48 (7) ^{b, d}	265±34 (4) ^c	404±66(4)	53

a) p<0.02 from CONTROL

c) p<0.05 from CONTROL

b) p<0.001 from CONTROL

d) p<0.001 from IPRONIAZID

* Phosphorylase activities are expressed in μ mole Pi/hr/100 mg of protein;
determinations were carried out in duplicate.

** Percentage stimulation by addition of AMP to incubation mixture.

5.2.2 Comments

Tissue protein concentrations were largely unaffected by the treatments. The decreased hepatic protein concentration (- 9%) of rats treated with L-dopa plus iproniazid (Tab. 4), is presumably of no great significance as the liver of these animals was slightly larger (+ 5,6%) than normal (Tab. 2).

The RBC count, the haemoglobin concentration and the haematocrit were lower than control values in rats treated with L-dopa plus either inhibitors and in rats treated with iproniazid alone. The mean cell volume and the mean cell haemoglobin were not markedly affected by these treatments, therefore the changes in haemoglobin concentration and haematocrit were presumably due to a decreased RBC count. This is in keeping with the observation that L-dopa binds to erythrocyte proteins in vivo (46), a property which may contribute to cellular breakdown. However, in rats treated with L-dopa plus iproniazid, the slight decreases in mean cell haemoglobin and in plasma proteins (Tab. 4), suggest that the treatment interferes with the homeostasis of blood proteins.

The increase in blood glucose, following treatment with L-dopa and a monoamine oxidase inhibitor, has been reported in another work (202) and may be linked to the release of glucagon induced by L-dopa treatment (203). Chronic treatment with L-dopa and a peripheral decarboxylase inhibitor does not have any effect on blood glucose concentration, an observation reported also after an acute study (118).

The decrease in liver glycogen observed after treatment with L-dopa plus iproniazid is presumably linked to hormonal changes occurring at high levels of monoamines (203). It is of interest that liver glycogen is significantly increased by Sinemet treatment. In rats treated with carbidopa or Sinemet, the activities of glycogen phosphorylase, a PLP dependent enzyme, is not below control levels (Tab. 6), suggesting that the increase in glycogen, observed in the liver of Sinemet treated rats, is not due a block in its utilization. This phenomenon might then be of a metabolic nature.

5.3 FREE AMINO ACID POOLS IN BRAIN, LIVER AND BLOOD PLASMA

One of the main catabolic routes of L-dopa and catecholamines is via O-methylation of the catechol ring.

Chronic treatment with carbidopa alone decreases pyridoxal phosphate concentration in the brain and peripheral tissues (82); the relationship between this vitamer and the remethylating process has been described in detail (see 2.4.2). Chronic treatment with an irreversible inhibitor of monoamine oxidase completely inhibits brain oxidative deamination (119), the major catabolic route of monoamines; thus O-methylation of catecholamines should be enhanced. Therefore, chronic treatment with L-dopa in conjunction with either inhibitor should severely strain the remethylating cycle. The major purpose of the experiments described below was to study the effect of treatments on the concentrations of methionine and of the amino acids metabolically related to methionine (Fig.11).

Interpretation of the amino acid changes that occur in the brain and periphery following treatment with L-dopa and either the monoamine oxidase or the peripheral decarboxylase inhibitors is complicated by several factors. Firstly, L-dopa renders brain polysomes more susceptible to disaggregation; this should result in the accumulation of at least some amino acids as protein synthesis is inhibited (204). Secondly, brain amino acids are believed to exist in many distinct pools with different metabolic roles (205); thus, a change in the concentration of a particular amino acid may reflect a change in only one of the many aspects of its metabolism, in distinct regions of the CNS (206). Thirdly, some amino acids are neurotransmitters but they also take part in the general metabolism and thus changes in their concentrations may reflect cumulative causes.

Furthermore, in peripheral tissues, the endocrine changes induced by L-dopa, such as the increase in the plasma levels of insulin and glucagon (203) may also affect the free amino acid pools; the former hormone stimulates the uptake of amino acids by tissues (207), the latter their release from muscles (230).

For all these reasons, any attempt to interpret individual variations in amino acid concentration is difficult. Thus, as it was done in other studies (177,209), we have attempted to draw general conclusions from all the data.

5.3.1 Results

The results are summarized in the following tables:

Table 7 Effect of treatments on brain free amino compounds.

Table 8 Significant differences in brain free amino compounds due to carbidopa and Sinemet treatments.

Table 9 Significant differences in brain free amino compounds due to iproniazid and L-dopa plus iproniazid treatments.

Table 10 Effect of treatments on blood plasma free amino compounds.

Table 11 Significant differences in blood plasma free amino compounds due to carbidopa and Sinemet treatments.

Table 12 Significant differences in blood plasma free amino compounds due to iproniazid and L-dopa plus iproniazid treatments.

Table 13 Effect of treatments on liver free amino compounds.

Table 14 Significant differences in liver free amino compounds
due to carbidopa and Sinemet treatments.

Table 15 Significant differences in liver free amino compounds
due to iproniazid and L-dopa plus iproniazid
treatments.

In the tables the following nomenclature is used:

N.E. not estimated

N.R. not resolved

N.D. not determined

TRACE detected but below the limit of measurability (about 5 nmole/g
of tissues under optimum conditions).

TABLE 7 -EFFECT OF TREATMENTS ON THE BRAIN FREE AMINO COMPOUNDS-

AMINO COMPOUND	CONTROL*	CARBIDOPA*	SINEMET*	IPRONIAZID*	L-DOPA+IPRONIAZID*
PHOSPHOETHANOLAMINE	1098 $\pm$ 109 (7)	1077 $\pm$ 217 (7)	1029 $\pm$ 96 (8)	1105 $\pm$ 184 (6)	686 $\pm$ 119 (7)
TAURINE	3486 $\pm$ 109 (7)	3873 $\pm$ 925 (7)	3259 $\pm$ 307 (6)	3846 $\pm$ 392 (6)	2998 $\pm$ 370 (7)
UREA	4114 $\pm$ 519 (7)	2679 $\pm$ 578 (7)	3924 $\pm$ 452 (7)	2577 $\pm$ 478 (6)	3010 $\pm$ 636 (7)
ASPARTIC ACID	1897 $\pm$ 144 (5)	2360 $\pm$ 502 (6)	2361 $\pm$ 402 (5)	2322 $\pm$ 382 (7)	2145 $\pm$ 165 (7)
THREONINE	572 $\pm$ 57 (4)	670 $\pm$ 77 (5)	551 $\pm$ 91 (5)	711 $\pm$ 79 (6)	548 $\pm$ 43 (7)
SERINE	778 $\pm$ 105 (7)	929 $\pm$ 127 (6)	664 $\pm$ 63 (7)	987 $\pm$ 142 (7)	888 $\pm$ 38 (7)
GLUTAMINE	6403 $\pm$ 713 (7)	7159 $\pm$ 586 (7)	6262 $\pm$ 816 (8)	6602 $\pm$ 464 (7)	4646 $\pm$ 1064 (7)
PROLINE	60.7 $\pm$ 9.9 (4)	N.R.	77.3 $\pm$ 19.8 (7)	N.R.	101 $\pm$ 20 (4)
GLUTAMIC ACID	7751 $\pm$ 727 (7)	8554 $\pm$ 605 (7)	7035 $\pm$ 196 (8)	8119 $\pm$ 710 (7)	6752 $\pm$ 565 (7)
GLYCINE	909 $\pm$ 65 (7)	1203 $\pm$ 122 (7)	905 $\pm$ 67 (8)	1131 $\pm$ 168 (6)	975 $\pm$ 52 (7)
ALANINE	536 $\pm$ 50 (7)	442 $\pm$ 57 (7)	703 $\pm$ 76 (8)	435 $\pm$ 56 (6)	596 $\pm$ 71 (7)
α -AMINOADIPIC ACID	TRACE	TRACE	TRACE	TRACE	TRACE
α -AMINO BUTYRIC ACID	TRACE	TRACE	TRACE	TRACE	TRACE
VALINE	69.0 $\pm$ 8.3 (7)	80.1 $\pm$ 8.1 (6)	50.3 $\pm$ 15.9 (6)	79.4 $\pm$ 12.2 (7)	101 $\pm$ 14 (7)
CYSTATHIONINE	65.5 $\pm$ 8.1 (6)	78.7 $\pm$ 10.6 (6)	96.5 $\pm$ 25.8 (7)	74.7 $\pm$ 8.2 (6)	38.9 $\pm$ 17.6 (7)
METHIONINE	43.5 $\pm$ 14.1 (3)	44.4 $\pm$ 14.7 (4)	32.4 $\pm$ 10.2 (4)	50.5 $\pm$ 11.9 (4)	50.7 $\pm$ 13 (7)
ISOLEUCINE	34.3 $\pm$ 6.1 (7)	35.2 $\pm$ 5.1 (6)	21.4 $\pm$ 2.6 (6)	34.8 $\pm$ 5.6 (6)	48.7 $\pm$ 8.3 (7)
LEUCINE	83.2 $\pm$ 11.1 (7)	80.3 $\pm$ 7.5 (6)	49.1 $\pm$ 4.1 (6)	73.6 $\pm$ 7.9 (6)	99.5 $\pm$ 20.9 (7)
TYROSINE	81.1 $\pm$ 9.0 (6)	254 $\pm$ 31 (7)	109 $\pm$ 18 (5)	104 $\pm$ 7 (6)	79.7 $\pm$ 16.1 (7)
PHENYLALANINE	55.1 $\pm$ 3.6 (6)	54.3 $\pm$ 4.8 (7)	N.R.	51.3 $\pm$ 8.8 (6)	66.7 $\pm$ 5.9 (6)
β -ALANINE	43.6 $\pm$ 3.9 (5)	N.R.	48.5 $\pm$ 10.2 (4)	N.R.	N.R.
γ -AMINO BUTYRIC ACID	2374 $\pm$ 247 (5)	2610 $\pm$ 162 (5)	2344 $\pm$ 320 (5)	2488 $\pm$ 191 (5)	3219 $\pm$ 203 (5)
ORNITHINE	48.8 $\pm$ 19.7 (5)	28.3 $\pm$ 4.6 (4)	27.8 $\pm$ 2.8 (4)	22.7 $\pm$ 3.0 (3)	33.4 $\pm$ 10.4 (4)
LYSINE	271 $\pm$ 23 (5)	256 $\pm$ 25 (5)	202 $\pm$ 28 (5)	280 $\pm$ 18 (5)	267 $\pm$ 25 (5)
1-METHYLHISTIDINE	TRACE	TRACE	TRACE	TRACE	TRACE
HISTIDINE	68.7 $\pm$ 14.8 (5)	71.7 $\pm$ 7.1 (5)	38.0 $\pm$ 8.5 (5)	74.8 $\pm$ 8.3 (5)	56.1 $\pm$ 13 (3)
L-DOPA	N.D.	N.D.	434 $\pm$ 71 (5)	N.D.	TRACE

* Results are expressed in nmol/g frozen tissue.

TABLE 8 -SIGNIFICANT DIFFERENCES IN BRAIN FREE AMINO COMPOUNDS
DUE TO CARBIDOPA AND SINEMET TREATMENTS-

AMINO COMPOUND	% DIFFERENCE FROM CONTROL RATS*		% DIFFERENCE FROM CARBIDOPA TREATED RATS *
	CARBIDOPA	SINEMET	SINEMET
PHOSPHOETHANOLAMINE			
TAURINE			
UREA			
ASPARTIC ACID	24	24	
THREONINE			
SERINE			-29 ^c
GLUTAMINE			
PROLINE		27	
GLUTAMIC ACID			-18 ^c
GLYCINE	32 ^c		-25 ^c
ALANINE	-18 ^a	31 ^c	59 ^c
d-AMINOADIPIC ACID			
d-AMINOBUTYRIC ACID			
VALINE		-27	-37 ^b
CYSTATHIONINE	20	47	23
METHIONINE		-26	-27
ISOLEUCINE		-38 ^c	-39 ^c
LEUCINE		-41 ^c	-39 ^c
TYROSINE	213 ^c	34 ^a	-57 ^c
PHENYLALANINE			
B-ALANINE			
γ-AMINOBUTYRIC ACID			
ORNITHINE	-42	-43	
LYSINE		-25 ^b	-21
1-METHYLHISTIDINE			
HISTIDINE		-45 ^b	-47 ^c

- a) $p < 0.01$
b) $p < 0.005$
c) $p < 0.001$

* Percentage differences are listed if greater or equal to ($\pm$) 20%
or if significant.

TABLE 9 -SIGNIFICANT DIFFERENCES IN BRAIN FREE AMINO COMPOUNDS
DUE TO IPRONIAZID AND L-DOPA PLUS IPRONIAZID TREATMENTS-

AMINO COMPOUND	% DIFFERENCE FROM CONTROL RATS*		% DIFFERENCE FROM IPRONIAZID TREATED RATS*
	IPRONIAZID	L-DOPA + IPRONIAZID	L-DOPA + IPRONIAZID
PHOSPHOETHANOLAMINE		-38 ^c	-38 ^c
TAURINE			-22 ^b
UREA			
ASPARTIC ACID	22		
THREONINE	24 ^a		-23 ^c
SERINE	27 ^a		
GLUTAMINE		-27 ^b	-30 ^c
PROLINE		66	
GLUTAMIC ACID			-17 ^c
GLYCINE	24 ^a		
ALANINE	-19 ^a		37 ^c
α -AMINOADIPIC ACID			
α -AMINOBTYRIC ACID			
VALINE		46 ^c	27
CYSTATHIONINE		-41 ^a	-48 ^c
METHIONINE			
ISOLEUCINE		42 ^b	40 ^a
LEUCINE		20	35
TYROSINE	28 ^c		-23 ^a
PHENYLALANINE		21 ^b	30 ^a
β -ALANINE			
γ -AMINOBTYRIC ACID		36 ^c	29 ^c
ORNITHINE	-53	-32	
LYSINE			
1-METHYLHISTIDINE			
HISTIDINE			-25

- a) $p < 0.01$
b) $p < 0.005$
c) $p < 0.001$

* Percentage differences are listed if greater or equal to (+)20% or if significant.

TABLE 10 -EFFECT OF TREATMENTS ON THE BLOOD PLASMA FREE AMINO COMPOUNDS-

AMINO COMPOUND	CONTROL*	CARBIDOPA*	SINEMET*	IPRONIAZID*	L-DOPA+IPRONIAZID*
PHOSPHOETHANOLAMINE	4.8 $\pm$ 2.0 (5)	7.7 $\pm$ 0.9 (4)	16.4 $\pm$ 7.9 (6)	4.8 (2)	32.4 $\pm$ 20.6 (7)
TAURINE	141 $\pm$ 54 (6)	173 $\pm$ 25 (6)	146 $\pm$ 16 (4)	206 $\pm$ 50 (5)	395 $\pm$ 164 (7)
UREA	3671 $\pm$ 1359(6)	4405 $\pm$ 720 (6)	6519 $\pm$ 1168(7)	4934 $\pm$ 1102(5)	38846 $\pm$ 26076(7)
ASPARTIC ACID	9.5 $\pm$ 1.5 (5)	11.1 $\pm$ 1.6 (5)	13.6 $\pm$ 5.5 (6)	7.6 $\pm$ 1.7 (4)	17 $\pm$ 6.4 (6)
THREONINE	185 $\pm$ 33 (6)	181 $\pm$ 20 (6)	207 $\pm$ 13 (6)	184 $\pm$ 27 (5)	149 $\pm$ 30 (7)
SERINE	191 $\pm$ 39 (6)	163 $\pm$ 19 (6)	190 $\pm$ 22 (6)	183 $\pm$ 33 (5)	157 $\pm$ 47 (6)
GLUTAMINE	634 $\pm$ 130 (6)	620 $\pm$ 66 (5)	601 $\pm$ 119 (6)	581 $\pm$ 85 (5)	607 $\pm$ 161 (7)
PROLINE	120 $\pm$ 15 (5)	87.1 $\pm$ 25.3(6)	87.4 $\pm$ 8.8 (6)	80.8 $\pm$ 5.8 (4)	93.3 $\pm$ 30.2 (6)
GLUTAMIC ACID	120 $\pm$ 34 (6)	141 $\pm$ 20 (6)	87.8 $\pm$ 16.8(7)	132 $\pm$ 13 (5)	142 $\pm$ 38 (6)
CITRULLINE	72.3 $\pm$ 10.6(6)	49.4 $\pm$ 7.2 (6)	81.9 $\pm$ 16.3(6)	45.9 $\pm$ 6.1 (5)	75.2 $\pm$ 17.2 (7)
GLYCINE	216 $\pm$ 36 (6)	265 $\pm$ 23 (6)	351 $\pm$ 47 (6)	146 $\pm$ 16 (5)	192 $\pm$ 44 (7)
ALANINE	175 $\pm$ 16 (6)	97.6 $\pm$ 12.9(5)	204 $\pm$ 44 (7)	130 $\pm$ 13 (5)	210 $\pm$ 41 (7)
α -AMINOADIPIC ACID	TRACE	TRACE	TRACE	TRACE	19.6 $\pm$ 5.7 (5)
α -AMINO BUTYRIC ACID	TRACE	TRACE	TRACE	TRACE	17.8 $\pm$ 3.3 (5)
VALINE	150 $\pm$ 14 (6)	129 $\pm$ 13 (6)	181 $\pm$ 28 (7)	150 $\pm$ 37 (5)	157 $\pm$ 29 (7)
HALF-CYSTINE	60.0 $\pm$ 13.6(4)	41.7 $\pm$ 6.2 (5)	57.1 $\pm$ 11.9(6)	N.R.	25.2 $\pm$ 7.5 (4)
CYSTATHIONINE	N.D.	N.D.	15.9 $\pm$ 1.9 (4)	N.D.	10.1 $\pm$ 2.6 (4)
METHIONINE	41.1 $\pm$ 4.2 (6)	30.1 $\pm$ 4.9 (6)	29.3 $\pm$ 6.8 (4)	31 $\pm$ 5.5 (5)	28.7 $\pm$ 9.3 (6)
ISOLEUCINE	74.5 $\pm$ 7.9 (6)	68.6 $\pm$ 7.8 (6)	89.1 $\pm$ 15.7(7)	75.2 $\pm$ 13 (5)	78.7 $\pm$ 19.4 (7)
LEUCINE	138 $\pm$ 15 (6)	122 $\pm$ 12 (6)	143 $\pm$ 19 (6)	134 $\pm$ 21 (5)	134 $\pm$ 34 (7)
TYROSINE	87.8 $\pm$ 7.1 (6)	176 $\pm$ 17 (6)	174 $\pm$ 75 (6)	61.2 $\pm$ 6.1 (5)	40.6 $\pm$ 10.6 (6)
PHENYLALANINE	68.3 $\pm$ 8.9 (6)	51.7 $\pm$ 7.7 (6)	N.R.	53.0 $\pm$ 8.2 (5)	56.9 $\pm$ 10.9 (6)
β -ALANINE	N.D.	N.D.	N.D.	N.D.	14.4 $\pm$ 4.7 (4)
ORNITHINE	61.7 $\pm$ 15.5(5)	78.5 $\pm$ 20.3(5)	80.7 $\pm$ 15 (5)	79.8 $\pm$ 11.5(3)	105 $\pm$ 16 (4)
LYSINE	400 $\pm$ 96 (5)	330 $\pm$ 26 (5)	246 $\pm$ 41 (4)	421 $\pm$ 57 (3)	332 $\pm$ 45 (4)
1-METHYLHISTIDINE	12.4 $\pm$ 5.7 (3)	9.1 (2)	17.6 (2)	5.9 $\pm$ 1.9 (3)	10.3 (2)
HISTIDINE	59.3 $\pm$ 11.8(4)	69.2 $\pm$ 6.1 (5)	50.3 $\pm$ 14.5(5)	68.4 $\pm$ 12.1(3)	58.8 $\pm$ 5.6 (4)
L-DOPA	N.D.	N.D.	1313 $\pm$ 210 (5)	N.D.	17.7 $\pm$ 14.3 (6)

* Results are expressed in nmol/ml

TABLE 11 -SIGNIFICANT DIFFERENCES IN BLOOD PLASMA FREE AMINO
COMPOUNDS DUE TO CARBIDOPA AND SINEMET TREATMENTS-

AMINO COMPOUND	% DIFFERENCE FROM CONTROL RATS*		% DIFFERENCE FROM CARBIDOPA TREATED RATS*
	CARBIDOPA	SINEMET	SINEMET
PHOSPHOETHANOLAMINE	60	242	113
TAURINE	23		
UREA	20	78 ^b	48 ^b
ASPARTIC ACID		43	23
THREONINE			
SERINE			
GLUTAMINE			
PROLINE	-27	-27 ^b	
GLUTAMIC ACID		-27 ^c	-38
CITRULLINE	-32 ^b		66 ^b
GLYCINE	23	63 ^c	32 ^b
ALANINE	-44 ^c		109 ^c
α-AMINOADIPIC ACID			
α-AMINOBUTYRIC ACID			
VALINE		21	40 ^b
HALF-CYSTINE	-31		37
CYSTATHIONINE		↑ ^c	↑ ^c
METHIONINE	-27 ^b	-29 ^a	
ISOLEUCINE		20	30
LEUCINE			
TYROSINE	100 ^c	98	
PHENYLALANINE	-24 ^a		
β-ALANINE			
ORNITHINE	27	31	
LYSINE		-39	-25 ^a
1-METHYLHISTIDINE			
HISTIDINE			-27

a) $p < 0.01$

b) $p < 0.005$

c) $p < 0.001$

* Percentage differences are listed if greater or equal to (+)20% or if significant.

TABLE 12 -SIGNIFICANT DIFFERENCES IN BLOOD PLASMA FREE AMINO COMPOUNDS
DUE TO IPRONIAZID AND L-DOPA PLUS IPRONIAZID TREATMENTS-

AMINO COMPOUND	% DIFFERENCE FROM CONTROL RATS*		% DIFFERENCE FROM IPRONIAZID TREATED RATS*
	IPRONIAZID	L-DOPA + IPRONIAZID	L-DOPA + IPRONIAZID
PHOSPHOETHANOLAMINE		575 _b	575
TAURINE	46	180	92
UREA	34	958 ^a	687
ASPARTIC ACID	-20	79	124
THREONINE			
SERINE			
GLUTAMINE			
PROLINE	-33 ^b	-22	
GLUTAMIC ACID			
CITRULLINE	-37 ^c		64 ^b
GLYCINE	-32		32
ALANINE	-26 ^c	20	62 ^b
Δ-AMINOADIPIC ACID		↑ ^c	↑ ^c
Δ-AMINOBUTYRIC ACID		↑ ^c	↑ ^c
VALINE			
HALF-CYSTINE		-58 ^a	
CYSTATHIONINE		↑ ^c	↑ ^c
METHIONINE	-25 ^a	-30	
ISOLEUCINE			
LEUCINE			
TYROSINE	-30 ^c	-54 ^c	-34 ^b
PHENYLALANINE	-22		
β-ALANINE			
ORNITHINE	29	70	32
LYSINE		-17 ^b	-21
1-METHYLHISTIDINE			
HISTIDINE			

a) $p < 0.01$

b) $p < 0.005$

c) $p < 0.001$

* Percentage differences are listed if greater or equal to ($\pm$)20%
or if significant.

TABLE 13 -EFFECT OF TREATMENTS ON LIVER FREE AMINO COMPOUNDS-

AMINO COMPOUNDS	CONTROL*	CARBIDOPA*	SINEMET*	IPRONIAZID*	L-DOPA+IPRONIAZID*
PHOSPHOETHANOLAMINE	85.1 $\pm$ 13.2(3)	177 $\pm$ 52 (4)	221 $\pm$ 95 (3)	184 $\pm$ 57 (3)	575 $\pm$ 159 (6)
TAURINE	1182 $\pm$ 691 (4)	1299 $\pm$ 302 (5)	2803 $\pm$ 1379(6)	2204 $\pm$ 1445(5)	6463 $\pm$ 2447(6)
UREA	2463 $\pm$ 1773(5)	2634 $\pm$ 1162(5)	6945 $\pm$ 718 (6)	2727 $\pm$ 893 (4)	10004 $\pm$ 3849(6)
ASPARTIC ACID	912 $\pm$ 183 (4)	1242 $\pm$ 238 (6)	1170 $\pm$ 138 (6)	817 $\pm$ 69 (4)	633 $\pm$ 316 (7)
THREONINE	510 $\pm$ 101 (4)	661 $\pm$ 138 (6)	809 $\pm$ 85 (6)	482 $\pm$ 81 (4)	405 $\pm$ 35 (6)
SERINE	686 $\pm$ 234 (5)	1044 $\pm$ 123 (6)	1180 $\pm$ 212 (6)	640 $\pm$ 86 (6)	596 $\pm$ 165 (7)
GLUTAMINE	5313 $\pm$ 991 (5)	6044 $\pm$ 1471(6)	1308 $\pm$ 161 (6)	6169 $\pm$ 931 (6)	7070 $\pm$ 1069(7)
PROLINE	202 $\pm$ 50 (5)	243 $\pm$ 68 (4)	269 $\pm$ 53 (4)	223 $\pm$ 59 (6)	303 $\pm$ 25 (7)
GLUTAMIC ACID	3484 $\pm$ 836 (5)	3362 $\pm$ 785 (6)	3356 $\pm$ 386 (5)	3363 $\pm$ 582 (6)	3580 $\pm$ 844 (7)
GLYCINE	2373 $\pm$ 276 (3)	3250 $\pm$ 246 (6)	5624 $\pm$ 406 (5)	2242 $\pm$ 431 (6)	2913 $\pm$ 404 (7)
ALANINE	2277 $\pm$ 1374(3)	1259 $\pm$ 234 (5)	3399 $\pm$ 308 (5)	2426 $\pm$ 240 (6)	4081 $\pm$ 622 (7)
α -AMINOADIPIC ACID	42.6 $\pm$ 17.4(6)	77.7 $\pm$ 29 (4)	167 $\pm$ 73 (6)	76.3 $\pm$ 29.7(4)	208 $\pm$ 107 (6)
α -AMINOBTYRIC ACID	107 $\pm$ 15 (4)	N.R.	62.1 $\pm$ 27.1(6)	N.R.	44.6 $\pm$ 16.2(6)
VALINE	429 $\pm$ 27 (6)	374 $\pm$ 100 (6)	409 $\pm$ 160 (6)	436 $\pm$ 29 (6)	359 $\pm$ 47 (6)
CYSTATHIONINE	N.D.	N.D.	47.6 $\pm$ 21.4(5)	N.D.	17.6 (2)
METHIONINE	85.3 $\pm$ 23.5(5)	84.3 $\pm$ 23 (4)	71 $\pm$ 26.5(6)	102 $\pm$ 16 (5)	77.8 $\pm$ 9.3 (4)
ISOLEUCINE	268 $\pm$ 18 (6)	241 $\pm$ 67 (6)	217 $\pm$ 76 (6)	261 $\pm$ 26 (6)	190 $\pm$ 39 (6)
LEUCINE	516 $\pm$ 16 (6)	464 $\pm$ 103 (6)	374 $\pm$ 134 (6)	558 $\pm$ 44 (6)	363 $\pm$ 63 (6)
TYROSINE	205 $\pm$ 21 (5)	357 $\pm$ 73 (6)	337 $\pm$ 38 (5)	196 $\pm$ 36 (5)	140 $\pm$ 17 (6)
PHENYLALANINE	202 $\pm$ 9 (5)	171 $\pm$ 40 (6)	N.R.	173 $\pm$ 34 (6)	131 $\pm$ 15 (6)
β -ALANINE	231 $\pm$ 25 (5)	108 (2)	343 $\pm$ 83 (6)	147 $\pm$ 12 (3)	558 $\pm$ 84 (5)
ORNITHINE	334 $\pm$ 28 (6)	749 $\pm$ 211 (5)	806 $\pm$ 40 (6)	620 $\pm$ 111 (3)	768 $\pm$ 71 (5)
LYSINE	454 $\pm$ 48 (6)	703 $\pm$ 130 (5)	613 $\pm$ 103 (6)	836 $\pm$ 103 (3)	664 $\pm$ 84 (6)
1-METHYLHISTIDINE	6.8 $\pm$ 2.4 (4)	12.8 $\pm$ 2.8 (3)	9.4 $\pm$ 2.5 (4)	N.R.	9.8 $\pm$ 4.7 (4)
HISTIDINE	684 $\pm$ 78 (6)	715 $\pm$ 67 (5)	364 $\pm$ 58 (6)	750 $\pm$ 105 (3)	444 $\pm$ 27 (7)
L-DOPA	N.D.	N.D.	675 $\pm$ 322 (6)	N.D.	TRACE

* Results are expressed in nmol/g frozen tissue.

TABLE 14 -SIGNIFICANT DIFFERENCES IN LIVER FREE AMINO COMPOUNDS DUE TO CARBIDOPA AND SINEMET TREATMENTS-

AMINO COMPOUND	% DIFFERENCE FROM CONTROL RATS*		% DIFFERENCE FROM CARBIDOPA TREATED RATS*
	CARBIDOPA	SINEMET	SINEMET
PHOSPHOETHANOLAMINE	108	160	25
TAURINE		137	116
UREA		182 ^c	164 ^c
ASPARTIC ACID	36	28	
THREONINE	30	59 ^c	22
SERINE	52 ^a	72 ^a	
GLUTAMINE		-75 ^c	-78 ^c
PROLINE	20	33	
GLUTAMIC ACID			
GLYCINE	37	137	73 ^c
ALANINE	-45	49	170 ^c
α-AMINOADIPIC ACID	82	292 ^b	115
α-AMINO BUTYRIC ACID		-42	
VALINE			
CYSTATHIONINE		↑ ^c	↑ ^c
METHIONINE			
ISOLEUCINE			
LEUCINE		-28	
TYROSINE	74 ^b	64 ^c	
PHENYLALANINE			
β-ALANINE	-53	48	218
ORNITHINE	124 ^c	141 ^c	
LYSINE	55 ^b	35 ^a	
1-METHYLHISTIDINE	88	38	-27
HISTIDINE		-47 ^c	-49 ^c

a) $p < 0.01$

b) $p < 0.005$

c) $p < 0.001$

* Percentage differences are listed if greater or equal to ($\pm$)20% or if significant.

TABLE 15 -SIGNIFICANT DIFFERENCES IN LIVER FREE AMINO COMPOUNDS DUE TO IPRONIAZID AND L-DOPA PLUS IPRONIAZID TREATMENTS-

AMINO COMPOUND	% DIFFERENCE FROM CONTROL RATS*		% DIFFERENCE FROM IPRONIAZID TREATED RATS*
	IPRONIAZID	L-DOPA + IPRONIAZID	L-DOPA + IPRONIAZID
PHOSPHOETHANOLAMINE	154	576 ^b	166 ^a
TAURINE		447 ^b	434 ^a
UREA		306 ^b	251 ^a
ASPARTIC ACID		-31	-23
THREONINE		-21	
SERINE			
GLUTAMINE		33	
PROLINE		50 ^c	36 ^a
GLUTAMIC ACID			
GLYCINE		23	30
ALANINE		79	68 ^c
α -AMINOADIPIC ACID	79	388 ^b	173
α -AMINOBUTYRIC ACID		-58 ^c	
VALINE		-16 ^a	-18 ^a
CYSTATHIONINE		↑	↑
METHIONINE	20		-24
ISOLEUCINE		-29 ^b	-27 ^b
LEUCINE		-30 ^c	-35 ^c
TYROSINE		-32 ^c	-29 ^a
PHENYLALANINE		-35 ^c	-24
β -ALANINE	-34	142 ^c	265 ^c
ORNITHINE	86	130 ^c	
LYSINE	84	46 ^c	-21
1-METHYLHISTIDINE		44	
HISTIDINE		-35 ^c	-41 ^c

a) $p < 0.01$

b) $p < 0.005$

c) $p < 0.001$

* Percentage differences are listed if greater or equal to ($\pm$)20% or if significant.

5.3.2 Comments

5.3.2.1 Brain free amino acid pools.

Carbidopa treatment induced a significant and marked increase in the concentration of tyrosine in cerebral tissue (Tab. 8). Carbidopa does not cross the blood-brain barrier (83) and cannot therefore directly interfere with the intracerebral metabolism of tyrosine. The increase in brain tyrosine is presumably due to inhibition of peripheral decarboxylation and accumulation of tyrosine in the blood plasma (Tab. 11), and in its increased availability for transport into the brain. This explanation is consistent with the observation that carbidopa treatment increases the concentration of striatal tyramines (210). It is noteworthy that the brain concentrations of the other amino acids, which share the same transport carrier with tyrosine (124), were not markedly affected. The increase in glycine level following carbidopa treatment is of interest in the context of the relationship between the concentration of this amino acid and the availability of PLP in the brain (151).

Treatment with iproniazid alone induced changes in the free amino acid levels similar to those observed with carbidopa treatment (Tab. 9). The slight increase in tyrosine concentration is in keeping with inhibition of tyrosine hydroxylase by high levels of catecholamines (211), resulting from this treatment. The significant increases in glycine and serine may suggest increased requirement of these amino acids for regeneration of methionine.

In the brain of Sinemet treated rats the concentrations of L-dopa and 3-O-methyldopa were very elevated 90 min after the last injection (Tab. 8); both compounds were found in high concentrations also in the plasma of these rats (Tab.11). As both L-dopa and 3-O-methyldopa are transported across the blood-brain barrier by the same carrier, specific for neutral amino acids (124,111), competition for the use of this carrier is the probable cause for the decrease in valine, leucine, isoleucine and histidine in the brains of the Sinemet treated rats. The elevated level

of brain tyrosine is due to an increase in the transport of tyrosine from the blood into the CNS resulting from inhibition of peripheral aromatic amino acid decarboxylase by the hydrazine derivative. However, the lower concentration of tyrosine in the brain of Sinemet treated rats, as compared to rats treated with the inhibitor alone, is also consistent with the competition in the transport of the neutral amino acids by L-dopa and its O-methylated derivative. A similar observation has been reported after an acute study (118). The concentration of methionine was only slightly depressed by Sinemet treatment. The conclusion that can be drawn is that neither competition by L-dopa for the neutral amino acid carrier, nor increased requirement of methyl groups for O-methylation of L-dopa and catecholamines, nor the depressed concentration of pyridoxal phosphate caused by carbidopa treatment (82) have a marked effect on methionine concentration. Thus, the remethylating cycle within the CNS was working efficiently, as it is also suggested by the decrease in serine and glycine and by the increase in cystathionine. It should be noted however that these changes are not always significant in relation to both control and carbidopa treated rats.

The increase in alanine concentration has been reported in the work of others in which similar conditions were used (212,118).

The concentrations of L-dopa and 3-O-methyldopa in both brain (Tab. 7) and blood plasma (Tab. 10) of rats treated with L-dopa plus iproniazid were below the level of accurate measurement by the procedure used in this work. Thus, neither compounds should interfere with the transport of the other neutral amino acids into the brain at the time of sacrifice. The cerebral concentrations of valine, isoleucine and leucine were higher than in control rats. The plasma levels of these amino acids were not affected by the treatment (Tab. 12), thus the transport rates were presumably not affected either. These structurally related amino acids are catabolized by certain common enzymes (213), therefore a decreased intracerebral activity of any of these enzymes could explain this observation (151).

Methionine concentration is not affected by L-dopa plus iproniazid treatment; thus, the remethylating cycle within the CNS and the transport of methionine across the blood-brain barrier, are sufficient to keep the concentration of brain methionine at a normal level. It is of interest that the concentration of cystathionine is markedly decreased, which suggests that homocysteine is preferentially used in the regeneration of methionine and it is not catabolized by cystathionine- β -synthase (Fig.11). Similarly, the concentration of taurine, an amino acid derived primarily from cysteine (214), shows a tendency to decrease, which further suggests that, under these conditions, formation of cystathionine is not favoured.

Surprisingly, tyrosine concentration is not different from normal, but it is lower than in rats treated with iproniazid alone. The increased concentration of phenylalanine can inhibit tyrosine hydroxylase and thus block catecholamine synthesis (215). A similar change in GABA has been reported in another work (202).

5.3.2.2 Blood plasma and liver free amino acid pools

Treatment with L-dopa in conjunction with either inhibitor, did not have a pronounced effect on liver methionine concentration as compared to control rats or to rats treated with the inhibitors alone. However, the turnover of methionine was greatly increased, as suggested by the marked changes in cystathionine and taurine levels, and by the decrease in α -aminobutyric acid (ABA) (Tab.14 & 15). The latter is a metabolite of threonine, which rat liver can use in the synthesis of SAM (216).

From a quantitative point of view, Sinemet treatment induced a greater increase in liver cystathionine concentration than L-dopa plus iproniazid treatment. With the former treatment, rats were treated with four times the dose of L-dopa used in the latter, therefore Sinemet treated rats should use more SAM in order to methylate the exogenous L-dopa. Alternatively, the difference can be explained by a block in

cystathionine catabolism, through the inhibition by carbidopa of cystathionine- γ -lyase, a PLP dependent enzyme. The hydrazine derivative appears to induce a partial deficiency of vitamin B6, as it is also suggested by the increase in hepatic glycine levels in rats treated with carbidopa and Sinemet (217).

In the plasma of rats treated with L-dopa and either inhibitor the concentrations of methionine tend to be significantly lower than in control rats (Tab. 11 & 12). However, these variations appear to be due to the inhibitors alone rather than to L-dopa.

The changes in plasma concentrations of other amino acids related to methionine metabolism, such as cystathionine, taurine and glycine, appear to be a reflection of the changes seen in the liver. However the plasma concentration of ABA is increased only with L-dopa plus iproniazid treatment. In this context, it is of interest that dopamine alone is solely responsible for the increase in hepatic polyamine synthesis (155). Thus, with animals treated with L-dopa and iproniazid the methionine-SAM-SAH-homocysteine cycle (Fig.11) will be under a double strain: to methylate L-dopa and its metabolites, and to increase the concentration of polyamines. ABA absorbed from the plasma into the liver could help to relieve such strains, as suggested by Fig.24.

The 5-methylthioadenosine (MTA) formed by the transfer of a propylamine group from decarboxylated SAM to putrescine or spermidine, could be quickly reutilized to form SAM, which is used to methylate L-dopa and its metabolites. Therefore, this mechanism would ensure polyamine synthesis, without any net loss of methyl groups.

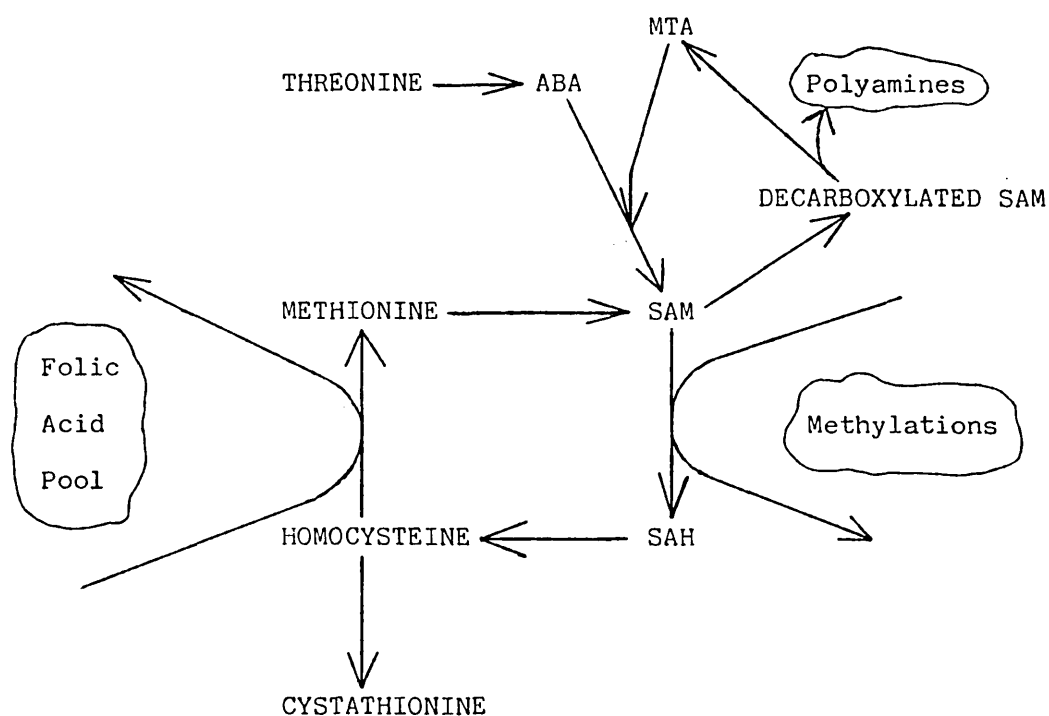


Fig.24 Putative role of α -aminobutyric acid in the metabolism of methionine and SAM.

The increases in serine, tyrosine, ornithine and lysine levels in the liver of Sinemet treated rats appeared to be mainly due to carbidopa alone (Tab 14). This suggests that the hydrazine derivative may inhibit certain PLP dependent enzymes involved in the catabolism of these amino acids. With respect to lysine metabolism, there is a marked increase in α -amino adipic acid, an intermediate in the formation of acetoacetyl-CoA from lysine (213).

The increase in urea and the dramatic decreases in glutamine and histidine in the liver of these rats appear to be mainly due to joint treatment with L-dopa and carbidopa (Tab. 14). Increased activity of the urea cycle may be part of a detoxification mechanism, linked to the removal of the amino group from administered L-dopa. The metabolic steps

could be via aminotransfer to α -ketoglutarate to yield glutamate and oxidative deamination of glutamate to liberate ammonia, which enters the urea cycle as carbamoyl phosphate. The decrease in hepatic histidine may suggest an increased catabolism of this amino acid by histidase. This reaction is part of a pathway which eventually leads to the formation of formimino glutamic acid. The formimino group can be transferred onto THF to form formimino-THF which is an intermediate in the synthesis of methylene-THF (218). Increased catabolism of histidine via this route, would provide extra one-carbon units for the regeneration of SAM and the O-methylation of L-dopa. The close relationship between histidine and SAM concentrations and transmethylation reactions has been shown in cells cultivated in a histidine free-medium, which resulted in a decreased SAM level and in a significant inhibition of ribosomal RNA methylation (219). In the plasma of Sinemet treated rats (Tab. 11), most changes seem to reflect those seen in the liver. The main exceptions are lysine, which shows a tendency to decrease, and glutamine and histidine which were largely unaffected by the treatment. The lack of effect on plasma glutamine, suggests that the large decrease in liver glutamine is a phenomenon mainly associated with hepatic metabolism.

The variations observed in the hepatic amino acid pool, following L-dopa plus iproniazid treatment, were usually similar whether compared to normal controls or to iproniazid treated rats (Tab. 15). The main exceptions were ornithine and lysine, whose increased hepatic concentrations appeared to be due to iproniazid alone. It is of interest that most essential amino acids were decreased by treatment with L-dopa plus iproniazid, when compared to treatment with the inhibitor alone. The large increase in hepatic urea, suggests a high turnover of the urea cycle.

The changes in the plasma amino acid pool of rats treated with L-dopa plus iproniazid do not closely resemble those observed in the liver of these rats (Tab. 12). This treatment markedly increased the levels of catecholamines (121); in this context, the very high concentration of plasma urea, suggests that the urea cycle may also play an important detoxifying role. It is noteworthy that treatment with iproniazid alone decreased by about one third the concentration of tyrosine observed in the plasma of control rats, whereas joint treatment with the MAO inhibitor and L-dopa decreased plasma tyrosine to about half of its normal level. These observations and the nature of the treatments suggest that the concentration of plasma tyrosine is inversely proportional to catecholamine levels. This relationship between plasma tyrosine and catecholamines has been described in another work, where a single injection of L-dopa or dopamine induced a decrease of tyrosine in the rat blood plasma (100).

5.4 METHYLTRANSFERASE ACTIVITIES

Brain monoamine oxidase activity is almost completely inhibited following chronic treatment with irreversible inhibitors (119). Furthermore chronic treatment with Sinemet causes accumulation of L-dopa in the CNS (Tab. 7).

It was therefore of interest to examine the effect of L-dopa in conjunction with iproniazid or carbidopa on brain catechol-O-methyltransferase, an enzyme which is complementary to MAO in the catabolism of catecholamines, and which also catalyses the O-methylation of L-dopa.

COMT activity in the erythrocytes was also examined, because it is assumed to reflect the enzyme activity in other tissues (112).

5.4.1 Results

The results are summarized in the following tables:

Table 16 Effect of treatments on methyltransferase activity in brain and erythrocytes.

Table 17 Effect of treatments on erythrocyte methyltransferase activities.

TABLE 16 -EFFECT OF TREATMENTS ON TOTAL METHYLTRANSFERASE
ACTIVITY IN BRAIN AND ERYTHROCYTES-

TREATMENT	BRAIN *	ERYTHROCYTES *
CONTROL	7.38 $\pm$ 0.44 (4)	6.08 $\pm$ 1.30 (4)
CARBIDOPA	7.89 $\pm$ 0.57 (4)	5.23 $\pm$ 1.08 (3)
SINEMET	7.95 $\pm$ 0.30 (4)	4.33 $\pm$ 0.56 (4) ^a
IPRONIAZID	7.76 $\pm$ 0.36 (4)	5.23 $\pm$ 1.20 (4)
L-DOPA + IPRONIAZID	7.82 $\pm$ 0.46 (4)	5.41 $\pm$ 1.03 (8)

a) $p < 0.05$ from CONTROL.

* Activities are expressed in nmol methylated product/hour per mg protein or haemoglobin; each determination was carried out in duplicate.

TABLE 17 -EFFECT OF TREATMENTS ON ERYTHROCYTE
METHYLTRANSFERASE ACTIVITIES-

TREATMENT	COMT *	SAM HYDROLASE *
CONTROL	4.11 $\pm$ 1.41 (4)	1.97 $\pm$ 0.18 (4)
CARBIDOPA	3.27 $\pm$ 0.74 (3)	1.96 $\pm$ 0.52 (3)
SINEMET	2.66 $\pm$ 0.20 (4) ^a	1.68 $\pm$ 0.48 (4)
IPRONIAZID	3.40 $\pm$ 1.14 (4)	1.83 $\pm$ 0.14 (4)
L-DOPA + IPRONIAZID	3.31 $\pm$ 0.63 (8)	2.10 $\pm$ 0.45 (8)

a) $p < 0.05$ from CONTROL

* Activities are expressed in nmol methylated product/hr/mg
haemoglobin; each determination was carried out in duplicate.

5.4.2 Comments

The results (Tab. 16) show that there was no marked change in brain COMT activity in animals treated with iproniazid or carbidopa either alone or in conjunction with L-dopa. By comparison, the activity of the enzyme in the erythrocytes was significantly inhibited by about 29% in Sinemet treated animals (Tab. 17).

Although no stimulation of enzyme activity was detected, it should be pointed out that in the present study, Sinemet administration depressed erythrocyte COMT activity less markedly than in other studies where L-dopa alone was administered (116). Furthermore, the enzyme activity was unaffected by chronic treatment with L-dopa plus iproniazid. These observations suggest that under these conditions COMT activity may be partially stimulated in the periphery.

COMT has a wide metabolic role (108) and thus it is difficult to assess the significance of the depressed erythrocyte activity in Sinemet treated rats. However, it is of interest that, despite the lower COMT activity, 3-O-methyldopa, the toxic product of the O-methylation of L-dopa, was detected in significant quantities only in the tissues of the rats treated with Sinemet.

5.5 S-ADENOSYL-L-METHIONINE AND S-ADENOSYLMHOMOCYSTEINE CONCENTRATIONS

The above experiments suggest that although neither brain nor liver methionine levels are significantly depressed by the L-dopa treatments, the remethylating cycle is under strain.

SAM metabolism is regulated by the ratio of its concentration to that of SAH (135,139). Under less severe conditions than those used in this study, a single L-dopa injection depressed SAM concentration in brain but not in the liver (126).

In the periphery, carbidopa may inhibit the metabolism of cystathionine, which is dependent on PLP requiring enzymes. In the CNS, the decreased availability of PLP induced by chronic carbidopa treatment, may have a similar effect. Both conditions may lead to an accumulation of homocysteine and this may interfere with the remethylating cycle. Iproniazid may indirectly increase the requirement of SAM, the cofactor of COMT, via its cumulative inhibition of MAO activities. The joint and chronic administration of dopa and either carbidopa or iproniazid may then result in altered SAM/SAH ratios.

5.5.1 Results

The results are summarized in the following tables:

Table 18 Effect of treatments on neural and hepatic SAM and SAH concentrations.

Table 19 Effect of treatments on neural and hepatic SAM/SAH ratios.

TABLE 18 -EFFECT OF TREATMENTS ON NEURAL AND HEPATIC SAM AND SAH CONCENTRATIONS-

TREATMENT	B R A I N				L I V E R			
	SAM*	%D**	SAH*	%D**	SAM*	%D**	SAH*	%D**
CONTROL	25.6±1.8(10)	-	5.3±1.0(6)	-	63.6±5.8(6)	-	47.4±4.0(6)	-
CARBIDOPA	24.8±3.3 (4)	-3	4.3±2.2(4)	-19	64.0±4.8(4)	1	45.4±3.2(4)	-4
SINEMET	13.3±1.7(10) ^{a,b}	-48	5.8±1.6(6)	9	69.3±8.0(5)	9	59.9±5.8(5) ^{f,g}	26
IPRONIAZID	28.2±5.1 (4)	10	3.5±0.8(4) ^d	-34	66.9±5.5(4)	5	56.6±9.7(4)	19
L-DOPA+IPRONIAZID	14.2±1.9 (8) ^{a,c}	-45	6.0±1.5(4) ^e	13	43.1±5.1(4) ^{a,c}	-32	42.6±3.5(5) ^h	-10

a) p<0.001 from CONTROL

b) p<0.001 from CARBIDOPA

c) p<0.001 from IPRONIAZID

d) p<0.02 from CONTROL

e) p<0.05 from IPRONIAZID

f) p<0.005 from CONTROL

g) p<0.005 from CARBIDOPA

h) p<0.02 from IPRONIAZID

* Results are expressed in nmole/g frozen tissue

** Percentage difference from control

TABLE 19 -EFFECT OF TREATMENTS ON NEURAL AND
HEPATIC SAM/SAH RATIOS-

TREATMENT	BRAIN	LIVER
CONTROL	4.78 $\pm$ 0.53 (6)	1.35 $\pm$ 0.17 (6)
CARBIDOPA	6.90 $\pm$ 3.50 (4)	1.41 $\pm$ 0.09 (4)
SINEMET	2.60 $\pm$ 0.85 (6) ^a ^b	1.17 $\pm$ 0.19 (5)
IPRONIAZID	8.21 $\pm$ 1.55 (4) ^a	1.21 $\pm$ 0.20 (4)
L-DOPA + IPRONIAZID	2.58 $\pm$ 1.11 (4) ^c ^d	1.01 $\pm$ 0.17 (4) ^e

a) $p < 0.001$ from CONTROL

b) $p < 0.02$ from CARBIDOPA

c) $p < 0.005$ from CONTROL

d) $p < 0.005$ from IPRONIAZID

e) $p < 0.02$ from CONTROL

5.5.2 Comments

5.5.2.1 Effect of treatments on brain S-adenosyl-L-methionine and S-adenosylhomocysteine concentrations.

Treatment with either iproniazid or carbidopa had no marked effect on cerebral SAM concentrations although some depression of SAH level in rats treated with iproniazid was observed. Chronic L-dopa treatments with either inhibitor decreased brain SAM levels but had no effect on SAH concentrations (Tab. 18) and thus the SAM/SAH ratios were significantly lower than in control rats (Tab. 19). Consequently, methylation of some endogenous substrates might be negatively affected.

A decrease in SAM concentration analogous to those found in our experiments has been reported within 45 min of a single L-dopa injection (126). However, under the more severe conditions used in the present study, the depression in the level of SAM was maintained for at least 90 min after the last injection.

Quantitatively, the decrease in brain SAM concentration was similar whether L-dopa was administered in conjunction with either the monoamine oxidase inhibitor or the peripheral decarboxylase inhibitor, although the dosage of L-dopa was four times greater in the latter treatment. The brain concentration of L-dopa was high (about 440 nmole/g tissue) in rats treated with Sinemet, but not in animals treated with the amino acid plus iproniazid (< 5 nmole/g tissue) (Tab. 7).

These observations suggest that the SAM level is more sensitive to high levels of catecholamines rather than to a high level of their amino acid precursor. This agrees with the suggestion that most of the 3-O-methyldopa found in the brain of Sinemet treated rats is of extracerebral origin (220).

The limited effect of the various treatments on SAH concentrations is presumably a consequence of the high turnover rate of this metabolite whose breakdown product, homocysteine, is required for the regeneration of SAM. The decreased availability of brain pyridoxal phosphate induced

by carbidopa treatment (82) does not appear to affect the catabolism of homocysteine and thus, indirectly, the concentration of SAH.

5.5.2.2 Effect of treatments on liver S-adenosyl-L-methionine and S-adenosylhomocysteine concentrations.

Treatment with either iproniazid or carbidopa had no marked effect on hepatic SAM or SAH concentrations. Treatment with Sinemet increased SAH but had no effect on SAM concentration.

Treatment with L-dopa plus iproniazid significantly decreased SAM concentration, while the SAH level was lowered only in comparison to treatment with the inhibitor alone (Tab. 18). In rats treated with L-dopa plus iproniazid the SAM/SAH ratio was significantly lower than in controls (Tab. 19).

The results obtained following treatment with L-dopa and either inhibitors suggest that the remethylation cycle was under strain. Different mechanisms could contribute to such a phenomenon.

In the case of Sinemet treatment, because of the block in peripheral decarboxylation by carbidopa, O-methylation becomes a major catabolic route of exogenous L-dopa. Furthermore, carbidopa may also partially inhibit other PLP dependent enzymes such as cystathionine- γ -lyase and cystathionine- β -synthase. This would explain the accumulation of SAH due to feedback inhibition of SAH hydrolase by homocysteine. A similar mechanism is suggested in another work, where the hepatic SAM and SAH levels were respectively increased and decreased in rats fed on a pyridoxine deficient diet (144). However, inhibition of these PLP dependent enzymes by carbidopa was not very strong, as it became apparent only under conditions of high SAM requirement (for example, when L-dopa was administered together with the hydrazine derivative).

With L-dopa plus iproniazid treatment, SAM concentration was decreased by about a third (Tab. 18), a change which is quantitatively higher than those reported in other works where the animals were treated with the amino acid alone (126,221). Also the SAH concentration showed a tendency

to decrease, suggesting that the rate of regeneration of methionine was presumably increased. Therefore, chronic inhibition of hepatic monoamine oxidase together with administration of L-dopa decreases SAM concentration, presumably by increasing the proportion of catecholamines which are O-methylated.

Methylation of endogenous substrates may be affected by the depressed SAM/SAH ratio in the liver of rats treated with L-dopa plus iproniazid (Tab. 19). This ratio was not significantly lower in the liver of Sinemet treated rats; even though certain transmethylating enzymes, more sensitive to SAH inhibition (132), may be selectively inhibited by this treatment.

5.6 NUCLEIC ACID CONCENTRATIONS

The methylation of nucleic acids utilizes SAM as the source of methyl groups (128) and SAH has been shown to inhibit many of the methyltransferases (131). Ribonucleic acids, particularly transfer RNA, undergo extensive post-transcriptional methylation in the course of formation of the mature transfer RNA molecules (159). A change in the degree of methylations of RNA molecules affects their stability (162). The experimental observations described in section 5.3 indicate that L-dopa, in conjunction with either a monoamine oxidase or a peripheral decarboxylase inhibitor, significantly interferes in the metabolism of methionine. The concentration of either SAM or SAH in liver and brain are affected and this usually results in altered SAM/SAH ratios (section 5.5). The report in the literature cited above and our own results prompted the study of the influence of L-dopa in conjunction with ipronia_zid or carbidopa, on the concentration of total RNA and transfer RNA molecules in brain and liver.

5.6.1 Results

The results are summarized in the following tables:

Table 20 Effect of treatments on total RNA and total transfer RNA in brain and liver.

Table 21 Effect of treatments on DNA and RNA in the liver.

TABLE 20 -EFFECT OF TREATMENTS ON TOTAL RNA AND TOTAL TRANSFER RNA
IN BRAIN AND LIVER-

TREATMENT	B R A I N *		L I V E R *	
	RNA	TRANSFER RNA	RNA	TRANSFER RNA
CONTROL	2.18±0.22(4)	0.112±0.004(4)	8.28±0.67(4)	0.232±0.027(4)
SINEMET	2.21±0.09(4)	0.111±0.004(4)	9.00±0.46(4)	0.228±0.028(5)
L-DOPA + IPRONIAZID	2.16±0.15(4)	0.111±0.005(4)	9.68±1.02(4)	0.265±0.020(5)

* Results are expressed in µg/mg of tissue.

TABLE 21 -EFFECT OF TREATMENTS ON DNA AND RNA
IN THE LIVER-

TREATMENT	DNA*	RNA*	RATIO DNA/RNA
CONTROL	2.62±0.19(4)	8.28±0.67(4)	0.317±0.015
SINEMET	2.66±0.16(4)	9.00±0.46(4)	0.295±0.012
L-DOPA + IPRONIAZID	2.54±0.52(4)	9.68±1.02(4)	0.261±0.027 ^a

a) $p < 0.02$ from CONTROL

* Results are expressed in $\mu\text{g}/\text{mg}$ of tissue

5.6.2 Comments

The concentrations of RNA and t-RNA in brain and liver of the rats were not significantly affected by any of the treatments (Tab. 20). However, in the liver of rats treated with L-dopa plus iproniazid, the total RNA and total transfer RNA levels had a tendency to increase. This was emphasized by the slightly enlarged liver of these animals (Tab. 2). Furthermore, the hepatic DNA/RNA ratio was significantly decreased by this same treatment (Tab. 21), suggesting an increased rate of transcription.

5.7 TRANSFER RNA BASE COMPOSITION

The treatments used in this study interfere with the metabolism of methionine and of SAM (see sections 5.3 & 5.5). Other experiments suggest that treatment with L-dopa plus iproniazid stimulates RNA synthesis (see section 5.6). Of the various ribonucleic acids, transfer RNA molecules, which are heavily methylated in their mature form, are affected by various treatments: nicotinamide (222) inhibits t-RNA methyltransferase in vitro, while polyamines stimulate methylation (223). These observations and the reported in vitro inhibition of liver t-RNA methyltransferase by dopamine (134), were the primary reasons for examining the degree of t-RNA methylation in brain and liver of L-dopa treated rats.

5.7.1 Results

The results are summarized in the following tables:

Table 22 Effect of treatments on the total base composition of brain t-RNA.

Table 23 Effect of treatments on the minor base composition of brain t-RNA.

Table 24 Effect of treatments on the total base composition of liver t-RNA

Table 25 Effect of treatments on the minor base composition of liver t-RNA.

In these tables the following nomenclature is used:

- Modified nucleosides (MOD): nucleoside triols derived from Y', hU',
m₁A', m₁G', m₂G', m₂²G', m₃C', m₅U', m₇G'.
- Methylated nucleosides (METH): nucleoside triols derived from m₁A', m₁G',
m₂G', m₂²G', m₃C', m₅U', m₇G'.
- Purine nucleosides (PUR): nucleoside triols derived from Adenosine,
Guanosine and their derivatives.
- Pyrimidine nucleosides (PYRIM): nucleoside triols derived from Uridine,
Cytidine and their derivatives.
- Major nucleosides (MAJOR): nucleoside triols derived from U', A', C'
and G'.

TABLE 22 -EFFECT OF TREATMENTS ON TOTAL BASE COMPOSITION OF BRAIN
TRANSFER RNA-

NUCLEOSIDE TRIOL				L-DOPA +	
	CONTROL*	SINEMET*	%D**	IPRONIAZID*	%D**
U'	15.60 $\pm$ 0.37	15.73 $\pm$ 0.30		15.44 $\pm$ 0.15	
A'	15.68 $\pm$ 0.56	15.21 $\pm$ 0.71		15.78 $\pm$ 0.74	
C'	25.45 $\pm$ 0.52	25.89 $\pm$ 0.17		25.59 $\pm$ 0.29	
G' + I'	30.32 $\pm$ 0.64	30.40 $\pm$ 0.43		30.26 $\pm$ 0.53	
Y'	3.255 $\pm$ 0.16	3.289 $\pm$ 0.092		3.085 $\pm$ 0.137	
hU'	2.880 $\pm$ 0.13	2.777 $\pm$ 0.048		2.878 $\pm$ 0.092	
m ₁ A'	1.661 $\pm$ 0.066	1.611 $\pm$ 0.117		1.709 $\pm$ 0.032	
m ₁ G'	0.856 $\pm$ 0.044	0.858 $\pm$ 0.068		0.829 $\pm$ 0.011	
m ₂ G'	0.695 $\pm$ 0.130	0.757 $\pm$ 0.154		0.830 $\pm$ 0.188	+19
m ₂ ² G'	0.638 $\pm$ 0.035	0.620 $\pm$ 0.040		0.596 $\pm$ 0.037	
m ₃ C'	0.544 $\pm$ 0.010	0.472 $\pm$ 0.082	-13	0.510 $\pm$ 0.067	
m ₅ C'	1.538 $\pm$ 0.035	1.550 $\pm$ 0.044		1.551 $\pm$ 0.140	
m ₅ U'	0.556 $\pm$ 0.121	0.588 $\pm$ 0.008		0.622 $\pm$ 0.054	+12
m ₇ G'	0.415 $\pm$ 0.063	0.493 $\pm$ 0.042	+19	0.482 $\pm$ 0.050	+16
MOD/MAJOR	0.180 $\pm$ 0.010	0.183 $\pm$ 0.005		0.180 $\pm$ 0.013	
METH/MAJOR	0.081 $\pm$ 0.002	0.082 $\pm$ 0.002		0.084 $\pm$ 0.004	
PUR/PYRIM	1.009 $\pm$ 0.006	0.989 $\pm$ 0.013 ^a	- 2	1.015 $\pm$ 0.013	

a) p<0.05

* The base composition of t-RNA is expressed as a percentage of the total base content of each molecule; 4 tissues were used for each group of animals. The t-RNA base composition of each tissue was determined in triplicate.

** Percentage differences from control are listed if greater or equal to ($\pm$)10% or if significant.

TABLE 23 -EFFECT OF TREATMENTS ON THE MINOR BASE COMPOSITION OF BRAIN
TRANSFER RNA-

NUCLEOSIDE TRIOL				L-DOPA +	
	CONTROL*	SINEMET*	%D**	IPRONIAZID*	%D**
Y'	24.99±0.77	25.26±0.85		23.30±0.47 ^a	- 7
hU'	22.11±0.57	21.32±0.40		22.11±1.18	
m ₁ A'	12.76±0.66	12.38±0.91		13.13±0.62	
m ₁ G'	6.58±0.39	6.59±0.53		6.36±0.20	
m ₂ G'	5.33±0.97	5.87±1.82	+10	6.34±1.23	+19
m ₂ G'	4.90±0.22	4.76±0.28		4.48±0.20 ^b	- 9
m ₃ C'	4.08±0.32	3.63±0.65	-11	3.93±0.58	
m ₅ C'	11.82±0.25	11.90±0.28		11.88±0.67	
m ₅ U'	4.27±0.86	4.52±0.09		4.78±0.50	+12
m ₇ G'	3.19±0.42	3.79±0.31	+19	3.69±0.27	+16

a) p<0.01 from CONTROL

b) p<0.05 from CONTROL

* The base composition of transfer RNA is expressed as a percentage of the total base content of each molecule excluding the four major bases.

** Percentage differences from control are listed if greater or equal to (±) 10% or if significant.

TABLE 24 -EFFECT OF TREATMENTS ON TOTAL BASE COMPOSITION OF LIVER
TRANSFER RNA-

NUCLEOSIDE TRIOL	CONTROL*		L-DOPA + IPRONIAZID*	
	CONTROL*	SINEMET*	%D**	%D**
U'	15.77 $\pm$ 0.49	15.34 $\pm$ 0.60	15.37 $\pm$ 0.38	
A'	16.28 $\pm$ 0.35	15.87 $\pm$ 0.93	15.66 $\pm$ 0.69	
C'	25.24 $\pm$ 0.22	25.64 $\pm$ 0.19	25.63 $\pm$ 0.39	
G'+I'	30.94 $\pm$ 0.48	31.16 $\pm$ 0.51	31.38 $\pm$ 0.99	
Y'	3.245 $\pm$ 0.242	3.209 $\pm$ 0.246	3.020 $\pm$ 0.192	
hU'	2.344 $\pm$ 0.329	2.392 $\pm$ 0.309	2.284 $\pm$ 0.227	
m ₁ A'	1.461 $\pm$ 0.139	1.334 $\pm$ 0.076	1.282 $\pm$ 0.059	-12
m ₁ G'	0.756 $\pm$ 0.115	0.740 $\pm$ 0.029	0.760 $\pm$ 0.042	
m ₂ G'	1.123 $\pm$ 0.127	1.111 $\pm$ 0.088	1.125 $\pm$ 0.160	
m ₂ G'	0.627 $\pm$ 0.074	0.619 $\pm$ 0.050	0.591 $\pm$ 0.036	
m ₃ C'	0.277 $\pm$ 0.049	0.304 $\pm$ 0.026	0.293 $\pm$ 0.035	
m ₅ C'	1.761 $\pm$ 0.249	1.668 $\pm$ 0.239	1.635 $\pm$ 0.153	
m ₅ U'	0.518 $\pm$ 0.101	0.513 $\pm$ 0.036	0.574 $\pm$ 0.079	+11
m ₇ G'	0.477 $\pm$ 0.127	0.410 $\pm$ 0.040	0.382 $\pm$ 0.046	-20
MOD/MAJOR	0.175 $\pm$ 0.009	0.170 $\pm$ 0.015	0.173 $\pm$ 0.017	
METH/MAJOR	0.081 $\pm$ 0.007	0.077 $\pm$ 0.005	0.078 $\pm$ 0.006	
PUR/PYRIM	1.047 $\pm$ 0.015	1.038 $\pm$ 0.038	1.045 $\pm$ 0.042	

* The base composition of transfer RNA is expressed as percentage of the total base content of each molecule; four tissues were used for each group of animals; the transfer RNA base composition of each tissue was determined in triplicate.

** Only percentage differences greater or equal to ($\pm$)10% of control values are listed.

TABLE 25 -EFFECT OF TREATMENTS ON THE MINOR BASE COMPOSITION OF LIVER
TRANSFER RNA-

NUCLEOSIDE TRIOL	CONTROL*		L-DOPA + IPRONIAZID*	
		SINEMET*	%D**	%D**
Y'	25.46 $\pm$ 1.74	25.81 $\pm$ 0.85		24.95 $\pm$ 1.32
hU'	19.52 $\pm$ 2.07	20.72 $\pm$ 1.69		20.30 $\pm$ 2.01
m ₁ A'	11.44 $\pm$ 0.52	10.77 $\pm$ 1.06		10.61 $\pm$ 0.79
m ₁ G'	6.07 $\pm$ 0.47	5.96 $\pm$ 0.27		6.28 $\pm$ 0.41
m ₂ G'	8.82 $\pm$ 1.01	8.64 $\pm$ 1.01		9.27 $\pm$ 1.01
m ₂ ² G'	4.91 $\pm$ 0.44	4.99 $\pm$ 0.47		4.88 $\pm$ 0.24
m ₃ C'	2.23 $\pm$ 0.30	2.35 $\pm$ 0.21		2.38 $\pm$ 0.30
m ₅ C'	13.79 $\pm$ 1.62	13.39 $\pm$ 1.38		13.57 $\pm$ 0.51
m ₅ U'	4.04 $\pm$ 0.63	4.34 $\pm$ 0.65		4.63 $\pm$ 0.72 +15
m ₇ G'	3.73 $\pm$ 0.70	3.04 $\pm$ 0.29	-18	3.15 $\pm$ 0.28 -16

* The base composition of transfer RNA is expressed as a percentage of the total base content of each molecule excluding the four major bases.

** Only percentage differences greater or equal to ($\pm$)10% of control values are listed.

5.7.2 Comments

Treatment with L-dopa in conjunction with either inhibitor did not have a generalized effect on the methylation of brain and liver t-RNA molecules. This is emphasized by the closeness of the modified to major and methylated to major base ratios among control and treated rats. The purine to pyrimidine base ratio of t-RNA in the brain of Sinemet treated rats is slightly depressed (Tab. 22). A more accurate picture of the modified base composition of t-RNA molecules is obtained by excluding the four major bases (185). No generalized decrease in the methylated base composition is apparent in either brain or liver; however, slight, but significant, decreases in $m_2^2 G'$ and Y' are found in brain t-RNA molecules of rats treated with L-dopa plus iproniazid (Tab. 23). A possibility is that the enzymes responsible for the methylation of guanosine are inhibited. For example, methionine sulfoxide, a drug which depletes brain SAM level, was shown to selectively inhibit the brain enzyme responsible for the methylation of adenosine (224).

The decrease in Y' may be of significance especially in relation to the function of t-RNA molecules as corepressor in the preferential expression of a gene within an operon (225).

Chronic L-dopa treatment is responsible for the induction and repression of different enzymes (37), and these effects, which are generally more prominent in the liver than in the brain, should also be reflected in a shift in t-RNA population. It is however surprising that the hepatic t-RNA composition is not affected by L-dopa treatments.

The failure of the elevated level of SAH found in the liver of Sinemet treated rats to inhibit transmethylation is presumably a reflection of the efficiency with which SAH is removed from the vicinity of any t-RNA methyltransferase. In mammalian tissues, a considerable proportion of t-RNA methyltransferases exist as complexes with SAH hydrolase and SAM synthetase (226). Such an arrangement would ensure a fast removal of any inhibiting SAH molecules and a constant supply of the methyl donor to the methyltransferase enzymes.

An overall effect of L-dopa treatment on t-RNA methylation is excluded by the results discussed above. Nevertheless, O-methylation of the ribose moiety of the nucleosides could be affected, but was not measured in our experiments.

5.8 AMINOTRANSFERASE ACTIVITIES

Under the present experimental conditions, the availability of pyridoxal phosphate can be depressed either by the condensation of the vitamer with the drugs (or one of their metabolites) used in this work, or by a change in the activity of pyridoxal kinase induced by the treatments. The reaction mechanisms have been described in details (see 2.2.1, 2.2.2 & 2.2.3).

A decrease in PLP availability would cause a diminution in the activity of some PLP-dependent enzymes, such as aminotransferases. In this study we measured the activity glutamate aminotransferase which has been described as a good index of pyridoxine availability (88,91).

The activity of tyrosine aminotransferase was also measured. This enzyme can be inhibited by the mechanisms described above, but its activity can also be stimulated under certain conditions. Some of the treatments used in this study caused an increase in the brain concentrations of tyrosine and L-dopa (Tab. 7); both amino acids induce the hepatic enzyme and may have a similar effect on the brain enzyme. Alternatively, the high levels of dopamine, resulting from L-dopa treatment in conjunction with either inhibitors, may induce tyrosine aminotransferase. This would represent a compensatory mechanism in order to decrease the concentration of tyrosine, the amino acid precursor of catecholamines.

5.8.1 Results

The results are summarized in the following tables and figures:

Fig. 25-27 In vitro condensation between pyridoxal phosphate and L-dopa or carbidopa.

Table 26 In vitro effect of pyridoxal phosphate, carbidopa, iproniazid and L-dopa on the activity of glutamate aminotransferase.

Tables 27-30 Effect of treatments on tissue aminotransferase activities and their stimulation by in vitro addition of pyridoxal phosphate.

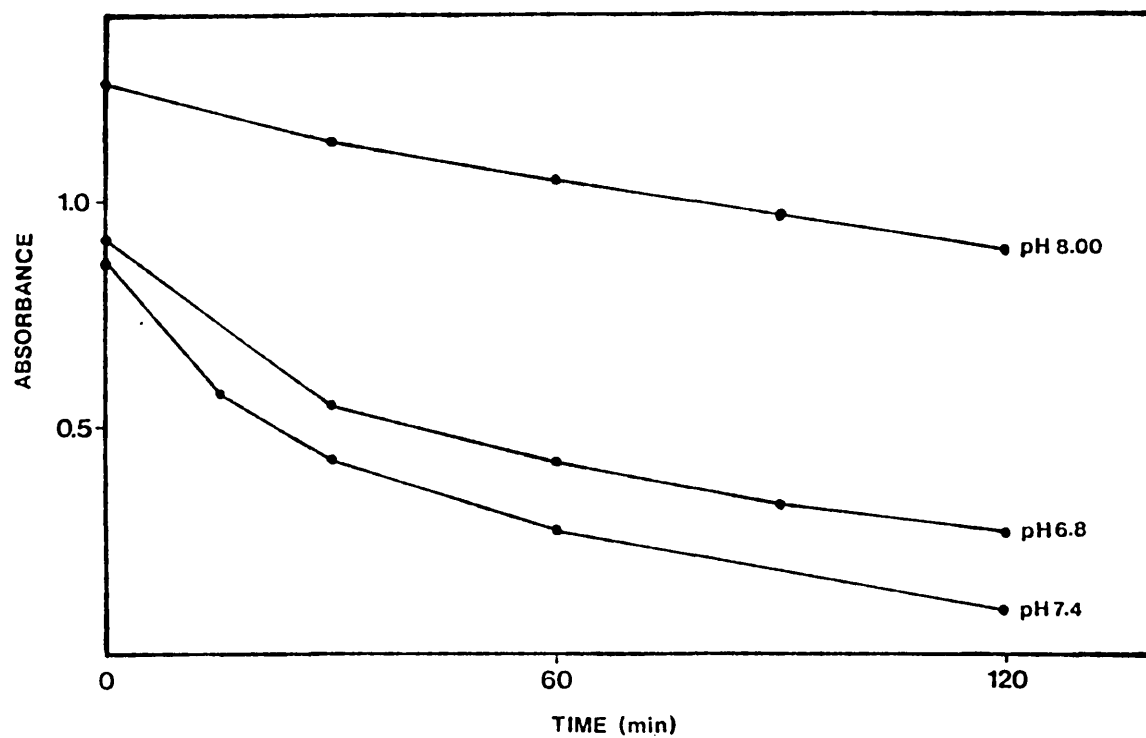


Fig.25 In vitro condensation of L-dopa and pyridoxal phosphate.

The disappearance of the pyridoxal phosphate absorption peak is followed at 416, 386 and 388 nm. for pH 8.0, 6.8 and 7.4 respectively.

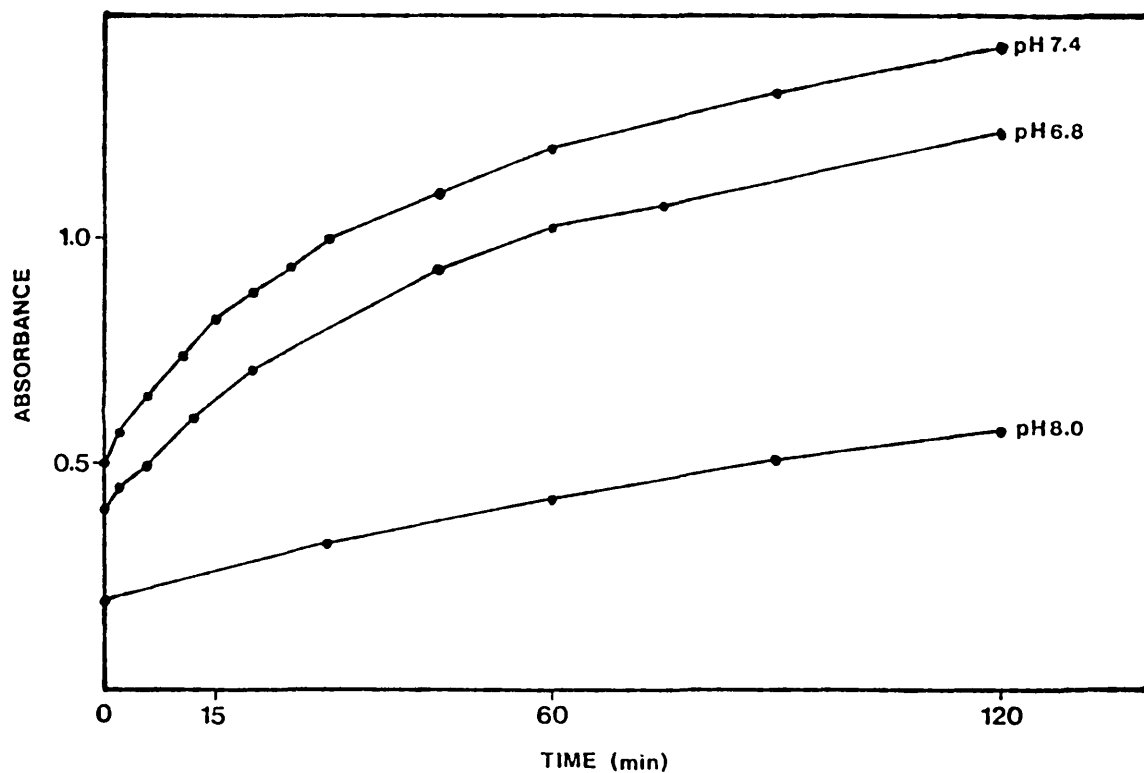


Fig.26 In vitro condensation of L-dopa and pyridoxal phosphate.

The appearance of the condensation product is followed at 325 nm. at pH 6.8 and 7.4, and at 312.5 nm. at pH 8.0

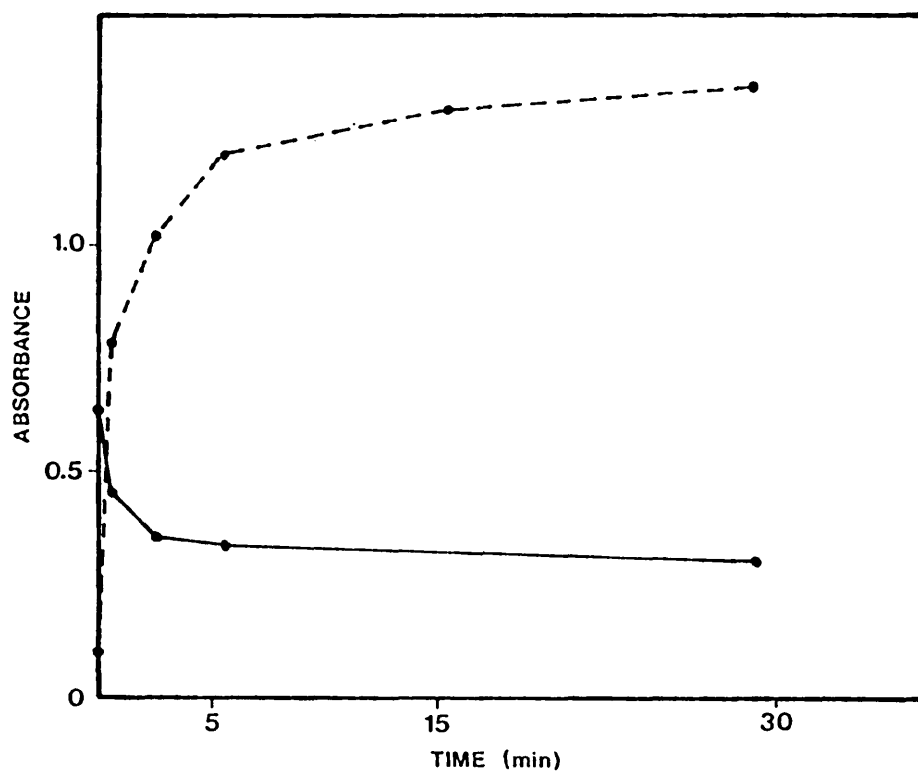


Fig.27 In vitro condensation of carbidopa and pyridoxal phosphate at pH 7.4. The rate of disappearance of pyridoxal phosphate (solid line) is followed at 388 nm.; the rate of appearance of the condensation product (broken line) is followed at 306 nm.

TABLE 26 -IN VITRO EFFECT OF PYRIDOXAL PHOSPHATE, CARBIDOPA,
IPRONIAZID AND L-DOPA ON THE ACTIVITY OF
GLUTAMATE AMINOTRANSFERASE*-

ADDITIONS TO INCUBATION		
MIXTURE	ACTIVITY**	%D §
NONE	6908 $\pm$ 365	-
PLP	6892 $\pm$ 267	-
PLP + IPRONIAZID	7192 $\pm$ 260	+ 4
PLP + IPRONIAZID + L-DOPA	6902 $\pm$ 153	-
PLP + L-DOPA	5787 $\pm$ 591	-16
L-DOPA	5502 $\pm$ 384	-20
PLP + CARBIDOPA	4124 $\pm$ 56	-40
PLP + CARBIDOPA + L-DOPA	3784 $\pm$ 198	-45
CARBIDOPA	3457 $\pm$ 231	-50
CARBIDOPA + L-DOPA	3312 $\pm$ 131	-52

* The source of the enzyme was the liver of untreated rats; each determination was carried out in triplicate; enzyme activity is expressed in μ mol of labelled α -ketoglutarate produced/hr/100 mg protein.

** When present: 25 μ M Pyridoxal phosphate (PLP)
500 μ M L-DOPA
50 μ M CARBIDOPA
50 μ M IPRONIAZID

§ Percentage difference from the activity obtained with no addition to the incubation mixture.

TABLE 27 -EFFECT OF TREATMENTS ON BRAIN AMINOTRANSFERASE ACTIVITIES AND THEIR STIMULATION BY IN VITRO
ADDITION OF PYRIDOXAL PHOSPHATE-

TREATMENT	GLUTAMATE AMINOTRANSFERASE*			TYROSINE AMINOTRANSFERASE*		
	WITHOUT	WITH	%D**	WITHOUT	WITH	%D**
	PLP	PLP		PLP	PLP	
CONTROL	11731 $\pm$ 1137(4)	11938 $\pm$ 690(4)	+ 2	2.64 $\pm$ 0.07(4)	3.06 $\pm$ 0.18(4)	+16 ^a
CARBIDOPA	12447 $\pm$ 1268(4)	12475 $\pm$ 1729(4)	-	2.79 $\pm$ 0.23(4)	3.22 $\pm$ 0.18(4)	+15 ^b
SINEMET	12407 $\pm$ 854(4)	12514 $\pm$ 1041(4)	+ 1	2.52 $\pm$ 0.19(4)	2.97 $\pm$ 0.13(4)	+18 ^c
IPRONIAZID	11364 $\pm$ 1091(4)	12041 $\pm$ 1202(4)	+ 6	2.82 $\pm$ 0.16(4)	3.28 $\pm$ 0.15(4)	+16 ^c
L-DOPA+IPRONIAZID	11193 $\pm$ 431(4)	12338 $\pm$ 1208(4)	+10	2.43 $\pm$ 0.31(4)	2.76 $\pm$ 0.12(4) ^d ^e	+14

a) $p < 0.005$ from activity without PLP

b) $p < 0.025$ from activity without PLP

c) $p < 0.01$ from activity without PLP

d) $p < 0.05$ from CONTROL

e) $p < 0.005$ from IPRONIAZID

* Activities are expressed in μ mole of labelled α -keto acids produced/hr/100 mg protein; each determination was carried out in duplicate.

** Percentage stimulation of enzyme activity resulting from in vitro addition of PLP.

TABLE 28 -EFFECT OF TREATMENTS ON LIVER AMINOTRANSFERASE ACTIVITIES AND THEIR STIMULATION BY IN VITRO
ADDITION OF PYRIDOXAL PHOSPHATE-

TREATMENT	GLUTAMATE AMINOTRANSFERASE*			TYROSINE AMINOTRANSFERASE*		
	WITHOUT PLP	WITH PLP	%D**	WITHOUT PLP	WITH PLP	%D**
CONTROL	6425+217(4)	6633+210(4)	+ 3	6.81+1.39(4)	10.47+2.30(4)	+ 54 ^c
CARBIDOPA	4919+524(5) ^a	5662+913(5)	+15	3.90+0.89(4) ^d	12.53+3.44(4)	+221 ^a
SINEMET	4288+319(5) ^b	5112+328(5) ^b	+19 ^a	4.17+1.09(5) ^d	17.66+3.30(5) ^f	+324 ^b
IPRONIAZID	5926+513(4)	6377+549(4)	+ 8	4.17+1.40(4) ^c	7.18+1.59(4)	+ 72 ^c
L-DOPA+IPRONIAZID	6813+824(4)	7526+992(5)	+11	16.27+2.00(5) ^{b,e}	27.07+4.34(5) ^{b,e}	+ 66 ^b

a) $p < 0.005$ from CONTROL or from activity without PLP

b) $p < 0.001$ from CONTROL or from activity without PLP

c) $p < 0.05$ from CONTROL or from activity without PLP

d) $p < 0.02$ from CONTROL

e) $p < 0.001$ from IPRONIAZID

f) $p < 0.01$ from CONTROL

* Activities are expressed in μ mole of labelled α -keto acid produced/hr/100 mg protein; each determination was carried out in duplicate.

** Percentage stimulation of enzyme activity resulting from in vitro addition of PLP.

TABLE 29 -EFFECT OF TREATMENTS ON BLOOD PLASMA AMINOTRANSFERASE ACTIVITIES AND THEIR STIMULATION BY
IN VITRO ADDITION OF PYRIDOXAL PHOSPHATE-

TREATMENT	GLUTAMATE AMINOTRANSFERASE*			TYROSINE AMINOTRANSFERASE*		
	WITHOUT	WITH	%D**	WITHOUT	WITH	%D**
	PLP	PLP		PLP	PLP	
CONTROL	58.1±5.9(4)	59.7±3.9(4)	+ 3	2.13±0.19(4)	2.33±0.05(4)	+ 9
CARBIDOPA	48.2±3.5(4) ^a	51.9±4.0(4) ^a	+ 8	2.26±0.06(3)	2.95±0.28(3)	+31
SINEMET	48.0±1.2(4) ^b	53.4±5.0(4)	+11	2.26±0.31(4)	2.72±0.42(4)	+20
IPRONIAZID	47.8±1.5(4) ^b	54.8±2.0(4)	+15 ^c	1.98±0.23(4)	2.07±0.13(4) ^e	+ 5
L-DOPA+IPRONIAZID	49.6±3.6(4)	55.7±1.9(4)	+12 ^d	1.65±0.26(5) ^b	2.31±0.26(5)	+40 ^c

a) p<0.05 from CONTROL

b) p<0.02 from CONTROL

c) p<0.005 from activity without PLP

d) p<0.025 from activity without PLP

e) p<0.01 from CONTROL

* Activities are expressed in μ mole of labelled α -keto acid produced/hr/100 mg protein; each determination was carried out in duplicate.

** Percentage stimulation of enzyme activity resulting from in vitro addition of PLP.

TABLE 30 -EFFECT OF TREATMENTS ON GLUTAMATE AMINOTRANSFERASE
ACTIVITY IN THE ERYTHROCYTES AND ITS STIMULATION
BY IN VITRO ADDITION OF PYRIDOXAL PHOSPHATE*-

TREATMENT	WITHOUT PLP	WITH PLP	%D**
CONTROL	1103 $\pm$ 89(4)	1121 $\pm$ 137(4)	+ 2
CARBIDOPA	675 $\pm$ 94(3)	774 $\pm$ 45(3)	+15
SINEMET	603 $\pm$ 21(4) ^b	734 $\pm$ 54(4) ^a	+22 ^a
IPRONIAZID	876 $\pm$ 121(4) ^c	970 $\pm$ 99(4)	+11
L-DOPA+IPRONIAZID	1264 $\pm$ 187(8) ^d	1242 $\pm$ 139(8) ^e	- 2

a) $p < 0.005$ from CONTROL or from activity without PLP

b) $p < 0.001$ from CONTROL

c) $p < 0.025$ from CONTROL

d) $p < 0.05$ from IPRONIAZID

e) $p < 0.01$ from IPRONIAZID

* Activities are expressed in $\mu\text{mole/hr/100 mg haemoglobin}$;
each analysis was carried out in duplicate.

** Percentage stimulation of enzyme activity resulting from
in vitro addition of PLP.

5.8.2 Comments

5.8.2.1 Condensation studies; in vitro effects of the drugs on hepatic glutamate aminotransferase activity.

The experimental observations recorded in Fig.25 & 26 indicate that the rate of formation of the condensation product of L-dopa and PLP was high at physiological pH. Under similar conditions, the condensation of PLP and carbidopa occurred at an even faster rate (Fig.27). No change in the absorption spectrum of a mixture of PLP and iproniazid occurred over the range of 190-450 nm. Therefore, the formation of a condensation product between PLP and iproniazid is unlikely.

The in vitro effect of the drugs on glutamate aminotransferase activity (Tab. 26) is in agreement with the findings from condensation studies. Since enzyme inhibition by carbidopa and L-dopa was partially relieved by pyridoxal phosphate, the mechanism of such inhibition is likely to occur via interaction between the drug and the cofactor. Furthermore, carbidopa is a stronger inhibitor than L-dopa, and iproniazid did not inhibit the enzyme.

Assuming that within the liver tissue the distributions of administered L-dopa and carbidopa are similar, the concentrations of the drugs studied in the in vitro experiments are presumably close to those obtained in vivo. For example, about 650 nmole of L-dopa/g of tissue were found in the liver of Sinemet treated rats (Tab. 13), and 25 nmole of PLP/g of tissue in the liver of rats treated with saline (82). However, these in vitro experiments are only indicative of the potential effects of the drugs in vivo, as they do not take into consideration the compartmentation within the cell of enzyme and cofactor, and the physical state of the latter, which in vivo is mainly bound to macromolecules (59).

5.8.2.2 Effect of treatments on the activities of aminotransferases
in vivo, and their stimulation by in vitro addition of pyridoxal
phosphate.

a) Glutamate aminotransferase.

Under the conditions used in this study, the activity of brain GOT was not altered by any of the treatments (Tab. 27). In the case of carbidopa this is due to the impermeability of the blood-brain barrier to the drug. The lack of effect of L-dopa cannot be ascribed to a progressive adjustment of peripheral and central metabolic mechanisms during chronic treatment (100,115,199,200), which would decrease uptake and decarboxylation of L-dopa by the brain and thus diminish the effect of L-dopa treatments on pyridoxine metabolism. Indeed, by doubling the Sinemet dose two days before sacrifice, metabolic adjustments should not play an important role. Moreover, because of the irreversible and cumulative nature of the inhibition of monoamine oxidase by iproniazid, catecholamine concentrations should be chronically high following treatment with L-dopa and this MAO inhibitor. These results show that neither chronic high levels of L-dopa nor of monoamines affect the availability of PLP to brain GOT.

GOT activities in peripheral tissues (liver, blood plasma and erythrocytes) were inhibited by carbidopa treatment. Addition of PLP in vitro restores the activity of the liver enzyme towards normal values, but in blood plasma, and particularly in the erythrocytes, GOT activity remains below control levels. These results may be explained in terms of two types of inhibition by the hydrazine derivative. Firstly, a decrease in the availability of PLP via the formation of hydrazones (Fig.7); this type of inhibition can be relieved in vitro by the addition of the cofactor. Secondly, an interaction with PLP at the active site (82); this form of inhibition cannot be relieved by addition of PLP in vitro, and it is particularly marked in the mature erythrocytes, where the apoenzyme cannot be synthesized.

Sinemet administration had similar effects on peripheral GOT activities as treatment with carbidopa alone. However, in vitro addition of PLP did not restore hepatic GOT activity to normal levels; moreover, in all the peripheral tissues examined, stimulation by addition of the cofactor was consistently greater following Sinemet than carbidopa treatment. This suggests cumulative inhibition by L-dopa and carbidopa. As L-dopa concentrations in the liver and plasma of Sinemet treated animals were high (Tab. 10 & 13), the amino acid and PLP could form a THIQ (see 2.2.1). Although the concentration of L-dopa in the tissues was presumably much higher than that of carbidopa, the additional inhibition due to the amino acid was small as PLP reacts far more readily with carbidopa than with L-dopa (see 5.8.2.1).

Following iproniazid treatment, the slight decreases in GOT activities seen in plasma and erythrocytes, were partially reversed by the addition of PLP in vitro. The possible effects of this drug on vitamin B6 metabolism have been described (see 2.2.3).

L-Dopa plus iproniazid treatment had a tendency to increase GOT activity in both liver and RBC, although none of the changes were significant in relation to control rats; the enzyme activity in the erythrocytes was significantly stimulated when compared to treatment with the MAO inhibitor alone. These results clearly show that the high levels of monoamines (and presumably of the corresponding THIQs) do not have a marked inhibitory effect on this PLP-dependent enzyme in vivo. We have observed that treatment with iproniazid alone had a tendency to decrease erythrocyte GOT activity whereas joint treatment with L-dopa plus iproniazid had no effect on enzyme activity. This would suggest that the high dopamine levels resulting from the latter treatment may stimulate GOT activity. In vitro addition of the cofactor does not stimulate the erythrocyte enzyme. This is surprising as the uptake of vitamin B6 from the plasma is dependent on its binding to haemoglobin (227), and the mean cell haemoglobin is slightly but significantly decreased by L-dopa plus iproniazid treatment (Tab. 5), and thus we might have expected that the cofactor does not saturate the enzyme in vivo.

b) Tyrosine aminotransferase.

In the present investigation no increase in TAT activity was observed following those treatments in which there was a significant increase in brain tyrosine concentration at the time of sacrifice (i.e., carbidopa, iproniazid and Sinemet treated rats, see Tab. 8 & 9). Our results do not support the suggestion that elevated tyrosine level in the brain stimulate TAT activity (92); this discrepancy may be due to an inadequate elevation of the brain tyrosine concentrations under our own experimental conditions. Although transamination is the main catabolic route of 3-O-methyldopa, neither elevated level of this amino acid, nor high level of L-dopa stimulated TAT activity in the brain of rats treated with Sinemet (Tab. 27). In rats treated with L-dopa plus iproniazid the activity of cerebral TAT is slightly lower than in control rats and in rats treated with the inhibitor alone. This effect is not however related to a decreased availability of PLP to the apoenzyme, as stimulation of the enzyme activity by addition of the cofactor is of a similar magnitude for all groups of rats (Tab. 27)

The level of TAT activity in erythrocytes was inadequate for accurate determination by the method used in this study, as it was lower than 0.3 $\mu\text{mol/hr/100 mg protein}$ (191).

Hepatic enzyme activity was depressed by about 40% by carbidopa treatment (Tab. 28). The activity was restored to normal by addition of pyridoxal phosphate to the assay medium. Following PLP addition in vitro, there was no evidence of an in vivo induction of the apoenzyme by either elevated level of tyrosine or by reduced availability of pyridoxal phosphate.

Sinemet treatment inhibited the liver enzyme, and addition of PLP caused a three fold stimulation of enzyme activity (Tab. 28). Induction of liver TAT is presumably due to the high concentrations of its substrates (i.e. tyrosine, L-dopa and 3-O-methyldopa; Tab. 13). However, hormonal effects due to L-dopa metabolism in the adrenal gland (46) cannot be entirely

excluded, as carbidopa does not readily enter this organ (47). The observation that treatment with Sinemet does not lead to increased peripheral catabolism of L-dopa by TAT, confirms what has been previously suggested (47).

Iproniazid treatment causes a slight depression in hepatic TAT activity, which can be attributed to some metabolites of iproniazid or to the formation of THIQ (see 2.2.3).

Joint treatment with L-dopa plus iproniazid dramatically stimulated hepatic tyrosine aminotransferase activity (Tab. 28). This effect is presumably induced by glucagon or insulin (228), whose secretions are stimulated by L-dopa administration (203). In the liver, the availability of PLP to the enzyme is not decreased, because the stimulation by the cofactor is similar to the one observed with either iproniazid treatment or with control rats.

The different effect of the drug treatments on liver and plasma TAT activities (Tab. 28 & 29) may appear surprising as most plasma proteins are synthesized in the liver and released into the plasma.

Aminotransferases have essentially an intracellular role and leak in the blood plasma during cellular turnover (229). Changes in the composition of plasma proteins do not reflect short-term metabolic changes in the liver (230); since the half-life of cytosolic hepatic TAT is about 2 to 4 hours (93), this may partly explain why the activities of plasma TAT did not parallel the changes observed in the liver.

Moreover, the short half-life of cytosolic TAT suggests that a significant proportion of the tyrosine transaminase activity in the plasma may be associated with the other non-specific enzymes. It has been reported that mitochondrial TAT activity is due to mitochondrial GOT (231), which has a plasma half-life of about 17 hours (232); moreover, cytoplasmic GOT and alanine aminotransferase with similar or longer plasma half-lives (103) can use tyrosine as substrate (231,233). The high TAT activity relative to the tyrosine level in the plasma can be explained by the ability of GOT and alanine aminotransferase to transaminate tyrosine.

Under normal circumstances, their TAT activity is presumably of no great physiological significance as their K_m for tyrosine are high (95) and the endogenous tyrosine concentration is relatively low (Tab. 10 & 13).

Treatment with either carbidopa or Sinemet did not decrease plasma TAT and in vitro addition of PLP appreciably stimulated this activity (Tab. 29). Since mitochondrial and cytoplasmic TAT have different sensitivities to inhibition by carbidopa (107), the lack of effect by the hydrazine derivative is in keeping with the suggestion that other aminotransferases are active in the transamination of aromatic amino acids in blood plasma. The significant stimulation of TAT activity by PLP in vitro, may suggest that more apoenzyme than in controls is present in the plasma of these rats, and that the additional activity, more easily inhibited by carbidopa, is mainly associated with cytosolic TAT. Alternatively, a higher activity is not necessarily due to higher amounts of the enzyme protein in blood plasma: TAT exists in at least three forms with different half-lives (93), therefore changes in the proportions of isoenzymes with different half-lives, as well as changes in the proportion of the non-specific enzymes which make up the total blood plasma TAT activity are possible.

The apparent lack of inhibitory effect on the plasma enzymes by the hydrazine derivative, was paralleled by an increase in plasma concentration of the endogenous substrates; namely, tyrosine for rats treated with carbidopa alone and tyrosine, L-dopa and 3-O-methyldopa for rats treated with Sinemet (Tab. 10). Conversely, plasma TAT activity and plasma tyrosine levels are reduced following treatment with L-dopa plus iproniazid. This suggests that there may be a correlation between TAT activity and substrate level in the blood plasma.

The relationship between plasma tyrosine concentration and plasma TAT activity following the different treatments is shown in Fig.28. To correct for the effect of the different treatments on total plasma protein (Tab. 4) and blood volume (118), the results are expressed as total tyrosine and total TAT activity in the whole plasma; the details of

the calculation are given in the legend of Fig.28.

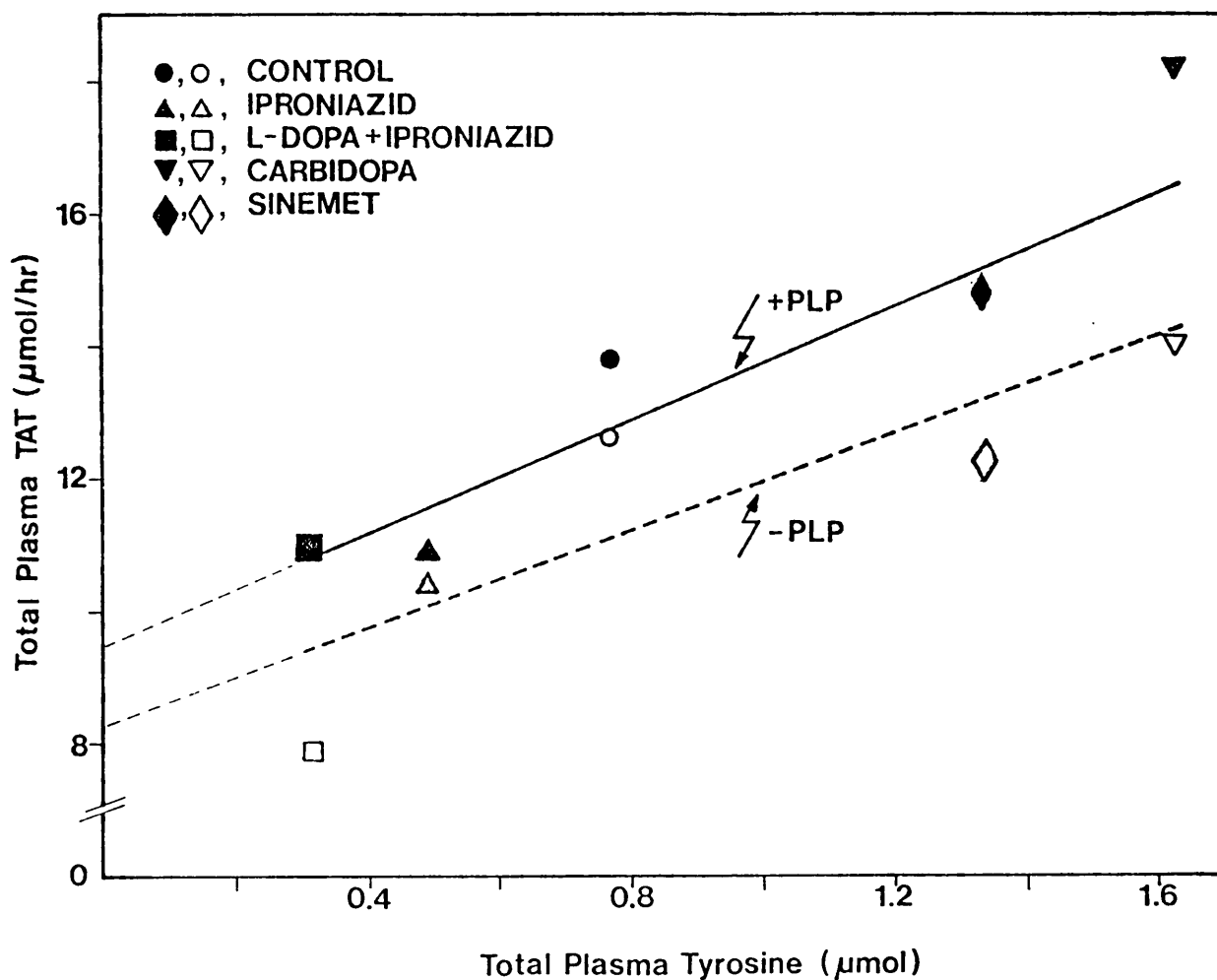


Fig.28 Relationship between tyrosine level and TAT activity in the blood plasma following the different drug treatments. The full symbols refer to TAT activity in the presence of PLP in vitro. (The body weights are shown in Tab. 2; the blood volumes of the rats are taken as 5.60% or 5.13% of the body weights if the treatment does not change or increases dopamine levels, respectively (118); plasma volumes are calculated from the haematocrit ratio shown in Tab. 5; protein concentrations are shown in Tab. 4)

The diagram indicates that the level of activity of plasma TAT is directly proportional to the plasma tyrosine concentration; this relationship may play a role in controlling the concentration of tyrosine in the blood plasma.

However, this interpretation should be considered with caution for the following reasons. The relationship between plasma tyrosine levels and TAT activities could be secondary and incidental to the pharmacological effects of each drug treatment. A change in the rate of cellular turnover, in the rate of exchange between intra- and extra-vascular plasma proteins, in the rate of synthesis, breakdown and secretion of albumin, to which B6 vitamers are bound in the blood plasma, can all have individual and cumulative effects (234).

CHAPTER 6) DISCUSSION

The studies reported in this thesis were aimed to determine whether administration of L-dopa, in conjunction with inhibitors of its catabolism, decreases the methylation of endogenous substrates and/or affects the activity of PLP dependent enzymes.

Analysis of the free amino acids in brain and liver has shown that methionine concentrations were not affected by treatment with L-dopa in conjunction with either inhibitors. However, the effect of the L-dopa treatments on cystathionine levels (as well as on the concentration of other amino acids closely related to methionine metabolism), suggests that there is an increase in the turnover of methionine in the tissues examined (see 5.3.2). This increase was not due to stimulation of COMT activity (see 5.4.2), but it is likely to result from an increased utilization of SAM for the catabolism of L-dopa and of catecholamines, as the changes in SAM and SAH concentrations suggest (see 5.5.2). The lower than normal concentration of SAM in rats which underwent the L-dopa treatments, probably limits the activity of COMT, which would explain why this catabolic enzyme was not stimulated by increased levels of L-dopa and catecholamines.

Other authors have reported that administration of L-dopa inhibits the methylation of endogenous substrates by decreasing the availability of the methyl donor or by increasing the concentration of SAH. Thus, in the CNS, a decrease in adrenaline concentration has been linked to the changes in SAM and SAH levels which follow a single injection of L-dopa (51). Similarly, administration of the amino acid diminishes the proportion of catecholamines which are catabolized by O-methylation (130). It has also been reported that a single injection of L-dopa inhibits protein synthesis (204). These observations prompted an investigation into the effect of L-dopa on the methylated base content of t-RNA, because methylation of these molecules is indispensable for normal protein synthesis.

Some changes in the methylated base composition of t-RNA molecules were detected following L-dopa administration, although no gross inhibition of t-RNA transmethylation was observed (see 5.7.2); furthermore these effects were more prominent in the brain rather than in the liver, although a tendency for increased t-RNA synthesis was noticed only in the hepatic tissue (Tab. 20). The failure to observe a generalized decrease in the methylation of t-RNA molecules may be explained in different ways. For example, the methyltransferase enzymes catalyzing the methylation of the different nucleosides may have different sensitivities to inhibition, which would explain the lack of an overall inhibitory effect by the L-dopa treatments. Alternatively, the enzyme complex formed by t-RNA methyltransferase, SAM synthetase and SAH hydrolase (226) may protect the methyltransferases from inhibition. In view of the important metabolic roles of t-RNA molecules (235), their transmethylation may be more resistant to inhibition than reactions involving the methylation of endogenous substrates which play a less important metabolic role.

Hepatic SAH concentration was increased following Sinemet treatment (see 5.5.2), which strongly suggests that the catabolism of homocysteine by some PLP dependent enzymes is partially blocked by the hydrazine derivative. This interpretation is in keeping with results showing that carbidopa inhibits PLP dependent enzymes other than the aromatic amino acid decarboxylase (79,80,81). To our knowledge, no previous reports of in vivo inhibition of peripheral GOT and TAT by carbidopa (see 5.8.2) have appeared in the literature. Another PLP dependent enzyme, glycogen phosphorylase, was not markedly affected by carbidopa (see 5.2.2). Furthermore the concentration of blood haemoglobin was not markedly decreased (see 5.2.2), which suggests that the process of hematopoiesis, which is strictly dependent on pyridoxine availability, was not affected. Alternatively, the decreased haemoglobin concentration in the blood of rat treated with L-dopa and either inhibitor may be linked to the decreased number of red blood cells (Tab. 5). These two effects, which result in no net change in the concentration of haemoglobin per cell may

be related through the central role of PLP as coenzyme of δ -aminolaevulinic acid synthetase, which may then result in a diminished life-span of the erythrocytes.

Early workers have investigated whether administration of carbidopa may induce a deficiency of pyridoxine (47,82). The relative ease with which L-dopa and PLP can form a complex in vitro (Fig.25 & 26) and the effect of catecholamines on pyridoxal kinase activity, stimulated other work on the effect of the amino acid on pyridoxine metabolism (37). The findings reported in this work do not support the view that chronic treatment with L-dopa, in conjunction with either carbidopa or iproniazid, decreases the availability of PLP to two brain aminotransferase enzymes (see 5.8.2.2). The strong binding of PLP to the GOT apoenzyme (236) may partially explain the lack of effect of L-dopa on the enzyme; the observation that the stimulation of TAT activity, by the addition of PLP in vitro, was similar for all groups of rats, suggests that the degree of saturation of the apoenzyme by the cofactor is similar in treated and untreated animals.

The results reported in this work show that the hepatic TAT apoenzyme is induced by treatment with L-dopa in conjunction with either inhibitor and that addition of PLP in vitro, stimulated TAT activity to above control values (Tab. 28). The fluctuation in response to L-dopa or Sinemet therapy observed in Parkinsonian patients treated also with pyridoxine (26,47), can then be explained as due to increased peripheral decarboxylation and transamination of the amino acid which would decrease the amount of L-dopa made available for transport into the CNS.

Moreover following chronic treatment with Sinemet the beneficial effects of drug holidays, during which the patients ingest an adequate diet with vitamin supplementation, may be related to the reversal of the inhibitory action of L-dopa and carbidopa on PLP dependent enzymes in the body.

It is of interest that blood plasma TAT activity was resistant to inhibition by carbidopa and Sinemet. The observation that in the blood

plasma these treatments inhibited GOT but not TAT, suggests that a good proportion of the latter activity is not due to the unspecific activity of blood plasma GOT to transaminate tyrosine (Tab. 29). This interpretation is also supported by the observation that the ratio of the activity of GOT to TAT was widely different in the blood plasma and erythrocytes (Tab. 29 & 30). The physiological significance of blood plasma TAT activity is unclear, and to our knowledge has not been previously investigated. Further studies are needed to determine whether the enzymes which contribute to the total TAT activity in the blood plasma have an affinity for tyrosine which is high enough to affect its concentration in the blood. It is however likely that treatment with Sinemet will raise the blood plasma concentration of L-dopa, and possibly tyrosine (Tab. 10), to levels where the transaminating pathway becomes quantitatively significant.

It is of interest that the linear relationship between tyrosine concentration and TAT activity in the blood plasma is observed with or without addition of PLP in vitro (Fig.28). Further studies are needed to establish whether this relationship is just coincidental to the effects of the drug treatments or whether it has a physiological significance. There is no evidence of the existence of specific mechanisms which export enzymes, whose known role is intracellular, from tissues into the blood. However it must be pointed out that, although TAT has been extensively studied, many recent observations have shown that our understanding of the physiological role of this activity is rather incomplete. For example, some workers have shown that a new aromatic aminotransferase activity appears in the small intestine of vitamin B6 deficient rats (237); others have been unable to identify an isoenzyme responsible for the transamination of tyrosine in the rat liver (238). The main source of blood PLP is the liver (73) and the mechanism controlling the transport of the cofactor from the hepatic tissue into the blood can be affected in some pathological conditions (239). Thus, the availability of PLP in the blood may be an important factor in controlling TAT activity by regulating the degree of the apoenzyme saturation.

Hepatic glycogen phosphorylase activity and blood glucose level were not affected by either carbidopa or Sinemet treatment, nevertheless treatment with Sinemet increased liver glycogen level (Tab. 6). This increase could be explained by the following metabolic adjustment which takes place within the liver during chronic treatment with Sinemet. When L-dopa is administered together with the peripheral decarboxylase inhibitor, O-methylation of the amino acid and of its transaminated metabolites (Fig.9) is a major catabolic route. This results in an increased utilization of SAM, which in turn should stimulate the synthesis of the methyl donor. It has been shown that when SAM synthesis is increased, the hepatic ATP concentration in the rat is decreased (240). Thus, chronic treatment with L-dopa and a peripheral decarboxylase inhibitor may progressively induce an increase in the level of liver glycogen, which would act as a storage of energy readily convertible into ATP for the peripheral catabolism of exogenous L-dopa. In the rats treated with Sinemet the changes in body weight and the changing responses to the daily administration of the drug during the 15 days of treatment (Fig.22), may also suggest that metabolic changes take place during the length of the experiment. The loss of body weight together with the weak behavioural response observed in the first few days of treatment suggest that more L-dopa was metabolized in the peripheral tissues and less was made available for transport into the CNS, than at a later stage of the treatment when the body weight was stable and the behavioural response became stronger (Fig.22). After about ten days from the start of the treatment, the weaker response of the rats to the drug may be due to changes in the number of dopamine receptors in the CNS (201), but also to stimulation of mechanisms like glucogenesis, which would decrease the amount of dopamine formed in the CNS, by increasing the proportion of amino acid which is catabolized in the periphery.

In summary, we have been unable to show any effect of the L-dopa treatments on either the methylation of endogenous substrates or on the availability of PLP in the brain, however the results presented in this

thesis can stimulate further work into the effects of L-dopa on peripheral metabolic adjustments which are induced by chronic treatment with this drug, and, namely, alteration in aromatic amino acid transaminating activity in the blood plasma and in glycogen metabolism in the liver.

Although under physiological conditions the role of glycogen phosphorylase is primarily glycogenolytic, in Sinemet rats there is a small but significant increase in liver phosphorylase activity yet a significant increase in liver glycogen concentration. This may be due to an induction of glycogen synthetase activity resulting from the slightly elevated blood glucose levels. It is certainly worthy of further investigations. The possible role of glucagon should also be examined. Investigations of cAMP levels should also be of interest.

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