SOME EFFECTS OF STEROID HORMONES AND KYNURENINE ON TRYPTOPHAN AND ITS CONVERSION TO 5-HYDROXYTRYPTAMINE

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Some effects of steroid hormones and kynurenine on tryptophan and its conversion to 5-hydroxytryptamine

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Summary

The relationship of certain steroid hormones to mood state in humans and to biochemical parameters related to 5-hydroxytryptamine function in the brains of mice have been studied.

Cortisol and "free" tryptophan showed significant relations with mood in puerperal women.

Cortisol, oestradiol and mestranol failed to affect the binding of tryptophan to albumin in vitro whereas a very high concentration of norethisterone increased "free" tryptophan.

Brain 5-HT turnover was measured in the mouse after cortisol, a combination of norethisterone and oestradiol, and in different stages of the oestrus cycle. A high dose of cortisol reduced turnover, after 24 hr. pretreatment whereas a lower dose, or repeated dosing with a low dose had no effect. A low-dose combination of norethisterone and oestradiol had no effect on turnover, after 14 day pretreatment. A slight reduction in brain 5-HT levels and an increase in turnover were found in oestrus, compared with dioestrus animals.

The effects of these hormones on plasma and brain levels of the tryptophan metabolite, kynurenine, were studied. A low-dose combination increased plasma kynurenine without affecting brain kynurenine. Cortisol was found to decrease plasma kynurenine levels after 24 hr. pretreatment, but had no effect on brain levels.

The mechanism of uptake of kynurenine into brain was investigated. There appear to be two mechanisms of uptake, one of which is active.

Kynurenine failed to affect the in vitro uptake of 5-hydroxytryptamine. DL-kynurenine was found to reduce plasma tryptophan after 2 hr. pretreatment, but increased brain tryptophan after 30 min. A biphasic effect of DL-kynurenine was found on brain 5-hydroxytryptamine turnover, a low dose reduced turnover, whereas higher doses had no effect on turnover.

Key words: cortisol; female hormones; 5-hydroxytryptamine; turnover; kynurenine

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List of Abbreviations

TP = tryptophan

5-HTP = 5-hydroxytryptophan

5-HT = 5-hydroxytryptamine

5-HIAA = 5-hydroxyindoleacetic acid

IAA = indoleacetic acid

MAO = monoamine oxidase

MAOI = monoamine oxidase inhibitor/inhibition

ECT = electroconvulsive therapy

CSF = cerebro-spinal fluid

LSD = lysergic acid diethylamide

UFA = unesterified fatty acids

ug = micrograms

OPT = o-pthalaldehyde

DCPX = dichloro-p-xylene

NA = noradrenaline

DA = dopamine

Terminology

cortisol ≡ hydrocortisone

tryptophan pyrrolase ≡ tryptophan 2,3 dioxygenase

INTRODUCTION

1. An Overview of depression

Mental illness has been known for centuries and affective illness is particularly tragic both for the patient and for his relatives and friends. As well as causing a great deal of misery, depression has a finite death rate by suicide. There is a large volume of evidence to support a biological basis for depression, including the associated disturbances of sleep, appetite, gastrointestinal function, sex-drive and sensation. There is often diurnal variation in the severity of depression, and it is often associated with other illnesses, such as endocrine and metabolic diseases. The likelihood of suffering from depression increases if there is a family history of the disorder. The fact that there is often no association between external "stress" factors and the onset of a serious depressive illness also indicates a disorder of the internal environment. Further support for a biological dysfunction in depressive illness is provided by the fact that organic therapies have been developed.

The first physical treatments were the "shock" therapies, including the use of insulin coma and camphor or pentylene-tetrazole induced seizures for major endogenous psychiatric illnesses (Baldessarini 1975). Later electric shock treatment was used, and modern electroconvulsive treatment remains the most effective treatment for severe depression (ibid.).

The isolation of reserpine from a crude extract of Rauwolfia serpentina in the 1950 s was important in the development of a biogenic amine hypothesis of depression. Reserpine was first used as an

antipsychotic agent, but the development of the phenothiazines, which were more effective in schizophrenia, caused its use to decline. However, it continued to be used as an antihypertensive, and depression was a commonly reported side effect (Muller et al 1955, Harris 1957). Reserpine also caused a syndrome of behavioural "depression" in laboratory animals. It was found to cause depletion of dopamine (DA), noradrenaline (NA) and 5-hydroxytryptamine (5-HT) by interfering with their storage (Holzbauer and Vogt 1956). This observation drew attention to the possible relationship between brain monoamines and behaviour.

Iproniazid was developed from the antituberculous drug, isoniazid, and was reported to elevate mood (Pare and Sandler 1959). It was found to cause inhibition of the enzyme monoamine oxidase (MAO) (Smith 1964), which is a major means of inactivating biogenic amines.

Imipramine was introduced in 1957, and it was the first tricyclic antidepressant. It was shown to inhibit the uptake of NA into nerve terminals (Glowinski and Axelrod 1964) and the uptake of 5-HT into brain slices (Blackburn et al 1967, Ross and Renyi 1967). Since reuptake is a major means of removing amines from the synapse, inhibition of uptake increases the availability of NA and 5-HT at receptors (Iversen et al 1975).

Lithium has been demonstrated to be effective in the treatment of mania in controlled trials (Shou 1968, Baastrup et al 1970) and improves depression in some patients (Mendels et al 1972a). Its mechanism of action is not well understood but it appears to decrease both

catecholaminergic and serotonergic activity in some test systems.

These observations indicated a possible role of amines in depressive illness. The metabolism of biogenic amines, particularly NA, DA and 5-HT, has often been found to be disturbed in depression. These amines are all accepted to be transmitter substances in the central nervous system (see Green and Grahame-Smith 1975). The functions and metabolism of these amines have been widely investigated in order to improve our understanding of the aetiology of depressive illness. It has been proposed that depression is caused by a deficiency of NA or 5-HT at specific receptor sites. On the basis of the fact that different patients appeared to have abnormalities of either NA or 5-HT function, it was suggested by Van Praag (1971) that there may be two types of depression, with an abnormality of either noradrenergic or serotonergic function.

During the research undertaken for this thesis particular attention was paid to the role of 5-HT in depressive illness. The synthesis of 5-HT takes place by a two-step process (see Fig. 1). Firstly tryptophan (TP) is converted to 5-hydroxytryptophan (5-HTP) by the enzyme TP hydroxylase in the presence of a pteridine cofactor. The 5-HTP is then decarboxylated to 5-HT in the presence of pyridoxine (vit B6). After release, 5-HT is deactivated by reuptake and metabolism via an aldehyde intermediate to 5-hydroxyindoleacetic acid (5-HIAA) by the action of MAO (see Baldessarini 1975).

Attempts have been made to test the hypothesis that depression is caused by a lack of 5-HT at specific receptor sites in the brain. Observations were made of the levels of 5-HT in the brains of depressed suicides and on the metabolites of 5-HT in brain and body fluids. Since 5-HT is not taken up into brain, the precursor amino acids, TP and 5-HTP

were administered to depressed patients in the hope that this would increase the availability of 5-HT in the brain and clinical improvement would be seen.

2. 5-HT dysfunction in depressive illness

2.1 Evidence from post-mortem studies

The levels of 5-HT in the hindbrains of suicide victims have been found to be lower than those in controls who died from accidents or acute illnesses (Shaw et al 1967). 5-HT levels were also found to be lower in the brainstem of a group of suicides than in normal controls (Pare et al 1969). In the latter study the suicides were suffering from reactive rather than endogenous depression. In another study no change in 5-HT levels was found but levels of 5-HIAA were lower in depressed patients after suicide than in control subjects after death from natural causes (Bourne et al 1968). However, no changes in 5-HIAA in the brainstem were reported by Pare (1969). Other authors found no difference in 5-HT levels in seven brain regions (Beskow et al 1976) or in thirty three different brain regions (Cochran et al 1976) examined in suicides versus controls.

Pare et al (1969) reported a positive correlation between the age of the subject and the brain 5-HT concentration, and these authors suggest that the decrease in 5-HT observed may be somewhat offset by the age differences between suicide and control groups. This observation was confirmed by Robinson et al (1971).

Interpretation of this type of study is difficult due to uncontrolled variables which may have influenced the results.

2.2 Investigations on cerebrospinal fluid (CSF)

Measurements of 5-HIAA levels in lumbar CSF may yield useful information about the cerebral metabolism of 5-HT (Moir 1970) even though it has been reported that some of the 5-HIAA is derived from the spinal cord (Schildkraut 1973). There have been several reports of reduced levels of 5-HIAA in the CSF of depressed patients compared with controls (e.g. Ashcroft et al 1966, Denker et al 1966, Coppen 1972, Van Praag 1970, 1971, McLeod 1971), though other workers found essentially normal levels of 5-HIAA. These conflicting results may be accounted for by the variable nature of the control groups, different conditions under which CSF was taken and relatively non-specific assay techniques. There is also variation between clinicians in the diagnosis of depression.

Probenecid has been used to block the efflux of carboxylic acids, including 5-HIAA, from the CSF. The accumulation of 5-HIAA in the CSF following the administration of probenecid was found to be decreased in a number of recent studies of depressed patients (Van Praag et al 1970, 1971, 1972, Goodwin et al 1973, Sjostrom and Roos 1972, Banki 1977). In most studies the decrease in CSF 5-HIAA levels persisted on recovery (Mendels et al 1972 (b), Coppen 1971). In contrast, noradrenaline metabolites in CSF seem to return to normal levels on recovery which led Kety (1971) to suggest that a 5-HT dysfunction may underlie the repeated attacks of depression while a NA dysfunction is responsible for the actual attacks.

2.3 Urinary metabolites

Urinary 5-HIAA was found to rise in the manic phase and fall

in the depressive phase in a longitudinal study of a group of manic-depressives (Strŏm Olsen et al 1948). Later reports suggested that 5-HIAA excretion might vary in a systematic way with consistent decreases in retardation and increases in agitation (Schildkraut 1973). Response to treatment with MAOI's may be more favourable in patients with relatively low urinary levels of 5-HIAA than in patients with higher urinary levels (Pare and Sandler 1959) but not all studies concur (see Schildkraut 1973). Interpretation of data from urine studies is difficult due to the dependance of 5-HIAA excretion on dietary factors.

2.4 Studies on Tryptophan

It has been reported that although the total serum TP levels are unchanged in depression, there may be a marked reduction in the fraction which is not bound to serum albumin (Coppen et al 1972, 1974(a)). This deficiency returns to normal on recovery (ibid.). However, this reduction in free TP has not been confirmed by other authors (Niskanen et al 1976, Peet et al 1976). It will be seen later that the "free" TP may be important in influencing brain TP and 5-HT synthesis.

There is a circadian rhythm of plasma free TP in normals with a minimum at midday and maximum at midnight. Endogenous depressives have been reported to show no variation in plasma free TP between 8 a.m. and 4 p.m. (Wirz-Justice 1975).

In another study by Coppen (1974(b)) there was no difference in the conversion of radioactive L-TP to 5-HIAA and indoleacetic acid (IAA) in urine, but the apparent volume of distribution of TP was reduced in depressed patients. This was confirmed by other workers (Ashcroft et al 1973 (a and b)).

CSF levels of TP have also been found to be lower in depressives (Coppen et al 1972) but this was not confirmed by other workers (Ashcroft et al 1973 (a and b)). Increased excretion of TP has been found in depressed patients in one study (Wood et al 1978).

It has been suggested that there may be a change in TP metabolism in depression, the activity of its major metabolic pathway, known as the kynurenine pathway, being increased. TP is thus thought to be diverted away from the pathway for synthesis of 5-HT in the brain (Curzon 1969).

2.5 Changes in TP metabolism in depression

There have been several reports of increased excretion of TP metabolites in depression, suggesting overactivity of the kynurenine pathway (see Fig. 2). Increased excretion of kynurenine (Rubin 1967, Curzon and Bridges 1970), 3-hydroxykynurenine (Curzon and Bridges 1970), and xanthurenic acid (Mangoni 1974, Cazzullo et al 1966) after a TP load have been reported. However, one group of workers found no alteration of TP metabolism (Frazer et al 1973). Changes in TP metabolism may be related to an increased activity of the initial rate-limiting enzyme of the kynurenine pathway in depression (Mangoni 1974). Increased urinary excretion of kynurenine paralleled increased plasma cortisol levels (Rubin 1967) suggesting that increased cortisol production in depression induces TP pyrrolase and increases the production of TP metabolites.

2.6 Uptake of 5-HT into platelets

The uptake of 5-HT into platelets has been reported to be lower in depressed patients (Pare et al 1974). Since the 5-HT uptake system of platelets seems to share some similarities with that of central 5-HT terminals (Smith et al 1978, Stahl and Meltzer

1978) this may indicate an abnormal uptake system in depressed patients.

2.7 Changes in enzyme activity

Monoamine oxidase (MAO)

Plasma MAO activity was found to be significantly higher in a group of premenopausal depressed women than in control subjects. Oral administration of conjugated oestrogens (Premarin) produced a significant decrease in MAO activity in the depressed patients, who also reported an improvement in their mood (Klaiber et al 1971). It was also reported that platelet MAO activity was higher in a large group of depressed patients compared with age-matched controls (Nies 1971).MAO activity was found to increase with age, and women had higher mean MAO activities than men. The authors speculate that this may explain the greater incidence of depressive illness in women than in men and the increase in incidence during middle age (Robinson et al 1971, 1972). However, further investigation is necessary to find out if platelet MAO activity gives any indication of brain MAO activity.

Platelet MAO was found to be lower in bipolar depressives than unipolar depressed patients or age-matched controls (Murphy et al 1972) but there was no consistent direction of change of platelet MAO activity in manic or depressed periods.

3. Precursor studies

3.1 Tryptophan

The administration of tryptophan has been reported by several groups of workers to improve depressive illness (Coppen et al 1967, 1972, Broadhurst 1970, Kline and Shah 1973), being favourably compared with ECT or imipramine. However, other workers report that TP was ineffective as an antidepressant in severe depression (Herrington et al 1974, Bowers 1970, Carroll et al 1970). Addition of an inhibitor of MAO (MAOI) with TP enhanced the response to TP (Coppen, Shaw and Farrell 1963, Glassman and Platman 1970, Pollin et al 1961), but caused hypomanic reactions in one study (Gayford et al 1973). It was also noted that TP could potentiate the therapeutic effect of tricyclic antidepressants, such as chlorimipramine (Walinder et al 1975) and MAOI (Coppen, Shaw and Farrell 1963).

The presence of TP increases its metabolism by induction of the pyrrolase enzyme. Thus,administered TP may be metabolised rapidly reducing the effective dose of TP. It was proposed that administration of TP in combination with allopurinol, which has been shown to inhibit pyrrolase *in vitro*, would increase TP availability. This combination has been shown to improve depression in one preliminary study (Shopsin 1978).

In several studies of the therapeutic efficacy of TP, pyridoxine was administered to ensure that there was no functional deficiency of this factor, since it is a co-enzyme in 5-HT synthesis (see Fig. 1).

It was noted by one group of workers that depressed patients treated with tryptophan had greater 5-HT content in their platelets than the same patients before TP treatment (Murphy 1972). This observation may indicate an improved ability to synthesise 5-HT after increasing TP availability.

Dunner and Goodwin (1972) reported that when L-TP was administered to depressed patients there was increased accumulation of 5-HIAA in the CSF but no improvement in mood. Similar observations were made by Bowers (1970), who found that four of six schizophrenics, compared with two of eight depressives, had increased CSF 5-HIAA after L-TP. It was suggested that some depressive patients were unable to utilise the increased TP made available to form 5-HT.

3.2 5-hydroxytryptophan (5-HTP)

Administration of 5-HTP was found to cause clinical improvement in some depressed patients (Van Praag 1972). The therapeutic response was found to be more favourable in patients with an apparent defect in 5-HT metabolism, as indicated by decreased accumulation of 5-HIAA after probenecid (see later chapters), though no correlation was found by other workers (Takahashi et al 1975). The effects of monoamine-oxidase inhibitors have been reported to be potentiated by 5-HTP (Kline et al 1964, Alino et al 1976) but not all reports indicate a therapeutic efficacy of 5-HTP as an antidepressant (Glassman 1969, Pare and Sandler 1959, Mendels et al 1975). In other investigations 5-HTP given in conjunction with a peripheral decarboxylase inhibitor produced mood elevation, disinhibition and neurological

changes (Puhringer et al 1976, Trimble et al 1975).

4. Cortisol secretion in depressive illness

There have been several reports that cortisol secretion is increased in depressed patients (Board et al 1956, 1957, Gibbons and McHugh 1962, Gibbons 1964, Bunney et al 1965, Hullin et al 1967, Rubin et al 1966), though not all authors concur (for review see Sachar 1967). The contradictions are likely to be partly due to differences in endocrine assessment techniques and the design of data collection. Different authors have assayed single samples of plasma, 24 hr. urinary excretion of 17-hydroxycorticosteroids and cortisol production by isotopic dilution techniques. In many of the studies control subjects were not well matched with depressed subjects. There may have also been interference in both the assay procedures and clinical assessments by the drugs many of the patients received. Twenty four hour urinary 17-hydroxycorticosteroids may give a better indication of total secretory activity than single determination of plasma cortisol, though early morning plasma cortisol levels may be more sensitive to milder stress. Many of the studies were performed on hospitalised patients and hospitalisation itself has been reported by some authors to increase cortisol secretion (Sachar 1967, Bunney et al 1969). This hypersecretion decreased as patients became used to the ward (Sachar 1967). However, recent controlled studies found no change in cortisol secretion on getting used to the ward (Shaw et al 1977, Carroll 1972). Studies on patients who would have adapted to hospitalisation have shown that some patients are hypersecretors of cortisol for extended periods (Bunney et al 1969, Hullin et al 1967). There is therefore

a subgroup of depressed patients who are hypersecretors of cortisol. These patients are all severely ill and some authors report that certain clinical characteristics correlate with increased cortisol production, such as suicidal impulses (Bunney et al 1969), high arousal (Sachar 1970) and anxiety (Gibbons 1962). Other authors, however, found no correlation with these characteristics (Carroll 1972).

That there is a significant fall in cortisol secretion on clinical recovery has been shown by many authors (Sachar 1967, 1970, Gibbons and McHugh 1962, Gibbons 1962, McClure 1966, Carroll1972, Shaw et al 1977).

The rhythm of cortisol secretion is often disturbed in depressed patients. In normal subjects who sleep from midnight to 8 a.m. there is virtually no secretion of cortisol for six hours, from late evening to early morning, then, beginning at about 2 a.m., cortisol is secreted in distinct bursts, the longest occurring between 5 a.m. and 9 a.m. There are usually seven to nine secretory episodes per day, with the maximum concentration at 8 a.m. The normal range of plasma cortisol is 6-26 ug/100ml (Scott 1978). In depressives, cortisol secretion does not cease in late evening, as in normals, so that there is relative hypersecretion in the late evening and early morning (Doig et al 1966, Sachar et al 1973, McClure 1966).

Dexamethasone, a potent synthetic corticosteroid, suppresses ACTH secretion in normal subjects and causes cortisol secretion to fall to zero after about twelve hours and remain suppressed for twenty four hours. Depressed patients with high evening cortisol levels are more resistant to suppression by dexamethasone than at other times of day. Late evening hypersecretors do not suppress until clinical recovery is almost complete (Schildkraut et al 1976).

The findings suggest that these abnormalities in cortisol secretion represent a primary neuroendocrine abnormality associated with depressive illness rather than a non-specific stress response.

The large volume of data on cortisol secretion in depressive illness is somewhat contradictory. There appears to be a subgroup of depressed patients who are hypersecretors of cortisol and others who are relative hypersecretors by the fact that their diurnal variation of secretion is abnormal. There is a large volume of evidence to show that cortisol secretion falls on clinical recovery. Some authors suggest a causal relationship between cortisol secretion and depressive illness, but this has not been substantiated.

Depression is frequent in patients with a disturbance of pituitary adrenal functions, and occurs paradoxically in both Cushing's disease (Trethowan and Cobb 1952) and Addison's disease (Cleghorn 1951).

Corticosteroid therapy has been reported to precipitate depressive reactions (Michael and Gibbons 1963, Nielsen and Fischer 1963), and has been a major reason for discontinuation of long term corticosteroid therapy in rheumatoid arthritis (Nielsen and Fischer 1963).

5. Female hormones and psychological state

Psychological state tends to fluctuate in women at times when the endogenous female hormones are in a state of change, such as during the menstrual cycle, at the menopause, during pregnancy, and the puerperium. Mood changes have also been reported in women taking synthetic oestrogen and progesterone preparations for oral contraception.

5.1 Menstrual cycle

Feelings of well-being, alertness and sexual arousal increase in the phase of the menstrual cycle leading up to ovulation, when

oestrogen, but not progesterone, is secreted. Thereafter in the luteal phase, when progesterone is secreted by the corpus luteum relaxation occurs. However, during the premenstrual phase, when progesterone levels are declining, dysphoria reaches a peak (Moos et al 1969). Many women experience a premenstrual syndrome, characterised by swelling of the breasts and abdomen and premenstrual "tension". The swelling is not readily explicable because there is little evidence of water retention at this time but these sensations may be related to redistribution of water and electrolytes between intracellular and extracellular compartments (Coppen and Kessel 1963). It is of interest that such redistribution is found in cases of severe depression (Coppen and Shaw 1963). The incidence of premenstrual tension is about one in sixteen women (Coppen and Kessel 1963) and the symptoms include depression, irritability and lethargy. There may be accompanying sleep disturbance and somatic symptoms, such as headache, migraine and epilepsy (Dalton 1964). In a small group of women the depression may reach clinical proportions. The tendency to commit suicide increases in the premenstrual and menstrual phase (paramenstruum) and at ovulation (Mandell and Mandell 1967, Dalton 1964).

5.2 Pregnancy and the puerperium

There is a significant reduction in the incidence of psychiatric admissions, including those for depressive illness, in pregnancy (Pugh et al 1963), though dysphoria has been reported in the third trimester (Treadway et al 1969). However, in the post-partum period there is an unusually high risk of depressive illness and admissions for depression increase in the nine months following childbirth (Pugh et al 1963). Dysphoria often occurs within the first ten post-partum days, and this syndrome has been called post-partum (Yalom et al

1968) or maternity (Pitt 1973) "blues". The incidence of "blues" has been reported to be about 50% of all post-partum women (Pitt 1973) and may be even greater. The "blues" may onset unexpectedly when lactation ceases (ibid.). The incidence of serious depressions has been reported to be one woman per thousand live births (Paffenberger 1961) but more recent reports indicate that it is 7-10% of women suffering "blues" (Pitt 1973). It was also noted that the infants of patients suffering post-partum psychoses had shorter gestation periods and weighed less (Paffenberger 1961). Of these psychotic patients 35% had recurrent psychoses in subsequent pregnancies (ibid.).

Plasma levels of oestrogens and progestagens rise throughout pregnancy and fall abruptly at parturition. Glucocorticoid levels also rise during human pregnancy, increase sharply at parturition, then fall during the puerperium (Plager et al 1964, Okada et al 1974, Batra and Grundsell 1978). The rise in cortisol levels is secondary to an induction of the formation of the cortisol-binding protein, transcortin, by oestrogen (Plager et al 1964, Treadway et al 1969). The occurrence of depressive symptoms has been correlated with progesterone withdrawal (Lorraine and Bell 1963, Hamburg 1966, Janowsky and Davies 1970). It may be significant that the uptake of 5-HT into brain slices is reduced during progesterone withdrawal in animals (Hackman et al 1973).

5.3 The Menopause

The discontinuation of menstruation at the menopause may be gradual, with decreasing loss at menstruation, or menstruation may be increasingly irregular, or end abruptly. Depression frequently occurs at the menopause, particularly if there has been an abrupt

end of menstruation, when there is a sudden fall in hormone secretion. The psychological symptoms associated with the menopause include anxiety, depression, irritability, emotional lability, headache, insomnia, fatigue and apathy. There are significantly more menopausal complaints in those who have borne children or had marked dysmenorrhoea.

5.4 Oral contraceptives

Depression has often been reported to be a side effect of oral contraceptive administration (Wynn et al 1975, Herzberg and Coppen 1970, Herzberg et al 1970, Adams et al 1973, Kane et al 1967, Nilson and Almgren 1968, Marcotte et al 1970). The reported incidence of depression in oral contraceptive users could be as much as 30% (Huffer et al 1970), though it is more likely to be 5-7% (Herzberg and Coppen 1970). The depression may improve after the first month of oral contraceptive use (Grounds et al 1970). Contraceptives with high progesterone/oestrogen ratios have been reported to be more likely to cause depression (Grant and Pryce-Davies 1968). Sequential contraceptives, where oestrogens are replaced by progestagens in the latter part of the month, reproduce the u-shaped curve of dysphoric affect seen in the normal menstrual cycle (see section 5.1), whereas the combination type obliterate the curve (Paige 1971).

6. Factors affecting the synthesis of 5-HT

6.1 Enzymes

The synthesis of 5-HT could be controlled by the activities of the enzyme TP hydroxylase or 5-HTP decarboxylase (see Fig. 1). Tryptophan hydroxylase is the rate-limiting enzyme in 5-HT

synthesis (Grahame-Smith 1964, Green and Sawyer 1966) and may not be saturated with its substrate.

6.1.1 Hydroxylase

Most of the studies of kinetic constants of TP hydroxylase have been made with the synthetic cofactor 6, 7 dimethyl-tetrahydropterin ($DMPH_4$). The Km of hydroxylase with this cofactor is 290uM, a finding which supports the view that the enzyme in vivo is unsaturated with TP, since its concentration in brain is about 50uM (Jequier et al 1969, Friedman et al 1972). The nature of the natural cofactor is uncertain as it has not been positively identified in brain. It is important that the natural cofactor is determined since tetrahydrobiopterin (BH_4), another possible cofactor, gives a Km for hydroxylase of 50uM, that is the actual brain TP concentration. The activity of TP hydroxylase can be modified by various treatments, and is possibly increased by morphine (Costa and Meek 1974) and decreased by LSD (ibid.) and lithium (Meek and Neff 1972). Reserpine has been reported to increase midbrain TP hydroxylase activity by increasing corticosteroid secretion. Pargyline prevents the action of reserpine on hydroxylase, possibly by preventing the increase of plasma corticosteroid (Costa and Meek 1974). Cortisol itself has been reported to increase TP hydroxylase activity (see section 8.2).

6.1.2 Decarboxylase

The enzyme which decarboxylates 5-HTP is probably the same enzyme which decarboxylates dopa, and it is often referred to as amino-acid decarboxylase. However, there are some reports that there are two enzymes which act on 5-HT and dopa, which

have different temperature and pH optima (for review see Green and Grahame-Smith 1975). Further investigations into the nature of decarboxylase are needed. In rat brain the concentration of 5-HTP is low, due to its rapid conversion to 5-HT, so that the enzyme is not rate-limiting. However, there has been one report (Rubin et al 1967) that the enzyme may not have the high activity in the human brain that it does in rat brain.

6.1.3 Monoamine oxidase (MAO)

An important route of degradation of 5-HT is by oxidative deamination by monoamine oxidase (for review see Costa and Sandler 1972, Youdim 1975). There may be two types of MAO, A and B, A being inhibited by clorgyline and having tyramine and 5-HT as substrate. Type B oxidises tyramine also, but does not act on 5-HT (see Neff and Yang 1973). There are many inhibitors of MAO, of which pargyline is one which inhibits the enzyme irreversibly. Many others are used clinically, as antidepressants.

It has been shown that the presence of 5-HT does not inhibit the activity of TP hydroxylase (McGeer and Peters 1969, Grahame-Smith 1964, Jequier et al 1969). This observation led various workers (e.g. Millard and Gal 1971) to suggest that 5-HT synthesis is not regulated by end product.

Some workers (Glowinski et al 1973, Macon et al 1971, Hamon et al 1972, 1973) have suggested that there is in fact a negative feedback mechanism which controls the synthesis of 5-HT. The evidence for this includes the fact that increasing the tissue levels of 5-HT threefold suppressed synthesis. This effect has

been observed both *in vivo* and *in vitro* and was thought to be due to inhibition of the first step of 5-HT synthesis, since no inhibition occurs if 5-HTP is substituted for TP. How this inhibition occurs is not clear, as 5-HT does not inhibit hydroxylase (McGeer and Peters 1969, Grahame-Smith 1964, Jequier et al 1969). Other workers (Carlsson 1972) have suggested that the inhibition of synthesis may be by stimulation of post synaptic 5-HT receptors which by a feedback loop reduces impulse flow.

However, Millard et al (1972) have disputed that there is feedback inhibition, suggesting that the effects of monoamine oxidase inhibition could be interpreted differently. It was suggested that after monoamine oxidase inhibition there is a rapid increase in synthesis followed by a return to normal rate, and it was this reduction toward normal turnover rate which had been observed.

There may be two active 5-HT pools (Glowinski et al 1973, Grahame-Smith 1974). One pool takes up newly acquired tryptophan and has a very high turnover rate of 5-HT. This is thought to be the functionally active pool. The other pool has a slower turnover of 5-HT and was suggested to be functionally inactive.

Synthesis inhibition appears to occur at brain 5-HT concentrations well outside the normal range. It is therefore unlikely that it has an important physiological role. Thus, since TP hydroxylase may not be saturated with its substrate, changes in TP concentration may be expected to affect brain

5-HT synthesis.

6.2 Changes in plasma TP concentration

There is a diurnal variation of plasma TP concentration which has a different pattern from that of other amino acids. The levels are lowest at 2-4 a.m. in humans, and increase by 50-80% to attain a plateau in the late morning or early afternoon. Rats, which feed at night, had the daily nadir and peak 8-10 hours later (Fernstrom and Wurtman 1971 (á)). The rhythm cannot be explained by food intake and is likely to be associated with changes in corticosteroid levels, the activity of the catabolising enzyme, TP pyrrolase, and the uptake of TP into tissues. The binding of TP to albumin may also be important, as it could protect plasma TP from catabolism or uptake. The rhythm of plasma TP is reflected by a diurnal rhythm of brain 5-HT levels. Mid-day administration of a dose of L-TP sufficient to increase plasma and brain TP within the nocturnal range of rats caused brain 5-HT to increase by 20-30% within one hour of treatment (Fernstrom and Wurtman 1971 (a)).

Other treatments which elevated plasma TP and brain TP increased 5-HT synthesis. A large single dose of TP rapidly elevated brain 5-HT and 5-HIAA levels (Ashcroft et al 1965), indicating increased 5-HT synthesis. Injection of the hormone insulin into fasting rats elevated serum and brain TP within two hours, and brain 5-HT levels also increased to a maximum after two hours (Fernstrom and Wurtman 1971 (b)). Administration of various drugs which alter plasma and brain tryptophan also change brain 5-HT (for review see Knott and Curzon 1972).

Changes of diet which affect plasma TP and the relative plasma concentrations of various amino acids, can also affect brain TP levels and 5-HT synthesis (see sections 6.3.2 and 6.4).

Thus, it can be seen that the rate of 5-HT synthesis is partly dependant on brain TP levels. The availability of TP to the brain is therefore very important.

Tryptophan is taken up into brain from the plasma by an active process, probably a facilitated carrier mechanism (Grahame-Smith 1964). There are two saturable uptake systems for TP and the high-affinity uptake is likely to be for transmitter synthesis. The availability of plasma TP and the efficiency of the uptake process are important factors in controlling brain TP concentration.

6.3 Factors affecting TP availability

6.3.1 Binding to serum albumin

TP is unusual in that it is highly bound to serum albumin (McMenamy and Oncley 1958, McMenamy et al 1957, Fuller and Roush 1973). It was suggested (Tagliamonte et al 1971a) that only the "free" portion is available for transport into brain (see Fig. 3). This suggestion is supported by the finding that the specific activity of ^{3}H-TP remains about twice as high in the serum as that in the brain, suggesting that a compartment of TP does not exchange with the brain, i.e. the TP bound to albumin (De Montis 1977). The binding of TP can be decreased by certain drug molecules, such as salicylate (McArthur and Dawkins 1969, Tagliamonte et al 1973), aspirin (Aylward and Maddock 1973, Smith and Lakatos 1971) and other rheumatic drugs(McArthur and Dawkins 1971)and benzodiazepines (Muller and Wollert 1975, Bourgoin et al 1975). The free TP

also increases when the concentration of unesterified fatty acids increases (Curzon and Knott 1974,1975, Bourgoin et al 1974, Curzon et al 1973, Curzon, Friedel and Knott 1973). As the plasma free TP increases, 5 - HT synthesis is stimulated (Gessa and Tagliamonte et al 1971, Knott and Curzon 1972).

Madras et al (1974) have questioned whether the plasma free TP directly influences the brain TP. On feeding rats a high fat, carbohydrate rich diet which increased* UFA and free TP brain TP was not increased. This observation could be explained (Yuwiler et al 1977) by the fact that the increase in free TP was overwhelmed by the aminoacid imbalance produced. Competition by other neutral aminoacids was found to have a greater influence on TP uptake than free TP because TP could be stripped from albumin in the cerebral blood vessels (ibid).

6.3.2. Competition by other amino acids

The presence of the large neutral amino acids, tyrosine, phenylalanine, leucine, isoleucine and valine, reduce brain tryptophan in vivo (Fernstrom and Wurtman 1972). These amino acids have been shown to compete with tryptophan for uptake into brain in vitro (Kiely and Sourkes 1972) and in vivo (Oldendorf 1971). Some tryptophan metabolites which are also neutral L-amino acids such as kynurenine, have also been found to reduce tryptophan uptake (Green and Curzon 1970)

Changes of diet which change the relative amounts of various amino acids in the plasma may also affect the uptake of tryptophan into brain (see section 6.4.)

* UFA = unesterified fatty acid

6.3.3 Extracerebral metabolism of TP

Tryptophan is metabolised by two major routes other than in protein synthesis (see Figs 1 and 2). The larger proportion of tryptophan is metabolised via the kynurenine pathway in the liver (see Fig. 2). The rate-limiting initial enzyme on this pathway is tryptophan pyrrolase. This enzyme is present in tissues other than the liver and is found at low levels in the brain (Gal 1974). Pyrrolase activity is increased by the presence of tryptophan and structurally related compounds e.g. α-methyl-tryptophan (Sourkes and Townsend 1955). This is not a substrate for the enzyme and its action is not by induction of enzyme formation but is on the formed enzyme (Civen and Knox 1960). The activity of pyrrolase can be induced by a variety of agents including cortisol (e.g. Thomson and Mikuta 1954, Knox and Auerbach 1955), ethanol (Badawy and Evans 1977), contraceptive steroids (e.g. Adams et al 1973, Rose 1966), barbiturates and sulphonamides (Badawy and Evans 1977).

High adreno-cortical activity during stress increases the activity of this pathway (Nomura 1965). This could result in diversion of tryptophan away from the important pathway for the synthesis of 5-HT in the brain (Curzon 1965).

Acceleration of the kynurenine pathway increases the utilisation of pyridoxine (Vitamin B_6) by the liver. This could result in a functional pyridoxine deficiency. 5-HT synthesis requires pyridoxine at the decarboxylation step, so that a deficiency would slow 5-HT synthesis. A deficiency of

pyridoxine has been reported to occur after administration of oral contraceptives (e.g. Price and Toseland 1969) (see section 9.1.3).

Control of tryptophan metabolism

Plasma corticoids have a well-defined diurnal rhythm (see section 4) and the activity of tryptophan pyrrolase roughly follows the rhythm of plasma cortisol, but about six hours later. The rhythm of plasma TP shows no clear relationship to that of pyrrolase activity (Hardeland and Rensing 1968). The pyrrolase rhythm is almost completely flattened out by adrenalectomy (ADX), whereas a TP rhythm is still apparent.

Pyrrolase influences the TP concentration more strikingly following TP administration. The rate of disappearance of injected TP from rat plasma is proportional to liver pyrrolase activity (Knox 1966). Conversely, when pyrrolase is decreased by adrenalectomy the plasma TP concentration remains high after TP injection much longer than in intact rats.

The protective action of pyrrolase against the toxic effects of administered tryptophan is consistent with this finding. The dependence of the fate of exogenous TP upon pyrrolase activity could be of therapeutic relevance. TP has been used in the treatment of depressive illness (Coppen et al 1967) and many depressives appear to have high pyrrolase activity (see section 3). These findings suggest that in some patients high pyrrolase activity might oppose the therapeutic efficiency of administered TP.

It is possible that TP can be diverted away from the 5-HT

synthetic pathway by increased pyrrolase activity (Curzon 1969). α-methyltryptophan is capable of decreasing brain 5-HT synthesis by this mechanism. It causes prolonged and extremely high pyrrolase activity, reduced brain TP, 5-HT and 5-HIAA levels, suggesting reduced 5-HT synthesis (Sourkes et al 1970).

Other procedures which increase pyrrolase activity, such as injection of adrenocorticoids, may also lead to decreased brain 5-HT (see section 8). There was found to be a temporal relationship between induction of pyrrolase and reduction of brain 5-HT (Curzon and Green 1968).

6.4 Effects of diet and insulin release

Food deprivation has been reported to increase brain TP and 5-HT synthesis. The increase in brain TP was not accompanied by an increase in total plasma TP (Curzon et al 1972) but the concentration of free TP increased considerably (Gessa and Tagliamonte 1974, Knott and Curzon 1972) apparently due to increased circulating unesterified fatty acids (UFA) (Knott and Curzon 1972). These changes may be due to increased lipolysis caused by the stress of food deprivation (see section 7). Administration of the antilipolytic agent nicotinic acid to fasting rats reduced plasma UFA, free TP, brain TP and 5-HIAA (Curzon and Knott 1974).

When insulin was administered to food-deprived animals, UFA and free TP decreased (Curzon 1974) but brain TP increased with increased 5-HT (Curzon 1974, Fernstrom and Wurtman 1971 (b)) and 5-HIAA levels (Curzon 1974), indicating increased 5-HT synthesis (Curzon 1974, Fernstrom and Lytle 1976). This effect may be due to increased TP uptake after insulin (Dickerson and Pao 1975). When insulin

was given to fed rats there was a decrease in serum UFA, free TP, brain TP, 5-HT and 5-HIAA (Curzon 1974).

When rats were fed a carbohydrate-fat (protein free) diet serum TP, brain TP and 5-HT concentration increased. These changes were presumably due to increased insulin secretion (Curzon and Knott 1977).

However, a low-protein diet decreased brain TP to 20% of normal and reduced 5-HT and 5-HIAA levels in regions of rat brain (Dickerson and Pao 1975). If the diet was supplemented with protein, 5-HT and 5-HIAA levels were restored to normal after seven days. On ingestion of a normal mixed diet brain TP did not rise, and in fact fell in some animals (Fernstrom, Larin and Wurtman 1973). These observations were later explained by the fact that the protein ingested would contain a mixture of amino acids, some of which could compete with TP for uptake into brain. When rats were given a diet including all amino acids, there was a slight increase in brain TP, whereas ingestion of a diet lacking the large neutral amino acids dramatically increased brain TP, 5-HT and 5-HIAA. The serum concentration of amino acids deleted from the diet fell relative to fasted controls. When animals ingested a diet lacking only acidic amino acids, the results were identical to those found for animals ingesting the complete diet, confirming that the neutral, but not acidic, amino acids compete with TP for uptake into the brain (Fernstrom and Wurtman 1972).

Chronic ingestion of a TP free diet causes brain 5-HT levels to be well below those of control animals (for review see Fernstrom

and Lytle 1976), and brain TP could be increased by feeding a diet supplemented with TP. Injecting L-TP into tryptophan-deficient rats also increased serum and brain TP, brain 5-HT and 5-HIAA.

Thus, the net brain TP concentration may result from a combination of the increase in TP uptake caused by insulin release, the degree of lipolysis occurring, and competition for uptake with other amino acids.

6.5 Influence of serum non-esterified fatty acids (UFA)

Various drug treatments e.g. L-dopa, aminophylline, theophyline, have been found to increase serum free TP, brain TP and 5-HT synthesis, and increase the plasma unesterified fatty acid (UFA) concentration (Curzon and Knott 1977). Since free fatty acids have previously been shown to displace TP from its binding to albumin an increased serum UFA concentration would be expected to increase plasma free TP, brain TP and 5-HT synthesis.

Anti-lipolytic drugs e.g. nicotinic acid have the opposite effects, decreasing UFA, free TP and brain TP (ibid.), brain 5-HT and 5-HIAA levels, suggesting decreased 5-HT synthesis (Curzon 1974).

The sympathomimetics isoprenaline and noradrenaline increase UFA and free TP. In experimental acute hepatic failure plasma UFA and free, but not total TP, increased, accompanied by increased brain TP and 5-HIAA, indicating increased 5-HT synthesis (Curzon et al 1973). In this situation no relation was found between competing amino acids and brain TP concentration, and the free TP was the determinant of brain TP.

7. Influence of stress

When an animal is subjected to adverse environmental conditions it suffers from stress. Severe stresses such as footshock have been applied experimentally and the response to stress consists of an increase in pituitary-adrenal activity producing increased secretion of adrenocorticotrophic hormone (ACTH) and increased plasma corticosteroids. There is also increased activity of the sympathetic nervous system, causing secretion of adrenaline and noradrenaline and a resultant increase in lipolysis.

Release of corticosteroids may increase TP pyrrolase activity (see section 8) which increases TP catabolism and reduces the availability of TP to the brain. ACTH and corticosteroids influence lipolysis *in vitro* and may be expected to increase plasma UFA and plasma free TP (Knott and Curzon 1977) but this was not confirmed by injection of hydrocortisone *in vivo*, when the UFA did not increase and the binding of TP to plasma albumin was enhanced (Green et al 1975).

Release of noradrenaline enhances lipolysis, increasing plasma UFA and free TP (Knott and Curzon 1972).

The effects of stress of various kinds on 5-HT synthesis have been determined. The results are often contradictory, and the effects observed may depend on the contributions made by the components of the stress response.

Thus, brain TP has been found to be increased (Curzon et al 1972, Curzon, Joseph and Knott 1972, Diez 1976, Neckers Ph.D. 1975) or unchanged (Curzon and Green 1971) after various stressors. Brain 5-HT levels have been reported to be little affected (Rosecrans 1969) in whole brain,

though a significant reduction in 5-HT was found in the telencephalon in rats. Other workers found a significant decrease in hypothalamic nuclei (Palkovits et al 1976) or whole brain (Green and Curzon 1975) or a slight decrease in brainstem-mesencephalon (Thierry et al 1968). Unfortunately the effect observed is not related to the type of stressor used, though one author has demonstrated a differential effect of two types of stressor on 5-HT turnover rate (Rosecrans 1969).

Some authors report increased 5-HT synthesis (Neckers Ph.D. 1975, Thierry et al 1968) after acute stress. Curzon and Green (1971) have demonstrated increased pyrrolase activity after immobilisation stress which would be expected to decrease turnover.

Chronic stress may also increase the activity of TP hydroxylase (Neckers Ph.D. 1975, Azmitia and McEwen 1974), though not all workers agree (Diez 1976). The significance of increased TP hydroxylase activity depends on whether or not the enzyme is saturated with its substrate (see section 6).

In psychiatric illness there is very often emotional stress. Emotional tension has been associated with a pronounced rise in the plasma unesterified fatty acids (Cardon and Mueller 1966, Van Praag and Leijnse 1966). However, depressed patients have been reported to have low plasma free TP (Coppen 1972) which may be expected to be associated with low plasma unesterified fatty acid (UFA) levels. Other authors report plasma free TP to be unchanged (Peet et al 1976) or increased (Niskanen 1976). Stein et al (1976) found that the free TP was reduced on day 6 post-partum in patients with the most severe depression. The reported increase in UFA in psychiatric illness was

thought to be due to increased levels of circulating catecholamines in depression, but this was disputed by one study, which found that the sympathetic activity was in fact lower in depression (Perez-Reyes 1969). The reasons for the anomaly between UFA and free TP are thus uncertain.

8. Effects of adrenocorticoids

8.1 Effects on brain TP and 5-HT synthesis

8.1.1 adrenalectomy

Adrenalectomy has been reported to decrease TP levels in the brainstem and striatal regions of rat brain, accompanied by decreased 5-HT and increased 5-HIAA levels, indicating increased 5-HT synthesis (Telegdy and Vermes 1975). Administration of corticosterone to adrenalectomised animals tended to normalise 5-HT synthesis, increasing brain TP and 5-HT levels and reducing 5-HIAA levels. Brain 5-HT was also found to be increased if corticosterone was administered to intact animals (ibid.). However, other workers have found decreased whole brain TP, 5-HT levels and synthesis after hydrocortisone was administered to adrenalectomised animals compared with adrenalectomised animals or sham-operated controls (Fuxe et al 1974, Azmitia et al 1970). Brain 5-HT levels have also been found to be unaffected by adrenalectomy (Shields and Eccleston 1973, Fuxe et al 1974). No consistent changes have therefore been observed after adrenalectomy which could be reversed by corticosteroid administration.

8.1.2 cortisol and corticosterone

Contradictory results have been reported concerning

the effects of cortisol on brain 5-HT levels and synthesis. The anomalies may be partly due to the fact that different doses and pretreatment intervals have been used. The importance of the dose used has been illustrated recently by Kovacs et al (1975) who showed that corticosterone at a dose of 1 - 2 mg/kg increased 5-HT levels in the hypothalamus after 30 min., whereas 5 mg/kg had no effect on 5-HT levels, and 10 mg/kg decreased them.

The synthesis of 5-HT was found to be increased after 90 min. pretreatment with cortisol 20 mg/kg in mice (Neckers and Sze 1975). This change was accompanied by increased brain TP, and increased uptake of TP into synaptosomes. Increased brain TP and 5-HT synthesis has also been reported by other workers (Hillier et al, Diez et al 1976, Millard et al 1971) after cortisol, adrenocorticotrophic hormone (ACTH) or corticosterone (Millard 1972). Other authors report brain TP to be unchanged (Azmitia and McEwen 1974, Thierry et al 1968) after corticosteroid treatment. There was found to be no change in 5-HT turnover rate when cultured raphe nuclei of newborn rats were cultured with cortisone or ACTH (Halgren and Varon 1972).

Recent reports indicate a fall in brain TP after cortisol (Green and Curzon 1968, Green et al 1975) accompanied by decreased liver TP, plasma free TP, brain 5-HT levels and synthesis (Green et al 1975). Many workers have reported a reduction in brain 5-HT levels (Curzon and Green, Green and Curzon 1968, Yuwiler et al 1971, Fuxe et al 1973, Yuwiler and Geller 1974)

after cortisol 5 - 20 mg/kg with pretreatment intervals of 3 - 10 hr. Brain 5-HIAA levels were also reduced, indicating reduced synthesis (Curzon and Green 1971). The fall in brain 5-HT was accompanied by increased activity of TP pyrrolase. It was suggested that an induction of pyrrolase which would increase TP catabolism, could reduce the availability of TP to the brain and reduce 5-HT synthesis. Injection of allopurinol into animals treated with cortisol prevents the fall in brain 5-HT (Green and Curzon 1968) and this was reported to be due to inhibition of pyrrolase by allopurinol. Allopurinol inhibits pyrrolase activity *in vitro*, but Joseph, Young and Curzon (1976) failed to demonstrate an inhibition of pyrrolase by allopurinol *in vivo*. On chronic dosing of animals with cortisol, brain 5-HT levels returned toward normal (Curzon and Green 1968). The reduction in brain 5-HT after cortisol, accompanied by pyrrolase induction, could not be demonstrated in older rats, which were more than 66 days old (Green and Curzon 1975). Maximal induction of pyrrolase occurred after different pretreatment intervals, depending on the salt of hydrocortisone injected. Hydrocortisone succinate gave the most rapid increase to peak pyrrolase activity (Green and Curzon 1975).

Cortisone (Shah et al 1968) and hydrocortisone (Joseph, Young and Curzon 1976) were found to inhibit the increase in brain 5-HT levels which normally occurs after TP loading.

8.2 Effects of cortisol on tryptophan hydroxylase

The activity of tryptophan hydroxylase has been found to be markedly decreased after adrenalectomy, and could be restored by treatment with corticosteroids (Azmitia and McEwen 1974, Rastogi and Singhal 1978). Cortisol has been found by some workers to increase tryptophan hydroxylase activity in adult intact (Azmitia and McEwen 1974) and neonatal (Sze et al 1976) rats, but other workers did not confirm the increases in activity in adult rats (Sze et al 1976, Diez et al 1976). Corticosterone and ACTH were also found to increase TP hydroxylase activity (Millard et al 1972).

TP hydroxylase activity is low in the newborn, and increases during postnatal development (Sze 1976). Adrenalectomy prevented the developmental rise in TP hydroxylase activity. If corticosterone 5 mg/kg was given to rats at 9 days old TP hydroxylase activity increased (Kuriyama et al 1971). The significance of these results may be limited if the enzyme is unsaturated.

8.3 Effects on the kynurenine pathway

Cortisol causes a marked increase in the activity of the kynurenine pathway. It induces the rate-limiting head enzyme tryptophan pyrrolase (see Fig. 2) in rats (Thomson and Mikuta 1954, Knox and Auerbach 1955, Hillier, Hillier and Redfern 1975, Green and Curzon 1975, Green, Woods and Joseph 1976), mice (Monroe 1968), humans (Altman and Greengard 1966) but not in gerbils (Green, Sourkes and Young 1975). The degree of induction can depend on the age of the animal, with younger rats showing greater induction of pyrrolase than older rats (Green and Curzon 1975). Increased pyrrolase activity correlates well with increased kynurenine production in vitro

(Green, Woods and Joseph) and excretion after a TP load in vivo (Altman and Greengard 1966).

Increased activity of this pathway decreases the availability of TP to the brain and may be expected to reduce synthesis.

8.4 Behavioral effects of cortisol

Parenteral 5-HTP or intracerebroventricular (icv) 5-HT causes behavioural changes in the mouse, including twitching of the head which is amenable to quantitative determination. Twenty four hour pretreatment with a very low dose of cortisol (75 ug/kg) markedly increased the twitch rate. With continued daily dosing there was a maintained fall in the 5-HTP head-twitch after 3 to 5 days treatment whereas the 5-HT twitch rate returned to normal (Handley and Miskin 1972). The increased twitch rate after 24 hr. pretreatment could be due to altered receptor sensitivity and the reduced response after 3 - 5 day pretreatment due to a functional lack of pyridoxine (ibid.).

Thirty minute or 4 hr. pretreatment with a higher dose of cortisol (1 mg/kg) was found to cause a significant reduction in the head twitch rate after 5-HTP (Brotherton and Doggett 1978). It appears that the effect on the head-twitch response may be dose and time-dependent and these authors (ibid.) suggest that the effects of cortisol may not be entirely due to an effect on brain 5-HT levels.

9. Relations between female hormones and 5-HT synthesis

9.1 Humans

9.1.1 Free tryptophan

The plasma free TP has been reported to be lower in the ovulatory phase of the menstrual cycle than during the premenstrual phase (Wirz-Justice et al 1975). This may be contrary to what would have been expected. Depression has often been reported to occur in the paramenstruum (Dalton 1964) and depression has been reported to be accompanied by low plasma free TP (Coppen et al 1974(a)though not all authors confirm this.

9.1.2 monoamine oxidase (MAO)

MAO activity was 20% lower in the premenstrual phase than at ovulation (Wirz-Justice et al 1975) and was lower during pregnancy than at six weeks post-partum (ibid.). Low MAO activity may be expected to cause increased 5-HT levels if platelet MAO activity is related to MAO activity in the central nervous system. MAO activity has been reported to be higher in women than in men and exhibits greater variability.

9.1.3 Effects of contraceptive steroids on TP metabolism

A disturbance of TP metabolism has often been found in women taking oral contraceptives (Green et al 1978, Brown et al 1975, Luhby et al 1971, Adams et al 1973, Leklem et al 1975, Rose and Adams 1972). Increased excretion of TP metabolites including 3-hydroxyanthranilic acid, 3-hydroxykynurenine, kynurenine and xanthurenic acid have been reported after a TP load. Price and Toseland (1969) reported an increase in urinary 3-hydroxyanthranilic acid without TP loading in women taking oral contraceptives. Some authors have attributed the change in TP metabolism to an induction of TP pyrrolase (Adams

et al 1973, Leklem et al 1975) which would increase the production of kynurenine pathway metabolites. However recent work disputes that pyrrolase is induced by oral contraceptives (Green et al 1978). Abnormal TP metabolism has also been reported in pregnancy (Sprince et al 1954) and following the administration of oestrogen, but not progesterone, to men (Wolf et al 1970).

Acceleration of the kynurenine pathway increases the utilisation of the co-factor pyridoxine which is essential to the first step of the pathway. This may lead to a functional deficiency of pyridoxine. It has been suggested that oral contraceptives cause depression by reducing the availability of the pyridoxine co-factor which is also required during 5-HT synthesis at the decarboxylation step (Toseland and Price 1969, Price and Toseland 1969). It was suggested therefore that depression could be corrected by giving pyridoxine to oral contraceptive users.

A deficiency of pyridoxine has been found in some women taking oral contraceptives (Adams et al 1973, Wynn et al 1975). The deficiency could be corrected by the administration of pyridoxine and the excretion of TP metabolites returned towards normal, although in some cases large doses of pyridoxine were needed (Wynn et al 1975). Administration of pyridoxine has also been shown to improve depression in some oral contraceptive users (ibid.).

The excretion of metabolites after an oral dose of L-kynurenine was also found to be elevated in oral contraceptives users indicating that abnormal TP metabolism may not only be due

to induction of pyrrolase. There may also be a change in the activity of enzymes beyond kynurenine in this metabolic pathway (Brown et al 1975)(see Fig. 2).

It was also observed that pyridoxine deficiency in baboons caused abnormal excretion of TP metabolites (Verjee 1975) indicating that the increased excretion of TP metabolites may be secondary to a pyridoxine deficiency.

A recent study has investigated the effects of oral contraceptives on the nutritional status of another B-group vitamin, riboflavin. The incidence of riboflavin deficiency was markedly increased in the contraceptive group compared with the control group, and the frequency of deficiency increased among those on oral contraceptives for a longer time (Newman et al 1978). This observation may indicate that oral contraceptives have other effects on nutrition.

9.2 Animal studies

Changes in TP and 5-HT levels in mouse brain have been found in different stages of the oestrous cycle (Greengrass and Tonge 1971). Brain 5-HT levels are at their maximum at dioestrus in the forebrain and midbrain. There is little hormonal activity at this time, oestrogen and progesterone levels being low. In proestrus, when oestrogen levels are increasing brain TP decreases and 5-HT levels are at their minimum at oestrus (ovulation). During metoestrus the progesterone levels increase and the brain amine levels return to dioestrus concentrations. Similar results have been observed in ewes (Wheaton et al 1972) and in the rat (Rozsahegyi et al 1973). The 5-HT concentration in the hypothalamus of the rat was found to vary with the oestrous cycle with

a sharp decline in 5-HT levels at proestrus. Castration decreased the morning hypothalamic serotonin secretion but progesterone and oestrogen increased it.

Removal of the ovaries of mice, so that the sex hormone levels in plasma declined, caused a significant decrease in 5-HT and 5-HIAA levels in all regions of mouse brain (Greengrass and Tonge 1975). When ovariectomised rats were treated with progesterone there was an increase in 5-HT levels in the mid and hindbrain. A combination of oestradiol and progesterone caused only a slight increase in 5-HT levels (ibid.). Chronic treatment with oestradiol in male rats produced a decrease in the 5-HT concentration in the midbrain (Leonard and Hamburger 1974) and this was accompanied by a slight rise in 5-HIAA levels. These effects were reversed by progesterone (ibid.).

Progesterone at a dose of 10 to 20 mg/kg increased 5-HT levels in some areas of rat brain and increased 5-HIAA levels in other regions (Ladisch 1977). These results were suggested to indicate an increase in 5-HT turnover after progesterone. Progesterone has also been found to increase brain TP and 5-HT synthesis in the brainstem of spayed female rats after a dose of 10 mg per animal (Glowinski et al 1973). Other effects on brain function have been found and progesterone was shown to increase the uptake of 5-HT into the preoptic region of rat brain (Wirz-Justice and Hackman 1972).

Contraceptive steroids have been used in sequence and turnover rate measured after inhibition of hydroxylase. Turnover rate was found to be decreased by oestrogen and restored by progesterone treatment (Fuxe et al 1974). In one study low doses of sex steroids were used in combination. Doses of 40 ug of oestrogen and 4 mg

progesterone per 100 g in rats failed to affect brain 5-HT synthesis in ovariectomised females (Hyyppa and Cardinali 1973).

In many studies the doses of steroids used have been very high and may have little relevance to the effects of oral contraceptives in man.

9.3 Behavioural effects of contraceptive steroids

Oestrogens were found to reduce the head-twitch response to 5-HTP in mice whereas a high dose of progesterone (250 mg/kg) potentiated the twitch response (Brotherton and Doggett 1978). A combination of an oestrogen and a progestagen in the same proportion as in the oral contraceptives, (ethinyloestradiol 0.025 mg/kg, norethisterone 1.5 mg/kg) also reduced the twitch response (ibid.). These observations possibly indicate a reduction in the availability of functional 5-HT after contraceptive combinations.

10. Kynurenine pathway metabolites

10.1 Effects of kynurenine pathway metabolites on 5-HT synthesis

Injection of L-kynurenine, 3-hydroxykynurenine and 3-hydroxyanthranilic acid 5 mg/kg decreased brain 5-HT after 2 hr. (Curzon and Green 1971). Metabolites formed subsequent to 3-hydroxyanthranilic acid had no effect at this dose (ibid.). None of these compounds affected the *in vitro* activity of TP hydroxylase or 5-HTP decarboxylase. L-kynurenine and 3-hydroxykynurenine 1 mM decreased the uptake of ^{14}C-TP into brain slices. TP metabolites which were not L-amino acids had no effect (Green and Curzon 1970). Kynurenine appears to compete with TP for uptake into brain. L-kynurenine was also reported to decrease brain 5-HIAA levels indicating decreased synthesis

(Curzon and Green 1971). This would be expected since it reduces TP uptake.

The effects of kynurenine on brain 5-HT synthesis deserve further investigation.

10.2 Influence of kynurenine on behaviour

Low doses of kynurenine (0.5 mg/kg) were found to cause marked potentiation of the head-twitch response to 5-HT and 5-HTP (Handley and Miskin 1977). Higher doses of kynurenine caused antagonism of both responses (ibid.). 3-hydroxykynurenine had a similar action on the head-twitch response to 5-HT and 5-HTP, a low dose causing potentiation of the behaviour and a higher dose inhibiting it.

This was a surprising observation since kynurenine appeared to decrease TP uptake and would be expected to decrease 5-HT synthesis in a dose-dependant manner.

Fig. 1

Synthesis of 5-Hydroxytryptamine

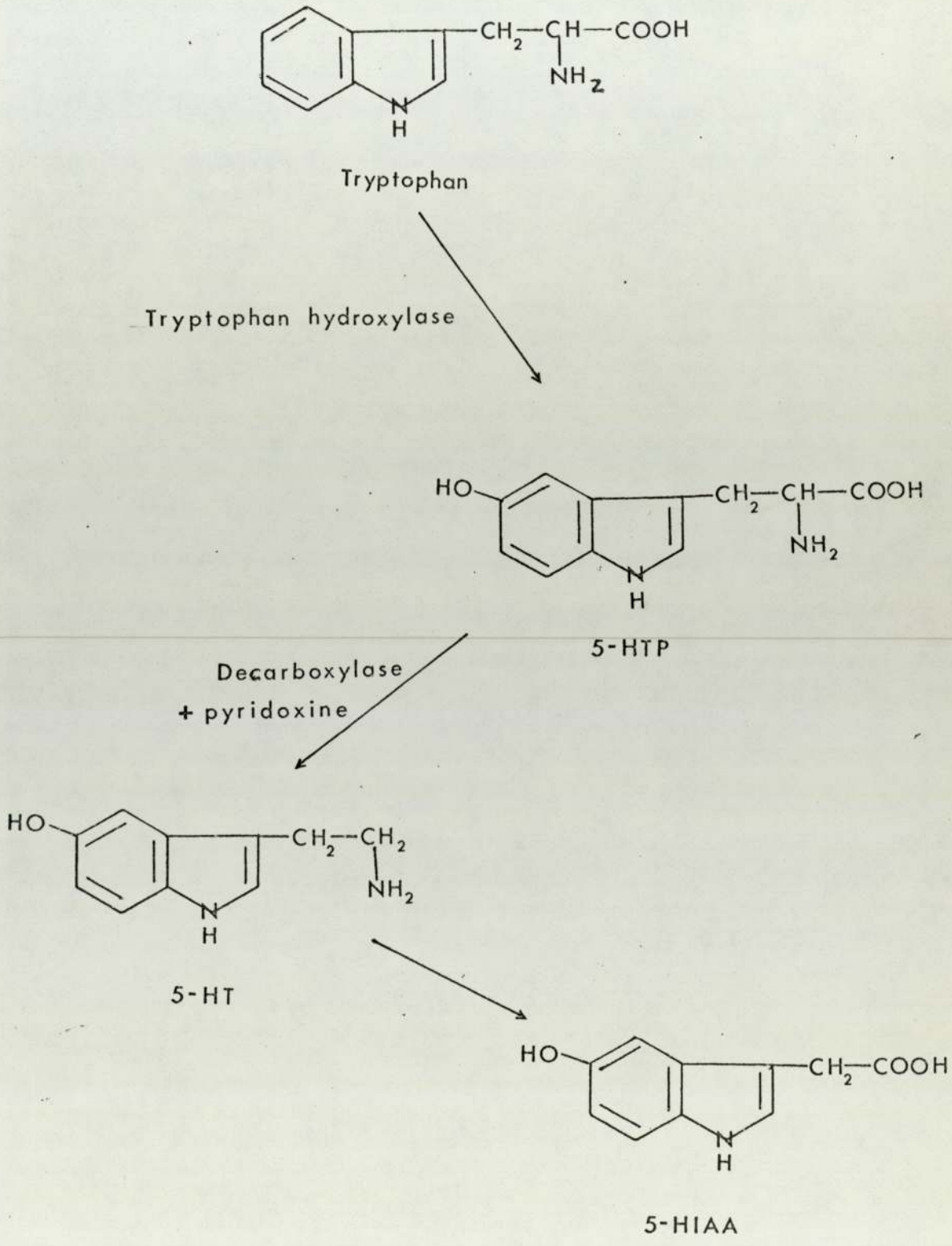
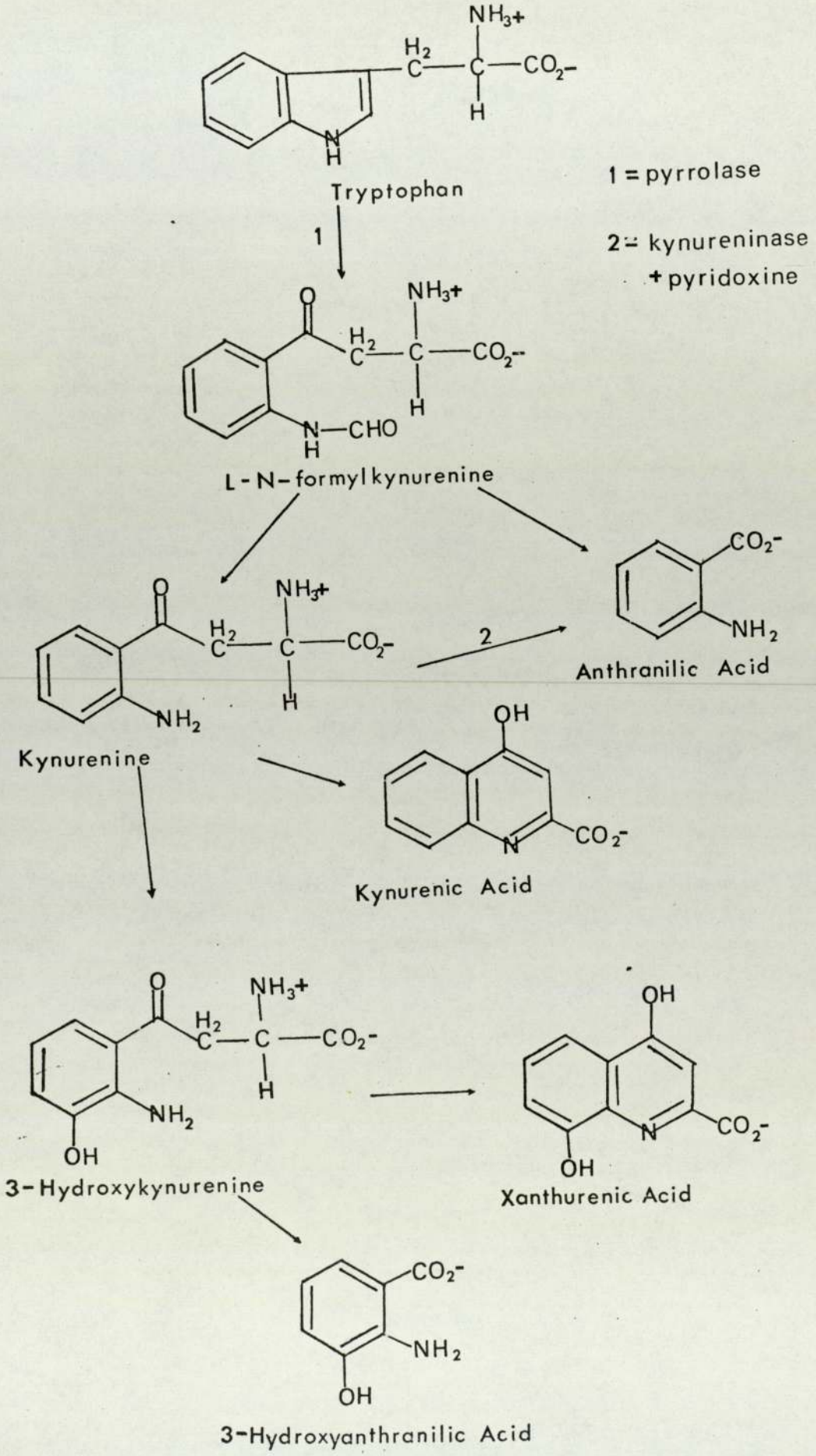


Fig. 2

The Kynurenine Pathway



Aims of the project

It had often been reported that both cortisol and female sex hormones affect brain 5-HT function.

Cortisol was found to reduce 5-HT levels and 5-HIAA levels, indicating reduced synthesis after 5 hr. pretreatment with a dose of 5 mg/kg (Curzon and Green 1968). Cortisol at a very low dose (75 ug/kg) was found to affect animal behaviour (Handley and Miskin 1972). The head-twitch response to 5-HTP and 5-HT was markedly increased after 24 hr. pre-treatment with cortisol whereas the twitch rate fell below normal after 5 day treatment with cortisol. It was planned to determine whether the change in behaviour after this low dose was accompanied by a change in 5-HT turnover rate.

The reduction in brain 5-HT levels after 5 hr. treatment with cortisol was accompanied by induction of TP pyrrolase (Curzon and Green 1968) and hence there was increased production of kynurenine (Green, Woods and Joseph 1976). Since kynurenine was found to decrease TP uptake (Green and Curzon 1970) into brain slices and brain 5-HT levels (ibid.) it was suggested that cortisol reduced brain 5-HT by increasing kynurenine levels. The effect of cortisol on plasma and brain kynurenine levels was to be investigated and the relationship between these and 5-HT turnover established.

Progesterone has usually been found to increase 5-HT turnover (Ladisich 1977, Leonard and Hamburger 1974) whereas oestradiol generally has the opposite effect decreasing 5-HT turnover (Leonard and Hamburger 1974, Greengrass and Tonge 1975). However very high doses of these hormones have been used and it was desirable to determine the effects of lower doses of these hormones, particularly in a combination, such as

occurs in the oral contraceptive pill. Oral contraceptives have been suggested to increase pyrrolase activity in women (Adams et al 1973, Rose 1966). Though changes in TP metabolism have often been reported, these have not always been attributed to pyrrolase induction.

It was possible that these steroids may have a similar mechanism of action on 5-HT turnover to cortisol, that is via increased kynurenine production. This theory was to be investigated by measurement of plasma and brain kynurenine and relating these to the 5-HT turnover.

Little work had previously been done on the mechanism by which kynurenine may affect 5-HT turnover. Dl-kynurenine had been shown to affect animal behaviour, a low dose (0.5 mg/kg) increasing the head-twitch rate after 5-HTP or 5-HT whereas a higher dose reduced it (Handley and Miskin 1977). L-kynurenine 5 mg/kg was also found to reduce brain 5-HT levels (Green and Curzon 1970). The actions of kynurenine on 5-HT turnover were further investigated.

It was possible that cortisol or female hormones affected the binding of TP to albumin, which could affect brain 5-HT synthesis. This idea was to be investigated.

Cortisol secretion had often been shown to be high in depressed patients (e.g. Board et al 1956, 1957, Gibbons and McHugh 1962) and % free TP had sometimes been reported to be lower in depression (Coppen et al 1974 Stein et al 1976). At parturition there is a high incidence of a dysphoric reaction (Yalom 1968, Pitt 1973) and it is a time when there are many hormonal changes occurring. This was thought to be a good model for studying relationships between biochemical variables, such as plasma cortisol, free and total TP, and mood state.

EXPERIMENTAL METHODS

1. Materials

Reagents were obtained from the following companies, and were of analytical grade, except for those obtained from Sigma, which were Sigma grade.

Sigma Chemicals Co. Ltd., Fancy Road, Poole, Dorset supplied

(i) human albumin, essentially fatty acid free prepared from fraction V

(ii) L-tryptophan

(iii) DL-kynurenine sulphate

(iv) L-kynurenine sulphate

(v) O-phthalaldehyde (OPT)

(vi) pargyline hydrochloride

(vii) hydrocortisone-21-phosphate

(viii) 5-hydroxytryptamine creatinine sulphate complex

(ix) 19-norethisterone acetate

(x) Probenecid (p-[dipropylsulphamoyl] benzoic acid

(xi) hydrocortisone

(xii) B-oestradiol-3-benzoate

(xiii) mestranol (ethinyl estradiol-3-methyl ether)

(xiv) Bovine serum albumin

Roche Products Ltd., Welwyn Garden City, Herts. donated

nitrazepam (Mogadon active substance)

Koch-Light, Colnbrook, Bucks. supplied

5-hydroxyindole-3-acetic acid

Baird and Tatlock, Unit 1, Millbook Industrial Estate, Moston Road, Sandbach, Cheshire supplied

(i) NCS tissue solubiliser

(ii) Triton X-100

Radiochemical Centre, Amersham, Bucks. supplied

(i) ^{3}H-hexadecane

containing 4.81×10^{6} dpm/g on 1/3/76

(ii) ^{14}C-tryptophan

s.a. 52mCi/mmol

(iii) ^{3}H-5-hydroxy [generally labelled

-^{3}H] tryptamine creatinine sulphate

s.a. 500 mCi/mmol

(iv) ^{3}H-Dextran 30mCi/g

MW 77,500 (average)

Phase Separations Ltd., Deeside Industrial Estate, Queensferry, Clwyd, N. Wales supplied

(i) silicone OV 225

(ii) chromosorb W AWDMCS 100-120 mesh

Packard Instrument Co. Ltd., Caversham House, 13-17 Church Road, Caversham, Berks. supplied

Dimilune 30 scintillation fluor

R. Emanuel Ltd., Wembley, England supplied

α,α_1 dichloro-p-xylene

J. Burroughs Ltd., 60 Montford Place, London, S.E.11 supplied

absolute ethanol

Fisons, 12 Derby Road, Loughborough, Leics. supplied

Tritium Absorber D.

All other reagents used were obtained from B.D.H. Chemicals Ltd., Poole, Dorset.

2. Instruments

2.1 spectrophotofluorimeter

An Aminco-Bowman spectrophotofluorimeter was used to measure fluorescence in the assays of tryptophan, cortisol, 5-hydroxytryptamine and 5-hydroxyindole-acetic acid. Light from the lamp passed via 3mm slits to excite the sample, which fluoresced at the emission wavelength. The emitted light was amplified by a photomultiplier tube and the fluorescence was read on a meter. Small glass cuvettes, matched for their light-emitting properties, were used.

2.2 Gas-Liquid Chromatograph (GLC)

A Pye-Unicam GCV chromatograph with ^{63}Ni electron-capture detector was used in the gas-liquid chromatographic assay for kynurenine. Various columns were prepared and examined, until a suitable one was found, which gave good resolution, little tailing of peaks, and a reasonable retention time. A 1.5m column of silicone OV 225 1.5% on chromosorb W (AWDMCS 100-120 mesh) at 123°C was used. The detector and injector were kept at 300°C. Nitrogen was used as carrier gas at 80 ml/min and for continual purging of the electron capture detector (ECD) at 30 ml/min.

Preparation of columns

The stationary phase, silicone OV 225 was dissolved in chloroform and coated onto the support, chromosorb W (AWDMCS 100-120 mesh). The chloroform was evaporated off with continual rotation, to ensure even coating of the support. The packing material was then dried under a flow of nitrogen on a fluidised bed. The glass

column (glass to metal seal) was packed under vacuum and vibrated to increase settling and eliminate cracking. The open end was filled with inert glass wool and the column conditioned at 300°C with a flow of nitrogen overnight.

2.3 Integrator

The GLC was linked to an Infotronics CRS 304 integrator, which was programmed to integrate the area under all peaks emerging from the column automatically. The areas of all sample peaks were related to that of the internal standard, dichloro-p-xylene peak on each run. The peak areas were used in all calculations.

2.4 Spectrometer (UV and visible light)

Protein determinations were performed by reading of the absorbance of light at 660nm on a Beckman spectrophotometer. The instrument was used with the tungsten lamp and 0.15mm slits. Due to the small sample volumes obtained, 1ml matched silica microcells were used.

2.5 Scintillation counter

The ^{14}C and ^{3}H isotopes were assayed for radioactivity by liquid scintillation counting on a Beckman instrument with ^{14}C and ^{3}H windows and direct printout on teletype. Various scintillation fluors were used, according to the function required, and these are described under individual methods.

2.6 Biological Materials Oxidiser

A Beckman BMO OX 100 was used for oxidation of blood and brain tissue. The oven temperature was 900°C with the catalyst bed at 700°C. The instrument was flushed with oxygen at 300ml/min

for a sample time of 4 mins. The tissue samples (about 100mg) were completely oxidised to $^{14}C\text{-}CO_2$ or $^{3}H\text{-}H_2O$.

3. Animal husbandry

All animals used were bred in our own laboratories and were albino BK/W strain mice. They were housed in a quiet, air-conditioned environment, with a fixed 12 hour light cycle (light time 07.00 to 19.00 hours) in a constant temperature of 70 $\pm$ 2°F. Animals were fed an ad libitum diet of Heygates modified 41B breeding diet and tap water. All animals used were healthy, and were usually males of the same age, and weighing between 22-30g, unless otherwise specified. Animals were randomly grouped in 6" x 9" cages a few hours before experimentation.

4. Determination of stage of oestrous cycle

Female mice have a four-day oestrous cycle, consisting of four stages namely, proestrus, oestrus (ovulation), metoestrus and dioestrus. Animals were subjected to vaginal smearing by a small glass rod dipped in normal saline. The smear was streaked on a clean glass slide and examined microscopically. The stage of the oestrus cycle was determined by comparison with characteristic smears as described by Simmons (1970). At proestrus the vaginal smear is characterised by sparse nucleated epithelial cells. At oestrus (ovulation) there are large numbers of anucleate, cornified epithelia on the slide. In metoestrus there is an even distribution of cornified epithelia interspersed with small leucocytes. Phase 1 of metoestrus shows a domination of cornified epithelia, and in metoestrus phase 2 leucocytes predominate. In dioestrus the smear shows only leucocytes.

5. Preparation of tissue samples

All experiments were performed at the same time of day (13.00 to 17.00 hours) to minimise variations in pyrolase activity (Hardeland and Rensing 1968) and brain 5-HT(Scheving et al 1968). All doses were expressed as base equivalents. Animals were killed by cervical dislocation and blood was collected from a neck incision via a heparinised funnel into heparinised tubes. Brains were rapidly removed and either assayed immediately or frozen and stored at -18°C for up to fourteen days. Blood samples were centrifuged at maximum speed on an MSE bench centrifuge for ten mins., and the plasma removed and frozen until assayed.

6. Combined assay for plasma tryptophan and kynurenine

6.1 Reagents

(i) stock solutions

tryptophan in 0.1N ammonium hydroxide buffered to pH 7 with sodium dihydrogen orthophosphate 1 mg/ml

dl-kynurenine sulphate 1 mg/ml (base equivalent) in distilled water

(ii) concentrated standard

The above stock solutions were diluted with distilled water to obtain a standard solution containing:-

(tryptophan 500ug/ml
(
(dl-kynurenine 25ug/ml

This solution was stored at -18°C and was used repeatedly without degradation. Distilled water was used to dilute the stock solution to the required concentration.

(iii) acidified butanol:- n-butanol containing 0.85ml concentrated hydrochloric acid per litre

(iv) n-heptane

(v) 0.1M Hydrochloric acid

(vi) 10M sodium hydroxide

(vii) Butyl acetate

(viii) dichloro-p-xylene in butyl acetate 200 ng/ml

(ix) sodium hydroxide/borate buffer containing 1.5g sodium tetraborate in 1.5M sodium hydroxide

(x) trifluoroacetic anydride

(xi) trichloroacetic acid (TCA) 10% in distilled water

(xii) 3.6% formaldehyde in 10% TCA) equal parts
6mM Ferric chloride in 10% TCA)

(xiii) "Tiron":- 1,2 dihydroxybenzene 3,5 disulphonic acid, disodium salt 100 mM

6.2 Method -

0.4 ml plasma was added to 4 ml ice cold acidified n-butanol, mixed and allowed to stand in a refrigerator for 10 mins. The proteins were then removed by centrifugation at 2,000 G in an MSE bench centrifuge for 5 mins. Since plasma contains approximately 75% water, 0.36 ml distilled water or standard solutions were added to the same volume of n-butanol to act as blank and standards respectively. The standards were prepared from the concentrated standard (see reagents) and at least three different concentrations were included in each assay (usually dilutions of 1/25 to 1/100 of concentrated standard). The supernatant butanol was added to 4 ml n-heptane containing 0.4ml 0.1N hydrochloric acid. The mixture was shaken for 10 mins. and centrifuged for 2 mins at

2,000 r.p.m. The organic phase was aspirated off. 300 ul of the acid layer was used for determination of kynurenine and two 50 ul samples were used for determination of tryptophan.

6.2.1 Kynurenine assay

Kynurenine was determined by the method of Joseph (1978). This gas-liquid chromatographic (GLC) method is specific for kynurenine. By using electron capture detection the method will assay very low levels of kynurenine, such as those found in brain, i.e. 100-200 ng/g. Kynurenine is converted by alkaline hydrolysis to o-aminoacetophenone, and this product is fluorinated with trifluoroacetic anhydride to produce an electron-capturing species (see Fig. 4).

To a 300 ul sample of the acid layer, obtained as above, was added 10N sodium hydroxide and 20ul of 100mM "Tiron" (see reagents) and the mixture shaken with 1 ml butyl acetate. 325 ul of the lower aqueous layer was removed and heated in a boiling waterbath in stoppered glass tubes for 20 mins. After cooling, 300 ul butyl acetate containing 200 ng/ml dichloro-p-xylene was added and the mixture shaken. (The dichloro-p-xylene acts as an internal standard, being detected by electron capture, and having a similar retention time to the product from kynurenine) 200 ul of the upper organic phase was added to 200 ul of sodium hydroxide/borate buffer (see reagents) and mixed. The mixture was allowed to settle, 25 ul of trifluoroacetic anhydride was added and immediately mixed, after centrifugation at 2,000 g for 2 mins. 100 ul of the upper organic phase was taken for injection into the GLC (for column and conditions see section "Instruments"). The retention time of the samples

was identical to that of authentic kynurenine (see Fig. 5). The standard curve of kynurenine over the concentrations in plasma is shown in Fig. 6.

6.2.2 Tryptophan assay

Tryptophan was determined fluorometrically by the method of Denckla and Dewey (1967). This method involves cyclisation of tryptophan with formaldehyde and a ferric chloride catalyst to form the highly fluorescent product norharman (see Fig. 7).

To a 50 ul sample of the acid layer, as obtained above, was added 1 ml 10% trichloracetic acid, and 100 ul formaldehyde/ferric chloride reagent (see reagents). The mixture was heated in tubes covered with marbles for one hour in a boiling waterbath. All samples were assayed in duplicate. After cooling, the fluorescence was read on a Aminco Bowman spectrophoto-fluorimeter at excitation 368 nm and emission 448 nm. The fluorescence maxima of plasma samples were identical with those of tryptophan standards and authentic norharman (see Figs. 8 and 9). The standard curve for TP is shown in Fig. 10.

It can be seen from the excitation/emission spectrum of norharman (Fig. 8) that the ratio of the peak height of the first and second maxima are constant. This ratio can be used to detect any quenching of sample fluorescence, since quenching decreases this peak height ratio.

7. Multiple assay for brain amines

(Joseph, personal communication)

7.1 Reagents

(i) stock solutions

(a) tryptophan dissolved in 0.1 M ammonium hydroxide and buffered to pH 7 with sodium dihydrogen orthophosphate 1 mg/ml

(b) kynurenine sulphate in distilled water 1 mg/ml (base equivalent)

(c) 5-hydroxyindoleacetic acid in distilled water 1 mg/ml

(d) 5-hydroxytryptamine creatine sulphate complex in distilled water 1 mg/ml (base equivalent)

(ii) concentrated standard

The above stock solutions were diluted with distilled water to give a concentrated standard containing

tryptophan 500 ug/ml)
kynurenine 25 ug/ml)
5-hydroxyindoleacetic acid 50 ug/ml)
5-hydroxytryptamine 50 ug/ml)

This solution was kept at 18°C and thawed only once, as the 5-HT was unstable to freezing and thawing.

(iii) acidified butanol

n-butanol containing 0.85 ml concentrated hydrochloric acid per litre

(iv) n-heptane

(v) 0.1 M hydrochloric acid

(vi) phosphate buffer 0.5M (pH 7.0)

disodium hydrogen orthophosphate 0.5 M

dihydrogen sodium orthophosphate 0.5 M

These were mixed together in suitable proportions to give pH 7.0.

(vii) o-phthalaldehyde reagent

o-phthalaldehyde (Sigma) crystals dissolved in concentrated hydrochloric acid 4 mg/ml

(viii) cysteine hydrochloride 1% in distilled water

This solution was kept frozen or used freshly prepared.

(ix) 10% trichloroacetic acid

(x) 3.6% formaldehyde in 10% TCA)) in equal parts
6mM Ferric chloride in 10% TCA)

(xi) "Tiron" (1, 2 dihydroxybenzene 3, 5 disulphonic acid, disodium salt) 100 mM

(xii) sodium hydroxide/borate buffer containing sodium tetraborate 1.5 g in 1.5 m sodium hydroxide

(xiii) trifluoroacetic anhydride

7.2 Method

Two brains were pooled for assay of 5-HT, 5-HIAA, tryptophan (TP) and kynurenine. The brains were weighed into 4 ml acidified butanol and homogenised. As 0.8 g of brain tissue contains approximately 0.6 ml of water, 0.6 ml distilled water or diluted standard solution (1/25 to 1/100) were added to 4 ml acidified butanol to act as blank and standard respectively. The butanol was back extracted into 4 ml heptane and 0.8 ml 0.1 N hydrochloric acid. The acid layer was

assayed for TP, 5-HT and kynurenine. A fixed volume of the organic phase was transferred to 0.8 ml phosphate buffer 0.5 M (pH 7.0). The organic phase was extracted into the buffer, which was then assayed for 5-HIAA.

7.2.1 5-HT and 5-HIAA

5-HT and 5-HIAA were assayed fluorometrically by the method of Maickel (1972). This method assays various indoles which are separated by solvent extraction, as above. The acid phase contains only 5-HT, whereas the organic phase contains N-acetyl-serotonin, 5-hydroxyindole-3-acetic acid and 5-methoxy indole-3 acetic acid. 5-HIAA is removed from the organic phase into the phosphate buffer. 5-HTP can also be assayed by extraction into di (2-ethylhexyl) phosphoric acid.

7.2.1 (i) 5-HT

To two 200 ul samples of the acid phase was added 0.65 ml o-phthalaldehyde reagent. 50 ul of cysteine hydrochloride 1% was added and the mixture heated in a boiling water bath for 20 mins. The fluorescence of the product was read on an Aminco-Bowman spectrophotofluorimeter at excitation 360 nm and emission 470 nm (see Figs. 11 and 12).

7.2.1 (ii) 5-HIAA

200 ul samples of the phosphate buffer were assayed for 5-HIAA by the OPT method as described above.

7.2.2 Tryptophan

Tryptophan was assayed fluometrically as described in section 6.

7.2.3 Kynurenine

Kynurenine was assayed by GLC as described in section 6. 50 ng/ml of dichloro-p-xylene (DCPX) in butyl acetate was used as internal standard. The standard curve for kynurenine over the concentration found in brain is shown in Fig. 13.

8. Measurement of 5-HT turnover rate

8.1 Introduction

The renewal rate of brain 5-HT is referred to as its "turnover". The turnover is generally considered to equal its synthesis rate under steady state conditions. Under certain conditions the tissue levels of amines have been found to remain constant, whereas the rate of turnover has changed. This measurement of turnover rate may yield more information concerning the functional state of 5-HT neurons.

Turnover rate may be measured by various means, including the use of non-isotopic and isotopic methods. Most of the isotopic methods are based on injection of labelled tryptophan of high specific activity and measuring its conversion to labelled 5-HT. One of these methods was attempted during this research, but no results could be obtained. The method involved injection of ^{3}H-TP into a peripheral vein in a mouse and after a suitable time interval the brain was removed, homogenised, and assayed for ^{3}H-TP and ^{3}H-5-HT (Costa et al 1968). Since labelled contaminants, such as ^{3}H-5-HTP and ^{3}H-5-HIAA were present, the ^{3}H-TP and ^{3}H-5-HT had to be separated both from each other and from these contaminants.

This was achieved by solvent extraction and ion-exchange chromatography. However, when this procedure was used the separation of ^{3}H-TP and ^{3}H-5-HT from each other was found to be incomplete. When column eluates were chromatographed on thin-layer plates, and visualised using a cinnamaldehyde reagent, cross-contamination of eluates was demonstrated. The recovery of ^{3}H-5-HT was found to be low, as this indole appeared to adhere to the column.

The non-isotopic methods are simpler and relatively low cost, but rely on the following assumptions

(i) 5-HT is formed by synthesis and lost by metabolism

(ii) The rate of formation and metabolism are equal

(iii) The turnover rates of TP and 5-HT are constant during measurement

(iv) No distinction is made between newly synthesised molecules of 5-HT during degredation.

One type of non-isotopic method depends on the use of irreversible inhibitors of monoamine oxidase (MAO), such as pargyline. After injection of pargyline, the brain levels of 5-HT increase linearly for a limited time. On the assumption that MAO inhibition is rapid and complete, and that 5-HT does not leave the brain or become metabolised by other pathways, the synthesis rate of 5-HT can be calculated (Tozer et al 1966).

A second method relies on the same assumptions. If they are correct, the rate of efflux of 5-HIAA equals the rate of 5-HT synthesis, and this can be calculated by the decline of 5-HIAA in brain (ibid.)

Another non-isotopic method makes use of probenecid, which inhibits the efflux of acid metabolites, including 5-HIAA, from the

brain. After probenecid, brain 5-HIAA increases linearly if a maximal dose of probenecid (above 200 mg/kg) is used, and the turnover rate is measured by the rate of increase of brain 5-HIAA.

These three methods showed good agreement for the turnover rate, indicating that the major assumptions were probably valid (Neff et al 1971). Two of these methods, the increase in brain 5-HT after pargyline and increase in 5-HIAA after probenecid, were used in this work. The decline of 5-HIAA after pargyline was investigated, but this proved difficult to measure accurately due to the low brain levels of 5-HIAA.

8.2 Reagents

(i) pargyline hydrochloride 75 mg/10 ml (base equivalent) in normal saline

(ii) probenecid 200 mg/10 ml. Probenecid was dissolved in molar sodium bicarbonate and the solution neutralised to pH 7.0 with potassium dihydrogen orthophosphate.

(iii) Other reagents - see Section 7.2

8.3 Methods

8.3.1 Pargyline Method

Pargyline at a dose of 75 mg/kg has previously been shown to completely inhibit mono-amine oxidase (MAO) in mice (Morot-Gaudry et al 1974). Pargyline i.p. in normal saline caused a linear increase in brain 5-HT levels up to one hour after treatment (see Fig. 14). Animals were killed at various times after pargyline, routinely 0, 20, 40 and 60 mins, the brains removed and assayed for 5-HT as described in

section 7.1. The results were expressed graphically and the nearest straight line fitted by regression analysis. The turnover rate can be calculated from the slope of the line.

8.3.2 Probenecid method

Probenecid 200 mg/kg was injected intraperitoneally (i.p.) with a treatment schedule similar to that described above. The brains were assayed for 5-HIAA as described in section 7.1. The results were treated statistically as above and the rate of increase of 5-HIAA is a measure of 5-HT turnover.

9. Binding studies

9.1 Reagents

(i) Kreb's improved bicarbonate buffer

	g/litre
sodium chloride	6.93
potassium chloride	0.354
calcium chloride (anhydrous)	0.28
potassium dihydrogen orthophosphate	0.162
sodium hydrogen carbonate	2.1
magnesium sulphate ($MgSO_4$ $7H_2O$)	0.29
Dextrose	2.0

Gas with 5% carbon dioxide/95% oxygen to pH 7.4

This buffer was made up without the calcium chloride and stored at 0-4°C. The calcium chloride was added and the solution gassed just before use.

(ii) Tryptophan solution

Tryptophan was dissolved in 0.1M ammonium hydroxide to a concentration of 1 mg/ml and diluted with buffer to a stock solution containing 250 ug/ml. This was

stored at 0-4°C.

(iii) Oestradiol benzoate 1 mg/ml in absolute ethanol was made up and stored at 0-4°C.

(iv) Mestranol 1 mg/ml in absolute ethanol was made up and stored at 0-4°C.

(v) Norethisterone acetate 1 mg/ml in absolute ethanol was stored at 0-4°C.

(vi) Acetylsalicylic acid (aspirin) was dissolved in phosphate buffer pH 7.4. The pH of the buffered solution was 7.2 and this solution was stored at 0-4°C.

(vii) Nitrazepam 1 mg/ml in absolute ethanol was stored at 0-4°C.

(viii) Oleic acid $C_{18}H_{34}O_2$, M.W. 282.5. This was dissolved in ethanol 0.15 mg/10 ml.

(ix) Amitriptyline hydrochloride 1 mg/ml (base equivalent) in distilled water was stored at 0-4°C.

(x) Maprotiline hydrochloride 1 mg/ml (base equivalent) in distilled water was stored at 0-4°C.

(xi) 10% trichloroacetic acid

(xii) 2% formaldehyde solution

(xiii) 6 mM Ferric chloride solution

(xiv) Norharman standard 0.01 mg/ml in 0.25% TCA

(xv) ^{14}C-TP 10 uCi/10 ml SA 52 mCi/mmol

(xvi) Phosphate buffer pH 7.0

Disodium hydrogen orthophosphate ($Na_2HPO_4.12H_2O$) 2.3 g/100 ml and potassium dihydrogen orthophosphate (KH_2PO_4) 0.908 G/100 ml

(xviii) Triton scintillation cocktail

PPO 5 g) in 1 l toluene
)
POPOP 0.1 g) = scintillant

2 parts scintillant were mixed with one part of Triton X - 100 to make the scintillation cocktail.

9.2 Methods

An equil ibrium dialysis adapted from the method of Bender et al (1975) was used to study the effects of various compounds on the binding of tryptophan to albumin.

A solution of fatty-acid free human albumin 3.78 g/100 ml in Krebs' improved bicarbonate buffer (see reagents) gassed with 95% O_2/5% CO_2 was used in all experiments.

50 ul of tryptophan solution (see reagents) was added to the albumin solution to give a final concentration of tryptophan of 11.9 ug/ml. The concentrations of tryptophan and albumin are within the range found in normal human plasma.

Cortisol (as base) was dissolved in ethanol to a concentration of 1 mg/ml, and this solution was diluted with distilled water to a suitable concentration to add 50 ul to the albumin solution. Oestradiol benzoate, mestranol and norethisterone acetate were dissolved in absolute ethanol. 100 ul aliquots of this solution were added to a flask and the ethanol evaporated off before addition of the albumin solution. Nitrazepam and oleic acid were also dissolved in ethanol and treated as above. 50 ul of distilled water was added to the albumin solution to maintain the same volume of solution. All other compounds were water soluble and were added as 50 ul aliquots. All doses are expressed as base equivalents, and in each

case the pH of the buffered solutions were checked to be approximately pH 7.4, since pH also affects binding (McMenamy and Oncley 1958). The albumin solutions were shaken at 37°C for five hours.

300 ul of the albumin solution was placed in small plastic cups and the volume displaced to 250 ul with a sheet of "Visking" dialysis tubing, which had previously been soaked in distilled water. The % free tryptophan was determined by two methods. A non-isotopic method was used first and some results were confirmed by a radiochemical assay.

9.2.1 Non-isotopic method

The albumin solution was equilibrated against 100 ul of Kreb's improved bicarbonate buffer overnight at 4°C. The next day 50 ul aliquots of albumin solution from the lower compartment were assayed fluorimetrically for tryptophan by the method of Denckla and Dewey (1967). The aliquots were added to 2 ml ice cold 10% trichloracetic acid (TCA) and the mixture centrifuged in an MSE high speed centrifuge at 10,000 G at 4°C for fifteen minutes to remove the protein. Aliquots of distilled water or tryptophan (10 ug/ml) solution in 10% TCA were included in the assay to act as blanks and standards respectively. All samples were assayed in duplicate. 200 ul of 2% formaldehyde solution were added to the TCA, fcllowed by 100 ul of 6 mM ferric chloride solution. The tubes were covered and boiled in a waterbath for one hour. The fluorescence of the reaction product, norharman was read in an Aminco-Bowman spectrophotofluorimeter at Excitation 368 nm and emission 448 nm. The fluorescence of

the samples were identical with that from tryptophan standards (see Fig. 8). The tryptophan in the top compartment could not be assayed by this method because the amount present was too low (about 0.3 ug/ml, below the limit of sensitivity of the instrument).

The tryptophan assayed in the bottom compartment represented the tryptophan bound to albumin plus a proportion of the free tryptophan. Since the total tryptophan is constant, changes in the % free could be calculated after addition of various compounds.

In this system human plasma or mouse plasma could be substituted for the albumin solution.

This method does not give an accurate measure of the actual free tryptophan, but will determine changes in the binding of tryptophan. The method involved addition of a physiological solution to the top compartment, which causes molecules of free TP to leave the bottom compartment until the concentration of free TP is equal on either side of the membrane separating the compartments. However, this flux disturbs the equillibrium between free and bound TP in the bottom compartment, and more molecules of tryptophan dissociate from the albumin to compensate. This dilution effect was not accounted for in the calculations. This effect was small and constant throughout these experiments. It can therefore be ignored, since only a comparison of the % free TP after various treatments was required.

The above system can be adapted for radioassay of the % free tryptophan.

9.2.2 Radioassay

The free TP could be determined radiochemically after the method of Bender et al(1975). The buffer in the top compartment was replaced by 100 ul of ^{14}C-tryptophan (0.1 uCi, S.A. 52 mCi/mmol) in 0.15 M sodium chloride. This was equilibrated overnight and the ^{14}C-TP became distributed in the albumin solution. The next day 50 ul of the solution from the top compartment was counted in "Triton" scintillation cocktail in a Beckman liquid scintillation counter.

The results were adjusted for background "blank" readings. These "blank" readings and the counting efficiency of the instrument varied slightly from day to day. For samples counted in 0.15 m saline and "Triton" scintillation cocktail the counting efficiency was about 80% for ^{14}C-TP. A 20 ul sample of standard solution of ^{14}C-TP in 0.15 m saline was counted with each batch of samples. The counts/min. were not converted to dpm and the % free TP was calculated directly. If the cpm/20 ul of ^{14}C-TP standard added to top compartment = x

Then total cpm in 100 ul of top compartment = 5x

cpm recovered from top compartment in 50 ul sample = y

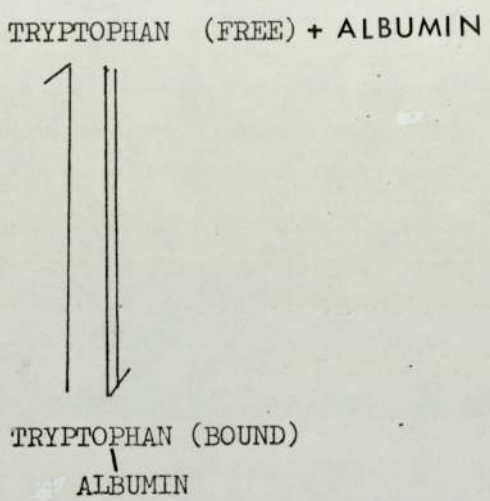
Therefore total cpm recovered in 100 ul of top compartment = 2y

But the labelled TP was distributed into a total of 350 ul

Therefore % free = $\frac{2y}{5x} \times \frac{350}{100} \times 100\%$

Fig. 3

THE BINDING OF TRYPTOPHAN TO ALBUMIN



10. Uptake experiments

10.1 Reagents

(i) Krebs' Ringer Phosphate buffer

Potassium chloride 4 mM

Magnesium chloride 2 mM

Calcium chloride 1 mM

Sodium chloride 175 mM

Glucose 10 mM

Sodium phosphate buffer 10 mM

pH 7.2

(ii) 0.32 M sucrose

(iii) ^{3}H - 5-HT (generally labelled) 1 mCi/100 ml

(SA 500 mCi/mmol

(iv) NCS Tissue solubiliser

(v) Dimulune 30 scintillation fluor

10.2 Preparation of synaptosomes

A crude synaptosomal fraction was prepared by the method of Whitakker. Routinely the cerebellum was removed from one mouse brain (wet weight about 0.4 g) and the cortex and brainstem homogenised in 4 ml ice-cold 0.32 M sucrose. The pestle was shaved down to produce a larger than usual gap between it and the homogeniser tube, so that the nerve endings were pinched off, producing synaptosomes. The homogenised tissue was centrifuged at 10,000 G for ten minutes at 4°C and the supernatant from this was taken and centrifuged again at 10,000 G for twenty minutes at 4°C. The supernatant was now discarded and the pellet (P_2 fraction) was the crude synaptosomal fraction, which is kept on ice at 0-4°C. The pellet was resuspended in 2 ml of phosphate buffer (see reagents). A small volume of synaptosomal suspension was kept for a protein determination to allow for variations in the concentration of synaptosomes in different experiments.

10.3 In Vitro experiments

In each experiment 0.1 ml synaptosomal suspension was used in each tube and the volume made up to 1 ml with Krebs' Ringer phosphate and kept on ice. 20 ul of ^{3}H-5-HT (0.4 nmol, SA 500 mCi/mmol) was added to each tube. Each treatment was duplicated. The effects of various concentrations of kynurenine on synaptosomal uptake of ^{3}H-5-HT was determined. Two tubes were incubated at 0-4°C for ten minutes to act as "blanks", since any labelled 5-HT taken up at this temperature may be expected to be non-active uptake. All other tubes were incubated at 37°C for ten minutes. Amitriptyline, which is known to inhibit the uptake of 5-HT, was used as a positive

control to ensure reliability of the method. A concentration of 10 uM completed inhibited ^{3}H-5-HT uptake. After incubation, the tubes were cooled in ice quickly, then centrifuged at maximum speed on an MSE bench centrifuge for 10 mins. The supernatant was discarded and the synaptosomes washed with 5 ml of buffer and centrifuged again. The supernatant was again discarded and 0.5 ml NCS tissue solubiliser added to the pellet, and incubated at 50°C until dissolved. The solubilised synaptosomes were added to 5 ml Dimilune scintillation fluor, and after keeping in the dark for 30 mins, the radioactivity present was counted by liquid scintillation in a Beckman counter.

10.4 In Vivo experiments

The effects of in vivo DL-kynurenine on synaptosomal uptake of ^{3}H-5-HT were also studied. Male mice (25±2 g) were pretreated with saline DL-kynurenine 0.5 mg/kg or DL-kynurenine 20 mg/kg for two hours. The animals were killed and the brains removed and synaptosomal fractions prepared from each as described previously. In each case a protein determination was done and the uptake per mg of tissue was compared.

10.5 Protein determination

10.5.1 Reagents

(i) Bovine serum albumin 2 mg/ml in distilled water was stored at 0-4°C.

(ii) Solution A was made up fresh and contained:-

sodium carbonate 3% in 0.1 N sodium hydroxide 5 ml

copper sulphate 1% in water 0.2 ml

sodium tartrate 2% in water 0.05 ml

(iii) Folin-Ciocalteau reagent 1 in 3, diluted with water. This was freshly prepared.

10.5.2 Method

20 ul samples of the synaptosomal suspension were assayed in triplicate by the method of Lowry (1951). The suspension was diluted to 100 ul with distilled water. At least three standard solutions of bovine serum albumin of suitable concentrations were included in the assay. Distilled water was used as the blank. One ml of solution A was added and the mixture allowed to stand for 10 mins. at room temperature. 0.1 ml Folin-Ciocalteau 1/3 reagent was added, and after 15 mins. the optical density was read at 660 nm on a Beckman UV/visible spectrophotometer. The standard curve was linear over the range used (see Fig. 15).

11. Estimation of residual blood volume in brain

11.1 Reagents

(i) Tritium Absorber D (Fisons) containing

Dioxan 70%

Toluene 30%

Naphthalene 20 g/l

scintillator (butyl PBD) 7 g/l

(ii) methanol

(iii) acetone/dry ice bath

(iv) ^{3}H-Dextran 30 mCi/g

MW 77,500 (average)

11.2 Method

100 ul of ^{3}H-dextran (30 mCi/g) 1 mg/ml was injected into a tail vein of an adult male mouse (20-25 g). Thirty minutes later the animal was killed by cervical dislocation and the neck severed for collection of blood into heparinised tubes. The brain was removed and roughly chopped. The blood and brain samples were freeze-dried overnight. The dry samples were weighed and oxidised in a biological materials oxidiser. The tissue was oxidised completely to ^{3}H-H_2O vapour, which was condensed in a glass trap immersed in an acetone/dry ice bath where it was frozen. The ice was thawed by immersing the trap in hot water, and 15 ml tritium absorber D was added. The catalyst bed was first primed with 200 ul of water. 200 ul of water was oxidised for the blank and 200 ul of water and 10 ul of ^{3}H-hexadecane acted as standard, to allow for the recovery of the instrument. The 100 mg samples of tissue were oxidised completely. The condenser trap was washed three times with methanol between samples. The samples in scintillant were counted by liquid scintillation.

The standard decays over a time period and tables from the Radiochemical Centre were used to determine the dpm/g for the standard at the time of use.

standard = 2.022×10^{3} dpm/mg

10 ul $\equiv$ 7.73 mg

The counts/min. were converted to dpm/g wet weight using the water content of blood and brain tissue

Brain $\equiv$ 77.54% water

Blood $\equiv$ 75.63% water

The dpm/ul blood was used to calculate the residual volume of blood in the brains.

12. Clinical pilot study

Tne study lasted for four weeks and took place at the maternity unit of Walsgrave Hospital, Coventry. Women presenting at the maternity unit during this time were considered for the study if they were born in the UK and were aged between 18 and 31. Women were selected who had given birth to live infants previously and were willing to cooperate. The day of parturition was considered as day 0 if birth occurred after 12.00 hours, and as day 1 if it occurred before 12.00 hours. Women were consulted on day 1 and consent to take part in the study obtained. Thereafter on postpartum days two to five psychiatric assessments and blood samples were taken. Four to five women per week were taken into the study and 18 subjects were accepted over the four-week period.

Subjects were asked to take a light, standard, low-protein breakfast each morning and drugs which have been shown to affect the binding of tryptophan, such as aspirin, were avoided. Samples of venous blood (10 ml) were obtained into heparinised tubes each day between 09.00 and 09.30 hours. The blood was centrifuged and the plasma transferred into coded tubes. The plasmas were kept on ice and transferred to the University of Aston, where biochemical assays for cortisol and tryptophan were undertaken.

Three self-rating scales were used for quantitative psychiatric assessment. These were administered immediately after blood sampling. The Multiple Affect Adjective Check List (MAACL) (Zuckerman and Lubin 1965) includes scales for depression, anxiety and hostility. It consists of a list of adjectives in randomised order and can be used daily. Patients were asked to mark adjectives which they thought applied to the way they felt today. Dr Gockshott, a psychiatric registrar, then

gave each patient a short interview and made an assessment of mood, noting other parameters, such as crying episodes, sleep disturbances, loss of appetite, etc. On two of the four days patients were issued with another two sets of self-rating scales - the Beck depression inventory (Metcalfe,Goldman 1965) with the modification of Pichot et al (1966) and the Hildreth (1946) feeling scale. These scales are short statements describing the way a subject may feel. The Beck/Pichot rates depression, and the Hildreth rates mania or elation. Dr Cockshott noted any other relevant obstetric history. Dr Cockshott was unaware of the biochemical results obtained and the scores for the rating scales were not calculated until the end of the study.

The plasma samples were assayed for cortisol, free tryptophan, and total tryptophan.

12.1 Total tryptophan

Total tryptophan was assayed fluorimetrically as described in section 9.

12.2 Free tryptophan

Free tryptophan was determined by the radioactive assay as described in section 9.

12.3 Cortisol

(i) fluorescence reagent

30 ml absolute alcohol)
)
70 ml conc. sulphuric acid)

This was mixed carefully in a bath of cold water and used immediately when cool.

(ii) dichloromethane (fluorescence grade)

(iii) cortisol standard 50 ug/100 ml in distilled water

Cortisol was assayed after the method of Mattingly (1962). This is a fluorimetric assay of 11-hydroxycorticoids, and assays both cortisol and corticosterone, though there is little interference from other steroids such as oestradiol.

0.5 ml blank (distilled water), standard cortisol solution, or plasma, was added to 5 ml dichloromethane. Due to the high volatility of dichloromethane, an automatic pipette could not be used and only glass measures could be used for dichloromethane. The mixture was shaken for 10 mins. and 2 ml of the dichloromethane layer was taken and 1 ml fluorescence reagent added. After mixing on a whirlimixer for 30 secs., the lower phase was removed. The fluorescence was read after 13 mins. on an Aminco-Bowman spectrofluorimeter at excitation 464 nm, emission 525 nm. The fluorescent product is unstable, but there was no significant change in fluorescence between 10-20 mins. after adding the fluorescence reagent, as was also found by Mattingley (1962).

Consent to initiate the project was obtained from the ethical committees of Walsgrave Hospital, Coventry and of the University of Aston, Birmingham.

The project had been initiated by Dr T. L. Dunn, Dr S. Handley and Dr J. Baker. The involvement of the author consisted mainly of practical help to the registrar psychiatrist, Dr C. Cockshott, at Walsgrave Hospital. The co-operation of the obstetrician and labour ward staff was gained in ensuring that a low-protein standard breakfast was obtaind and no aspirin was administered to women taking part in the study. Other relevant patient history was noted from the patient records. Dr Cockshott

conducted short clinical interviews and took blood samples. The blood samples were processed and the frozen plasma was transported to the University of Aston by the author. Most of the assay work was carried out by Dr S. Handley, Dr J. Baker and Mrs P. Marklew (technician), though assistance was given by the author when required. Some of the initial analysis of results was performed by the author.

13. Statistical Methods

Unless otherwise quoted, all errors were expressed by the standard error of the mean (SEM). The means of two groups of data were compared by the two-tailed student "t"-test for independent variables.

In the turnover studies the best straight line was fitted by regression analysis.

The equation for the line for the 1st treatment is

$$y = a_1 + b_1 x$$

where a = intercept, b = slope of the line. The regression coefficient (r) indicates how good the fit is. The slope of the line gives the turnover in ug/g/hr. This can be converted to nmol/g/hr by dividing by the molecular weight of 5-HT if the pargyline method is used or the molecular weight of 5-HIAA if the probenecid method is used.

The error in measurement of the slope can be described by the 95% confidence limits.

$$\text{Variance (b)} = \frac{\sigma^2}{\Sigma(x_1 - \bar{x})^2}$$

σ^2 is the variance of the data

$$\text{s.e. (b)} = \frac{\delta}{\sqrt{\Sigma(x_1 - \bar{x})^2}}$$

where s.e. (b) is the standard deviation of the slope.

An estimate of s.e. (b) is given by

$$\frac{\hat{\delta}}{\sqrt{\Sigma(x_1 - \bar{x})^2}}$$

where $\hat{\delta}$ is an estimate of the standard deviation of the data.

Confidence limits can then be found by

$$b \pm \frac{t\,(n - 2,\ 1 - \tfrac{1}{2}\alpha)\hat{\delta}}{\sqrt{\Sigma(x_1 - \bar{x})^2}}$$

When $\alpha = 0.05$ the 95% confidence limits are obtained.

$t\,(n - 2,\ 1 - \tfrac{1}{2}\alpha)$ is approximately = 2

for n = 20 to n = 32

The 95% confidence limits approximate to

$$b \pm \frac{2.\hat{\delta}}{\sqrt{\Sigma(x_1 - \bar{x})^2}}$$

The slopes of the regression lines obtained from the control and test groups were compared by the following analysis.

(1) If $y = a_1 + b_1x$ for the control group

(2) and $y = a_2 + b_2x$ for the test group

r_1 and r_2 are the regression coefficients for the lines (1) and (2).

$$\hat{\delta} = \frac{(n_1 - 2)(1 - r_1^2)(\Sigma(y_1 - \bar{y})^2 = (n_2 - 2)(1 - r_2^2)(\Sigma(y_2 - \bar{y})^2}{n_1 + n_2 - 4}$$

where $\hat{\delta}$ is the estimate of the standard deviation of the data.

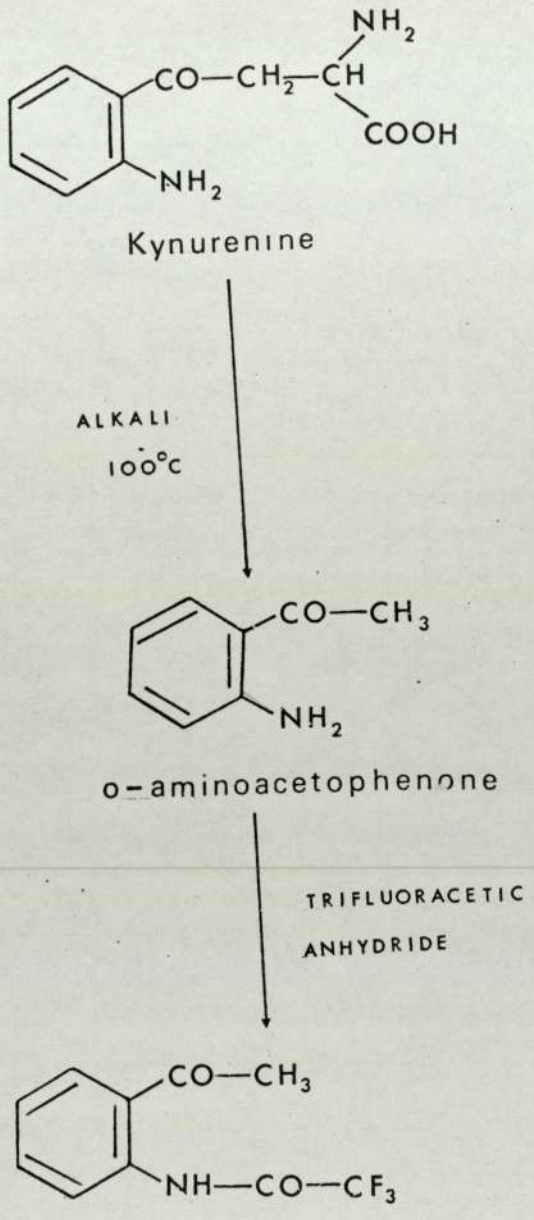
Proposing the hypothesis that $b_1 = b_2$ this is evaluated by the student "t" test when

$$t_{n_1 + n_2 - 4} = (\hat{b}_1 - \hat{b}_2) \sqrt{\frac{\frac{1}{\Sigma(x_1 - \bar{x})^2} + \frac{1}{\Sigma(x_2 - \bar{x})^2}}{\hat{\delta}}}$$

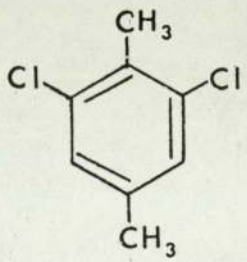
where $\hat{b}_1$ and $\hat{b}_2$ are the estimates of the slopes of the lines (1) and (2).

Fig. 4

Reactions occurring during kynurenine assay



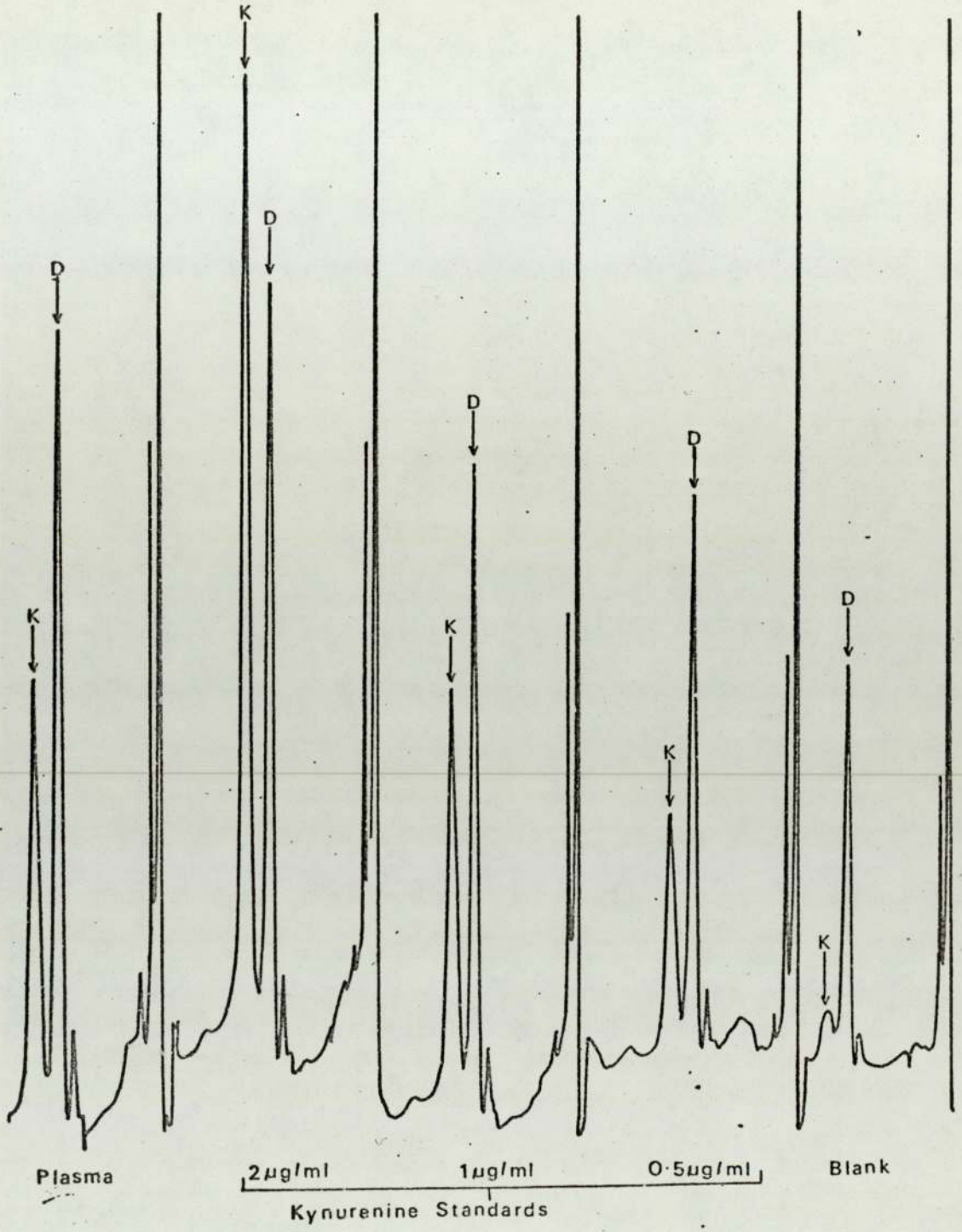
Internal Standard



$\alpha\alpha_1$-Dichloro-p-xylene

Fig. 5

Photograph of GLC Trace

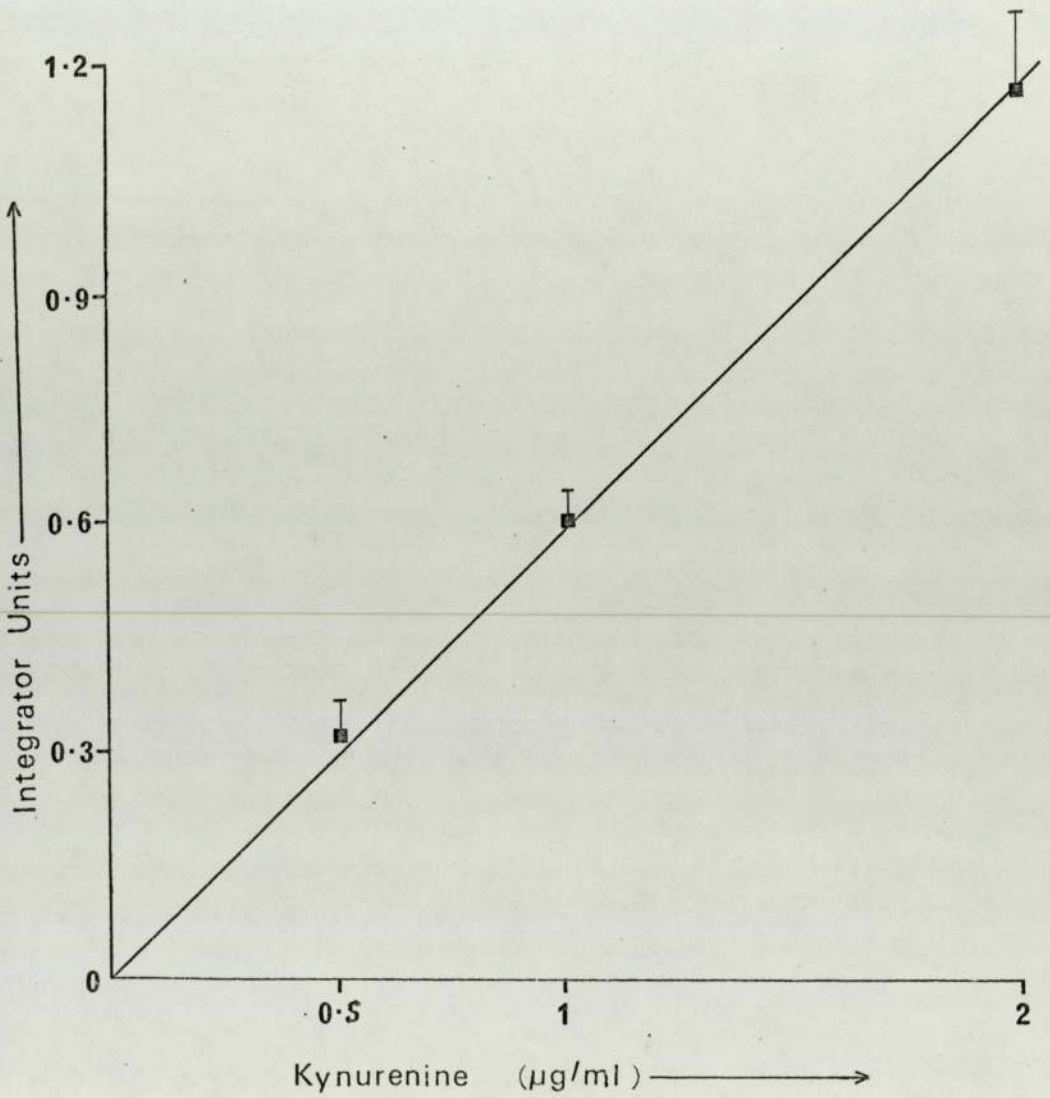


D = dichloro-p-xylene

K = kynurenine

Fig. 6

Standard curve for kynurenine over the range of concentrations found in plasma

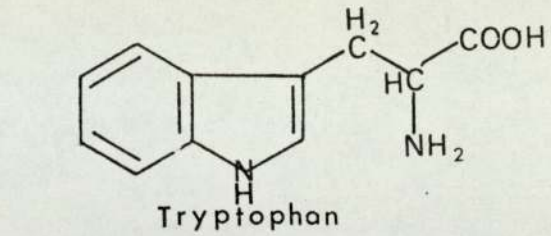


Each symbol is the mean of at least six determinations.

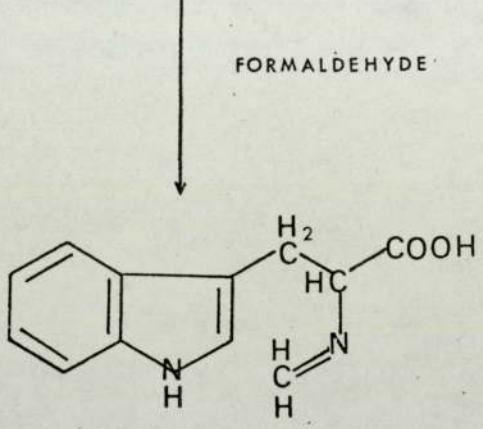
Concentration of dichloro-p-xylene (DCPX) 200ng/ml

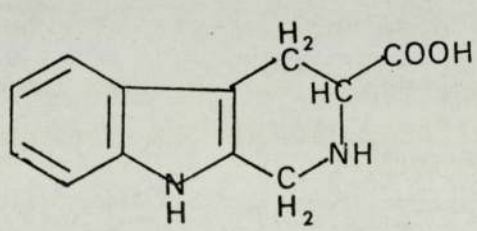
Fig. 7

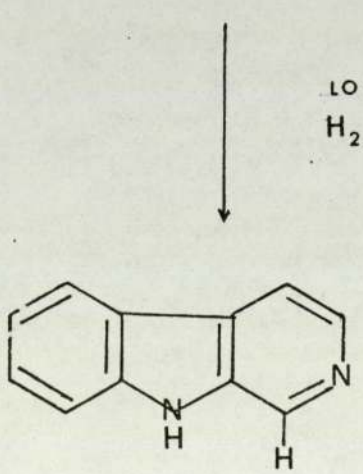
Reactions occurring in Tryptophan assay



Tryptophan







Norharman

Fig. 8

Excitation/Emission spectrum of Norharman formed from Standard Tryptophan

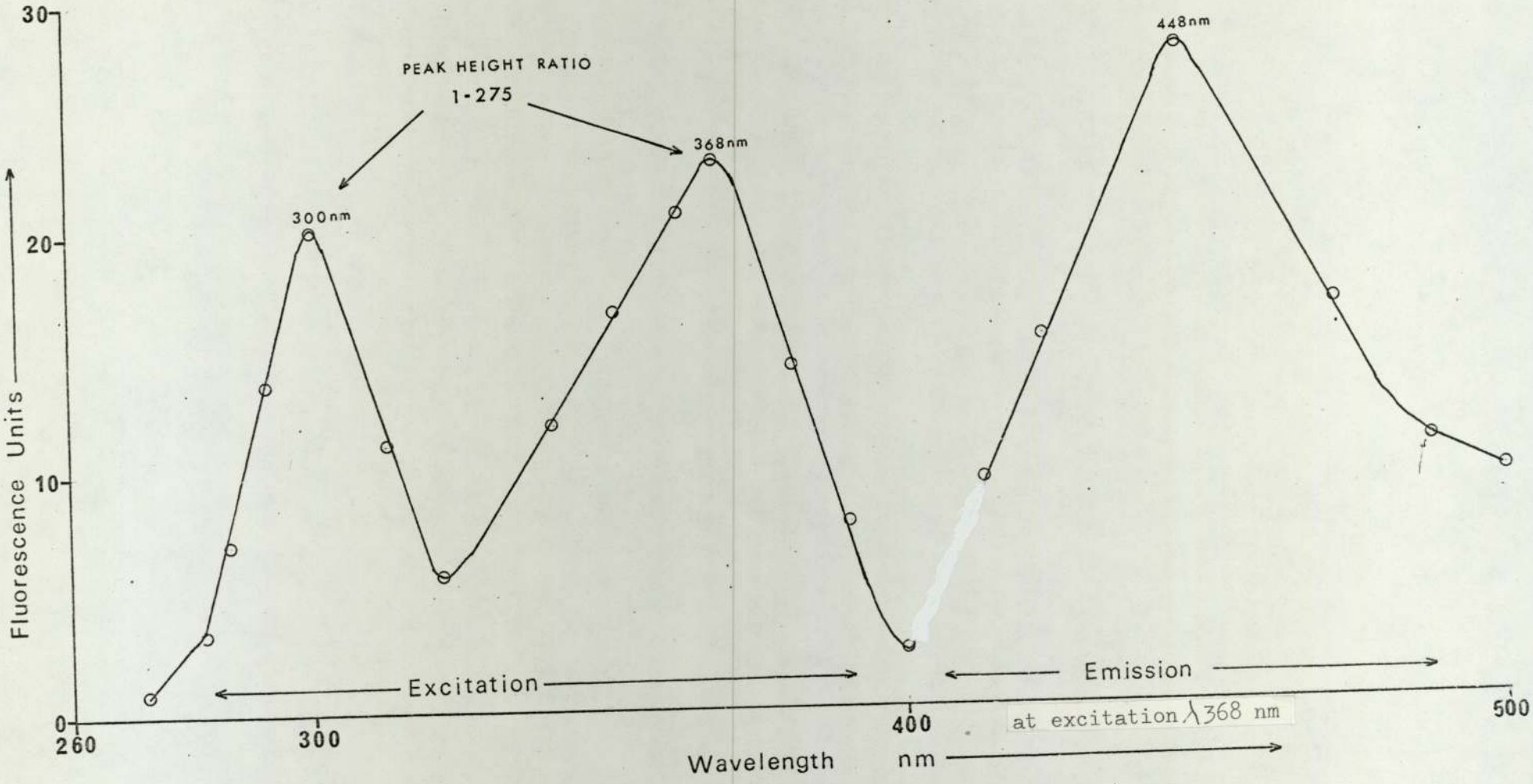


Fig. 9

Excitation/Emission Spectrum of Norharman formed from a sample containing Tryptophan

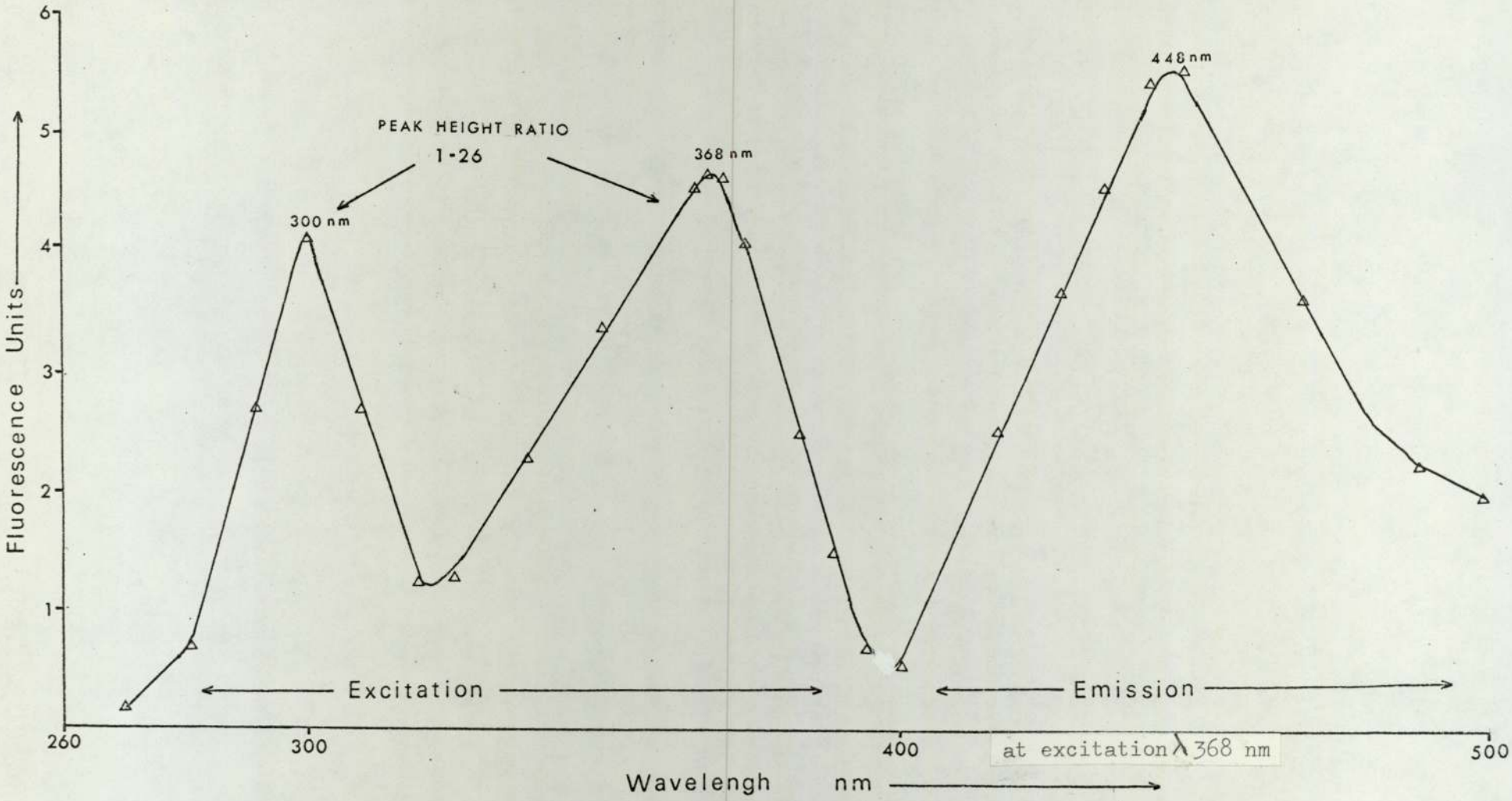
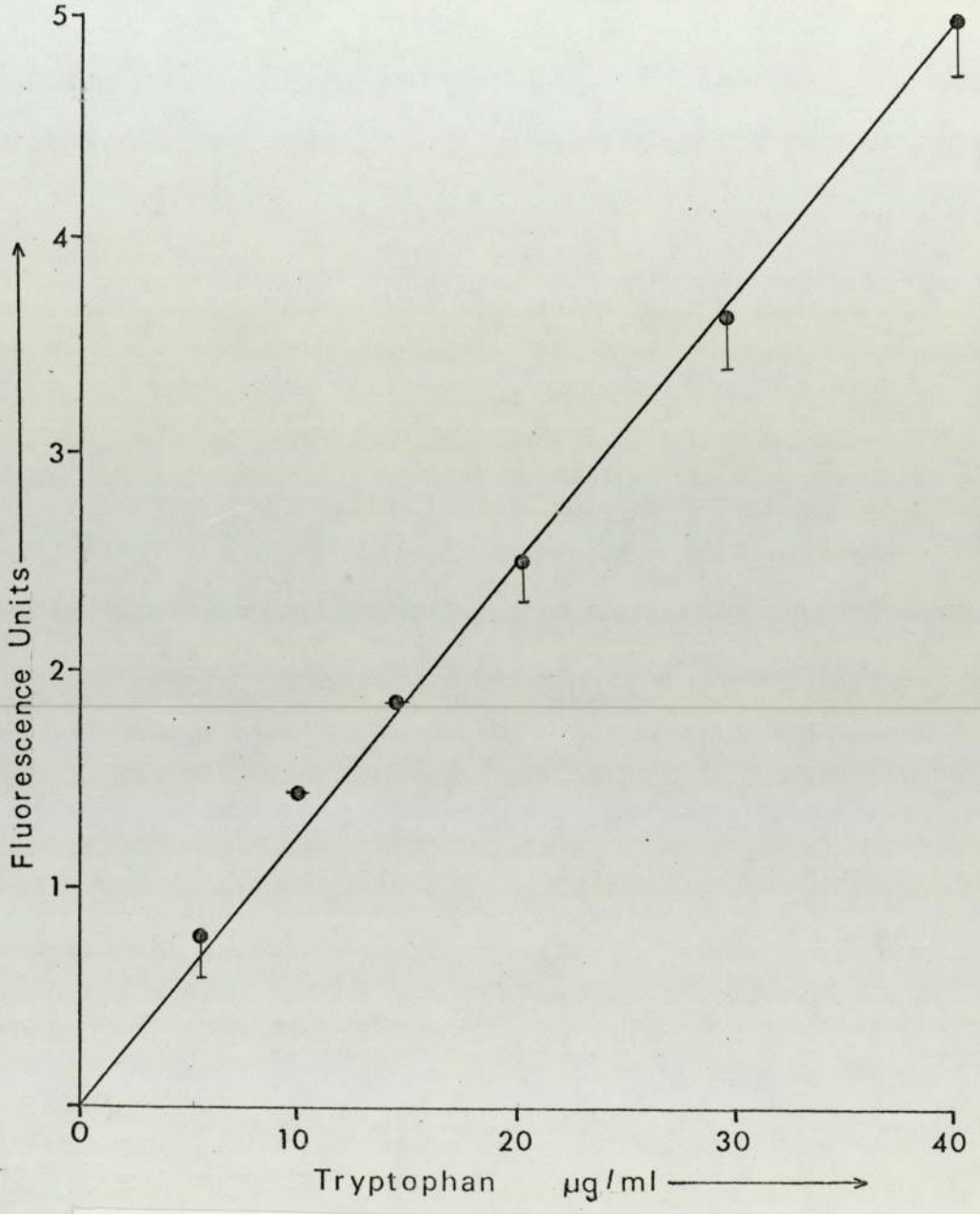


Fig. 10

Standard curve for tryptophan



Each symbol is the mean of 2 determinations.

Excitation λ_{max} 368 nm

Emission λ_{max} 448 nm

Fig. 11

Excitation/Emission spectrum of 5-Hydroxytryptamine in o-pthalaldehyde

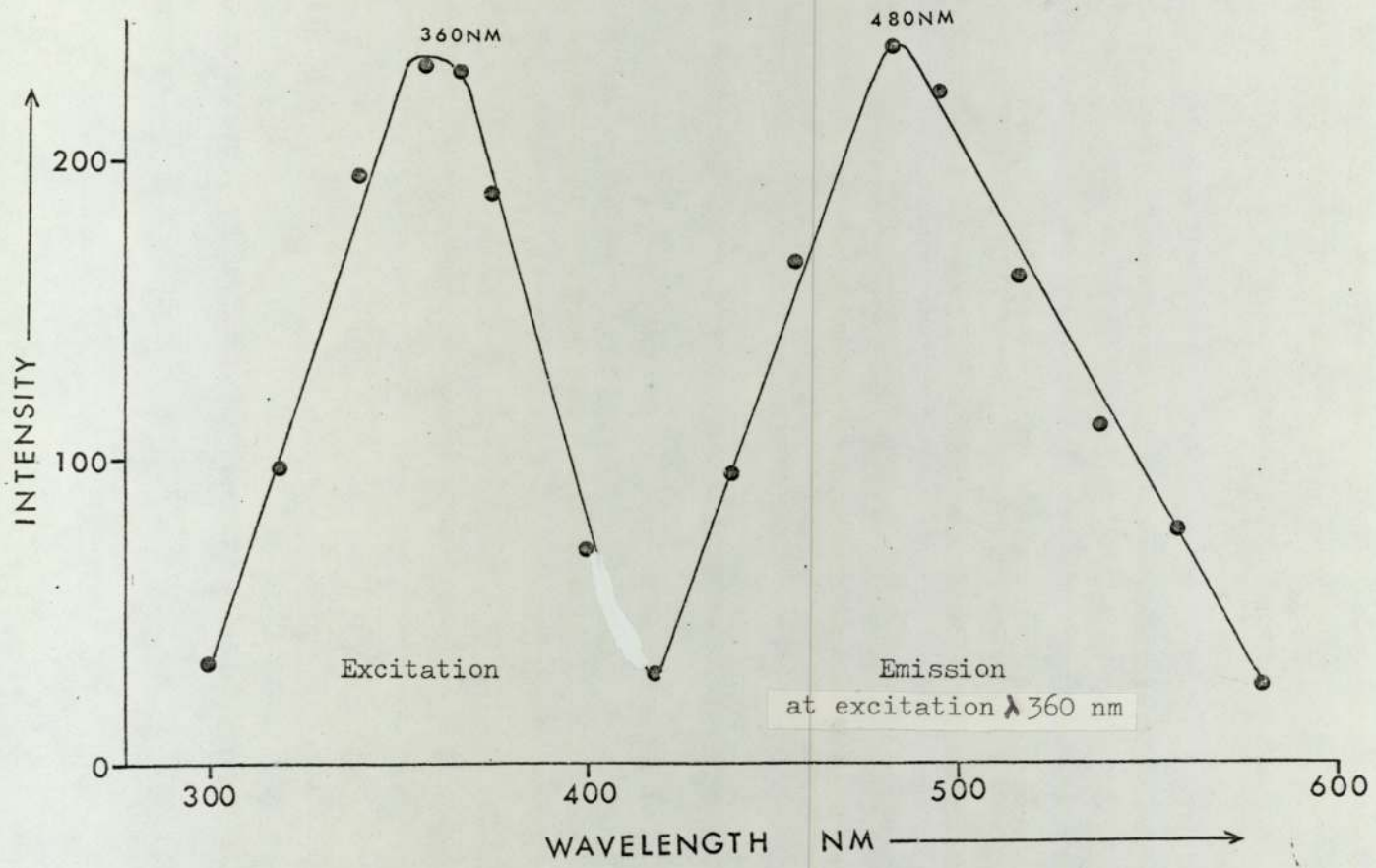
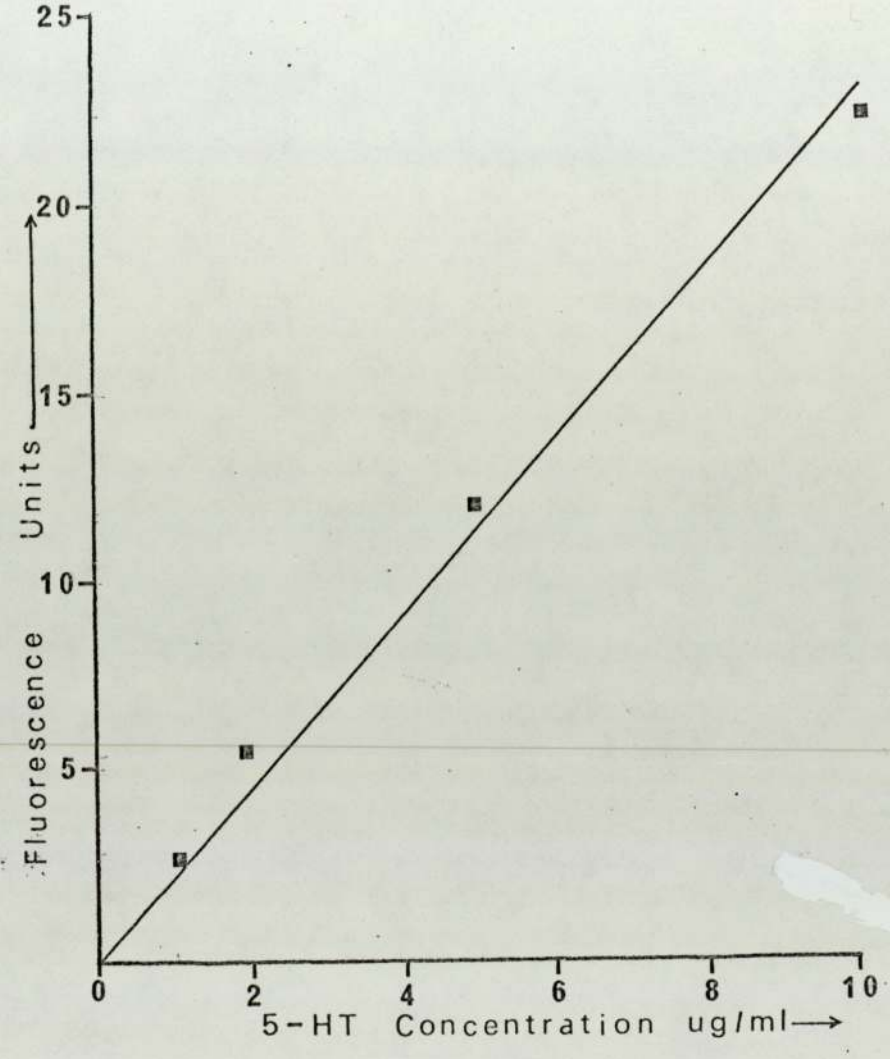


Fig. 12

Standard curve for 5 - hydroxytryptamine



Excitation λ_{max} 360 nm

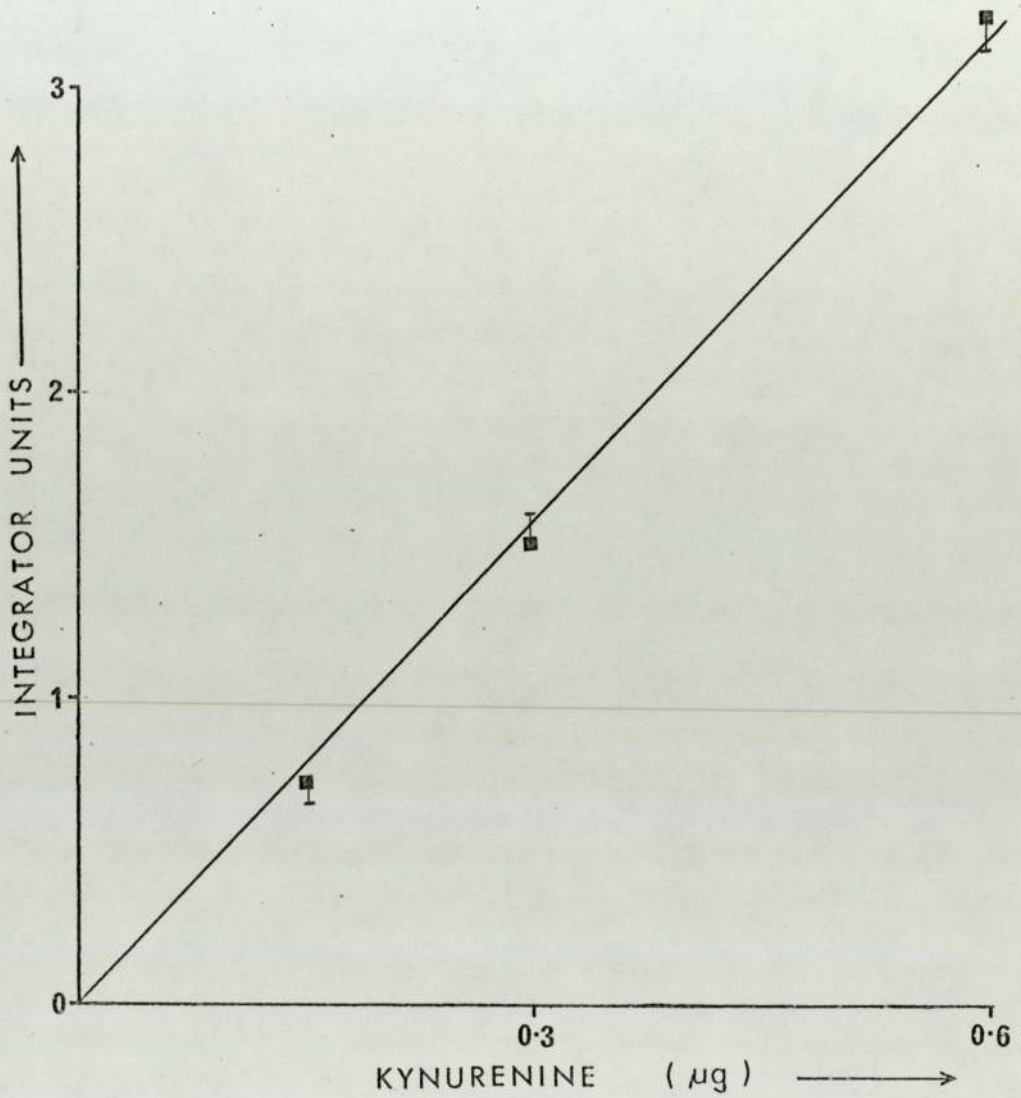
Emission λ_{max} 470 nm

Each symbol is the mean of two determinations

The standard errors are too small to be shown.

Fig. 13

Standard curve for kynurenine over the range of concentrations found in brain



Each symbol is the mean of at least six determinations.

Concentrations of dichloro-p-xylene (DCPX) 50 ng/ml

Fig. 14

Increase in brain 5-HT levels after pargyline

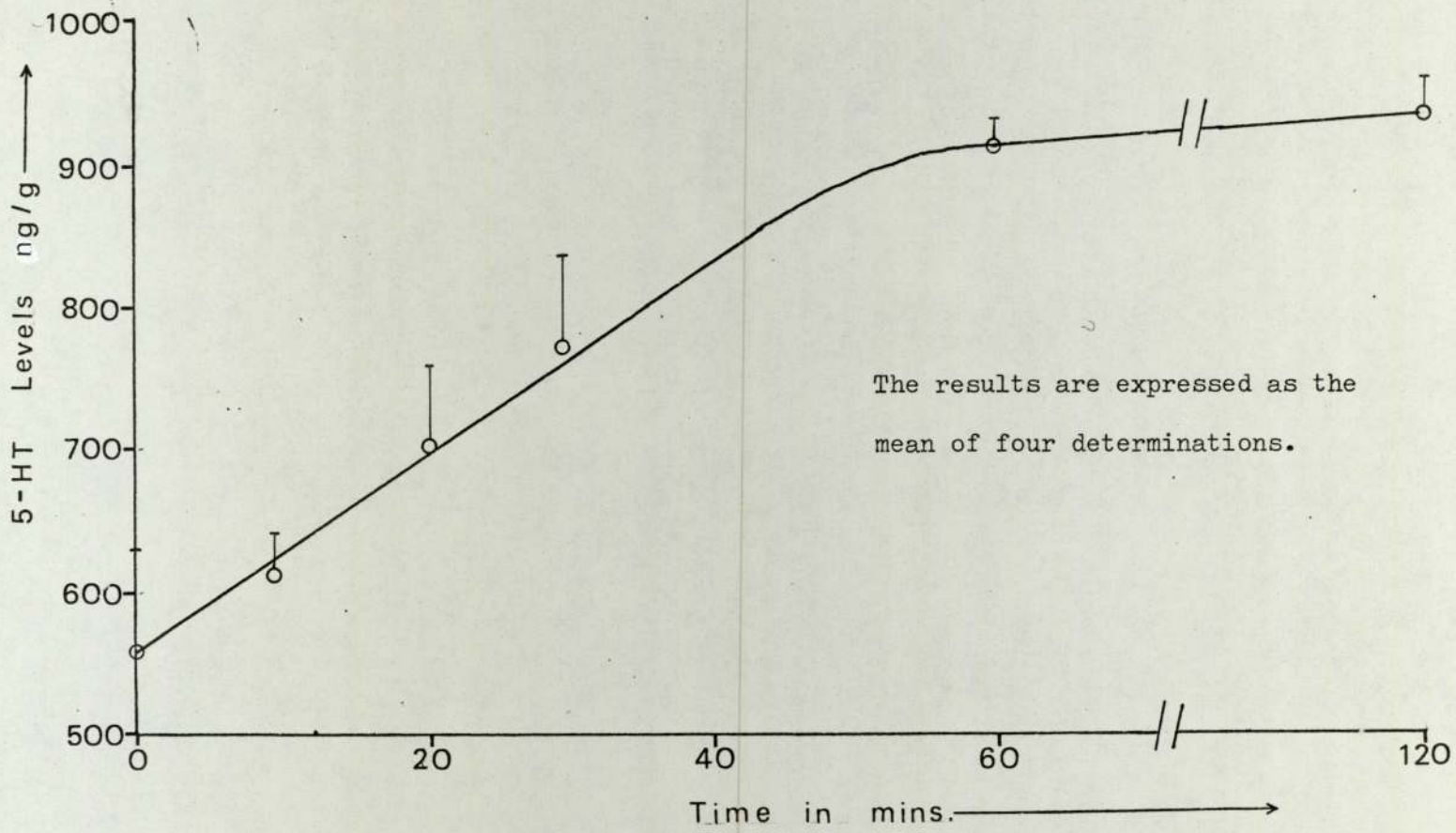
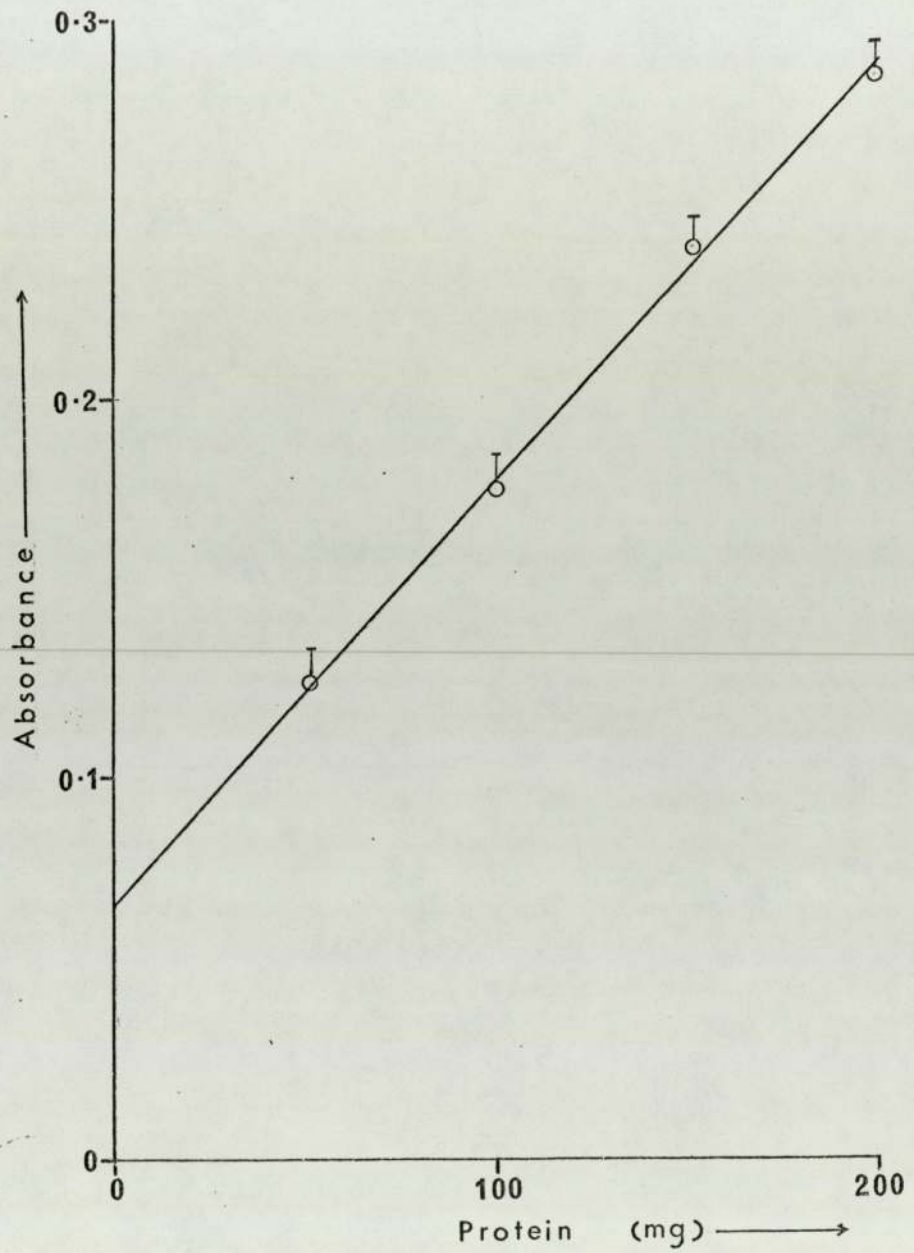


Fig. 15

Standard curve for protein determination



Each symbol is the mean of at least three determinations.

RESULTS

CHAPTER 1

CLINICAL PILOT STUDY

ON POST-PARTUM DEPRESSION

About 50% of post-partum women suffer a mild depressive reaction after childbirth (Pitt 1973) and this has been called post-partum "blues" (Yalom 1968). At this time there are profound biochemical changes occurring, and the puerperium may be an appropriate model for investigating possible links between biochemical changes and mood state. In this preliminary study the relation between plasma cortisol concentration, free tryptophan concentration and emotional state during the five days immediately post-partum were investigated.

This work was done in conjunction with other workers (see Experimental methods section 12).

Results

A total of 18 women took part in the study, which lasted four weeks. The women were studied on post-partum days one to five and venous blood samples (10 ml) were taken on each of these days between 9 - 9.30 a.m. The plasma was assayed for total and free tryptophan, and cortisol as described under experimental methods section. Changes in mood state over the immediate post-partum period were measured by a short clinical interview by a psychiatrist and by the administration of the psychiatric self rating scales (see experimental methods section 12).

The mean plasma cortisol was high after parturition, as was expected. Plasma cortisol steadily declined over the five post-partum days studied from a mean of 44.42 ug/100 ml to 23.15 ug/100 ml. The total plasma tryptophan rose over this period from 8.5 ug/ml to 10.04 ug/ml and the free TP also increased from 1.38 ug/ml to 1.80 ug/ml. None of the women was diagnosed as suffering from clinically significant depressive illness at clinical interview. Three women exhibited elevation of mood throughout the study but this was not clinical hypomania in the opinion of the

psychiatrist. Patients completed the multiple affect adjective check list (MAACL) which rated anxiety, hostility and depression on each of four days. The mean scores in the psychiatric rating scales are shown in Fig. 16. Data for the three women showing consistent elevation of mood are plotted separately and show that for these women the Beck-Pichot scores were zero throughout. The MAACL "D" (depression) scores were significantly lower and the Hildreth (elation) scores significantly higher than those for the remainder of the patients; MAACL "A" (anxiety) and "H" (hostility) scores were also significantly lower ($p < 0.001$ in all cases, Mann-Whitney "U" test). Biochemically these three women were distinguished by unusually high cortisol concentrations on day 2 (over 70 ug/100 ml compared with an average of 30 ug/100 ml for the remainder of the patients). The relation between the biochemical values and scores on the mood-rating scales was investigated by regression analysis. Spearman's rank correlation coefficient was used because of the non-parametric nature of the rating scale data. These analyses were performed for all patients across all days and checked by plotting scattergrams to ensure that significant associations were not due to outlying points.

Plasma free tryptophan concentration showed a significant correlation with the MAACL depression score ($r = - 0.30$; $p < 0.01$) and a significant positive correlation with the Hildreth (elation) score ($r = + 0.31$; $p < 0.05$). Plasma cortisol was also positively correlated with the Hildreth score ($r = + 0.35$; $p < 0.05$). Cortisol concentrations were not related to anxiety as measured by the MAACL "A" scale nor was there any significant correlation between cortisol and either free or total tryptophan concentration.

It was found that the concentration of free TP correlated with total TP ($r = + 0.75$; $p < 0.005$) but not with the percentage of free TP, suggesting that albumin binding was below saturation levels.

Nitrazepam was administered to nine patients on the night before blood was taken. Since nitrazepam has been shown to displace TP from its binding to albumin (e.g. Bourgoin et al 1975, see also "Introduction") it was thought this may affect the plasma % free TP. However, there were no consistent differences between the % free TP on mornings after subjects received nitrazepam as a hypnotic and mornings when no nitrazepam was received (see table 2).

Discussion

None of the 18 women studied were diagnosed as clinically depressed, though they showed mood changes of a depressive nature at interview, which were reflected in the rating-scale scores. The Beck-Pichot and Hildreth scales are generally applied to patients suffering from depressive illnss, so in this population, who were not clinically depressed, only a restricted range of scores occurred. The "MAACL" appeared to be more useful in rating changes in mood within the normal range.

It was found that the concentration of free TP correlated with total TP but not with % free. This observation was unexpected, since it indicated that the albumin binding may not be saturated. This result was not reflected in the main study, which continued from these investigations, (Handley, personal communication) when the % free was found to be more highly dependant on the concentration of free than on the total TP. Possibly the contrast in these results indicates that a small sample is not necessarily representative of a population.

Nitrazepam was found to be without effect on the free TP the following

morning. This result was confirmed in the main study when nitrazepam was also found to have no consistent effect on free TP.

Free TP was significantly correlated with mood state, in agreement with the findings of Stein et al (1976) who reported reduced % free TP on day 6 post-partum in patients with the most severe depression. This correlation between free TP and mood state also extended to patients who were elated. Three of the patients showed elation of mood throughout the study but were not considered by the psychiatrist to be clinically hypomanic. In the main study, 28 out of 74 patients were considered to be depressed post-partum. In these patients there was no increase in total TP on day 1 and no consistent relation between depression scores and free TP was found.

The plasma cortisol levels declined rapidly after parturition and were normal by the third post-partum day. In the main study cortisol was found to be high antenatally in agreement with previous findings. This rise is due to an increase in the cortisol-binding globulin transcortin caused by oestrogen (Treadway et al 1969, Plager et al 1964). Levels were found to peak on day one and declined slowly. However, at day 6 post-partum cortisol levels were still significantly above the levels found 6 weeks post-partum. Levels of cortisol have previously been found to peak during labour and fall to normal within the following week (Okada et al 1974, Batra and Grundsell 1978).

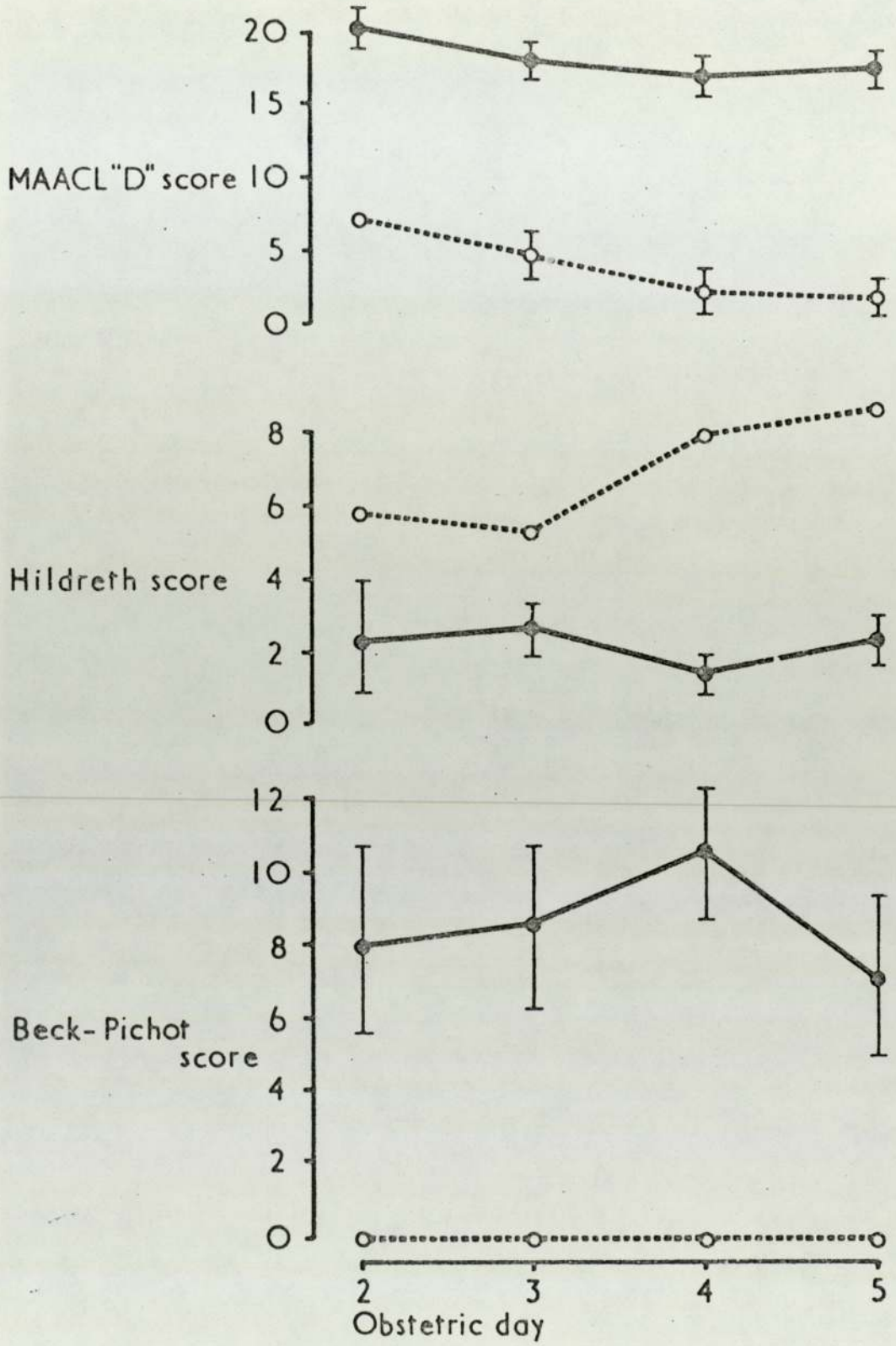
Cortisol levels appeared to be positively correlated with the degree of mood elevation. The relation between cortisol levels and mood state has not previously been studied in patients who were not diagnosed as mentally ill. Cortisol secretion has often been found to be elevated in depressed patients (e,g. Sachar 1967, 1970, Carroll 1972,

Gibbons and McHugh). In the main study depressed mood in the first week post-partum was consistently related to high antenatal cortisol but was not related to post-partum cortisol levels.

Thus certain biochemical variables were correlated with changes in mood state in the early puerperium, in the absence of clinical depression or mania, in a small sample of 18 women.

Fig. 16

Changes in mean score on psychiatric rating scales



Variation in mean scores (+ SE) on psychiatric rating scales for obstetric days 2-5. Open circles represent patients with greatly elevated mood. Closed circles represent all other patients.

Table 1

Plasma concentrations of cortisol, total and free Tryptophan during obstetric days 2-5 (expressed as mean ± standard error)

Obstetric day	cortisol (ug/100 ml)	Tryptophan (ug/ml) Total	Free
2	44.42 ± 9.76	8.52 ± 0.82	1.38 ± 0.28
3	29.12 ± 3.28	9.58 ± 0.60	1.60 ± 0.14
4	26.24 ± 2.99	9.56 ± 0.56	1.58 ± 0.10
5	23.15 ± 1.58	10.04 ± 0.58	1.80 ± 0.14

Table 2

Influence of Nitrazepam on plasma % free tryptophan

		% free tryptophan
(i)	After a night free of nitrazepam	15.58 ± 0.95 (6)
(ii)	After a night with nitrazepam in the same patients as (i)	16.17 ± 1.36 (6)
(iii)	After a night with nitrazepam	13.86 ± 1.83 (4)
(iv)	After a night free of nitrazepam in the same patients as (iii)	16.05 ± 1.875 (4)

The results are expressed as the mean ± standard error with the number of patients in brackets. All blood samples were taken between 9 - 9.30 a.m. the morning after night sedation with nitrazepam compared with mornings after no night sedation, in the same patients.

CHAPTER 2

EFFECTS OF STEROID HORMONES AND OTHER DRUGS ON TRYPTOPHAN BINDING

Tryptophan is highly bound to serum albumin, up to 90% in humans. This binding can be shown to occur in human plasma at 37°C (McMenamy and Oncley 1958, McMenamy et al 1957, Fuller and Rousch 1973). The degree of binding is affected by temperature and pH (McMenamy and Oncley 1958) and the presence of various drug molecules (Bourgoin et al 1975, McArthur and Dawkins 1969, 1971). The binding of TP to albumin in vivo may be important since it has been suggested that only the "free" portion enters the brain (Tagliamonte et al 1971(a)).

Cortisol is mainly bound to the globulin, transcortin, but there is a weaker binding to albumin. It is therefore possible that the presence of cortisol may cause displacement of TP from its binding to albumin. This possibility was therefore investigated.

Plasma % free TP has been demonstrated to be positively correlated with plasma oestrogens in perimenopausal women (Aylward 1976). It is possible therefore that oestrogens influence TP binding directly. The effects of both oestrogens and progestagens on TP binding was investigated.

2. Results

The binding of TP to a solution of human fatty-acid free albumin was measured by two methods, a non-isotopic method and a radiochemical method (see experimental methods section 9). The first method gave a semi-quantitative estimate of the effects of various compounds on the binding. Since the concentration of free tryptophan was very small (about 1 ug/ml) this fluorescence assay was not sufficiently sensitive to determine the actual % free TP accurately but changes in binding were indicated. The results were confirmed quantitatively by the radiochemical method. There was good agreement between the results obtained from the two methods.

A significant increase in free TP was found after the addition of nitrazepam to the albumin solution by both methods (table 3).

The binding of tryptophan to the albumin solution was found to be in the same range as that found physiologically in human plasma (i.e. 80 - 90% bound).

All binding experiments were performed at 37°C pH 7.4. The tryptophan and albumin solution was shaken for 5 hours to ensure equilibrium between tryptophan and albumin. The TP in the bottom compartment was equilibrated against buffer at 4°C overnight to ensure that equilibrium had been reached.

2.1 Effect of aspirin on TP binding

The presence of aspirin (acetyl-salicylic acid) in the in vitro system decreased the binding of TP to albumin in a concentration-dependant manner (Fig. 17, table 4). A concentration of 25 ug/ml significantly increased % free TP by the non-isotopic method ($p < 0.05$, "t"-test, one-tailed). The % free TP was further increased after a concentration of 250 ug/ml aspirin ($p < 0.001$) (table 4).

2.2 Effects of oleic acid

Oleic acid is a naturally occurring fatty acid. The concentration of free fatty acids in human plasma is 500 uM/l (Documenta - Geigy scientific tables) or approximately 0.15 ug/ml. In preliminary experiments oleic acid 0.15 ug/ml was added to a solution of fatty acid free albumin. There appeared to be no change in TP binding. However a concentration of 1.5 ug/ml of oleic acid significantly increased % free TP ($p < 0.001$)(table 6) as determined

by the non-isotopic method.

2.3 Benzodiazepines

Addition of the benzodiazepine, nitrazepam 100 ug/ml caused a significant increase in plasma % free TP by the non-isotopic method ($p < 0.01$)(table 5) and this change was confirmed by the radiochemical method ($p < 0.05$)(see Fig. 18, table 5).

These preliminary experiments demonstrated the reliability of the technique. The actions of some steroid hormones on tryptophan binding were determined.

2.4 Cortisol

Cortisol is present in human plasma at a concentration of 6 - 26 ug/100 ml (Scott 1978). Cortisol was added to the albumin solution to give concentrations of 10 to 1,000 ug/100 ml. There was no significant change found in the binding determined by either method, (table 3, table 7). In combination with aspirin, cortisol produced no significant change in binding from that after aspirin alone (table 4)(non-isotopic method). In combination with oleic acid the free tryptophan appeared to be further increased over that with oleic acid alone (table 6)($p < 0.01$). However the sample numbers in these experiments were small ($n = 4$).

2.5 Female hormones

The oestrogens, oestradiol (50 ug/ml) and mestranol (50 ug/ml) and the progestagen, norethisterone (50 ug/ml) were added to the albumin solution. Oestradiol and mestranol had no significant effect on the binding as determined by either method (Fig. 19, table 7). Norethisterone slightly increased the % free and this increase was

found to be statistically significant when the isotopic method was used ($p < 0.05$)(Fig. 19, table 7). The doses of these hormones are well outside even the therapeutic range but only a small effect was seen with norethisterone and no effect was seen with the oestrogens.

2.6 Antidepressant drugs

The effects of the antidepressant compounds maprotyline and amitriptyline on tryptophan binding were determined. Maprotyline 100 ug/ml had no effect on the binding whereas amitriptyline 100 ug/ml produced a significant increase in the % free which was demonstrated by both methods ($p = 0.01$ non-isotopic method, and $p < 0.05$, isotopic method)(table 5, Fig. 18).

Discussion

Aspirin was found to displace TP from its binding to albumin in a dose-dependant manner in this in vitro system. A concentration of 25 ug/ml caused a significant increase in % free TP. Since the plasma concentration of aspirin has been found to be 30 - 50 ug/ml after a therapeutic dose of 1 g (Hollister and Levy 1965) these observations are possibly of therapeutic significance. Aspirin has previously been observed to displace TP in vivo (Aylward and Maddock, 1973, Smith and Lakatos 1971).

Addition of a physiological concentration of oleic acid to the in vitro system had no effect on TP binding, possibly due to lack of sensitivity of the technique. At a concentration of ten times the normal plasma levels, oleic acid caused a marked displacement of TP. This observation was expected in view of previous reports that increased

plasma free fatty acids displaced TP *in vivo* (Curzon and Knott 1972).

Benzodiazepines have also previously been reported to increase plasma free TP (Bourgoin et al 1975, Müller and Wollert 1975). Nitrazepam was found to displace TP, causing a significant increase in % free, at a concentration of 100 ug/ml. Plasma levels of nitrazepam of 61 - 124 ng/ml have been reported in Man (Reider 1973). The concentration used in this system was therefore well outside the therapeutic range. However this concentration was of the same order as that used by other authors (Müller and Wollert 1975) and this experiment was intended only to confirm the reliability of the methodology. In the previous chapter (1) a therapeutic dose of nitrazepam at night was found to have no consistent effect on the % free TP in the plasma the following morning.

It had previously been shown that cortisol affected brain TP and 5-HT levels, though the results were somewhat inconsistent (see "Introduction" and Chapter 3). Brain TP has been found to be increased after 90 min. pretreatment, and this change was accompanied by increased 5-HT synthesis (Neckers and Sze 1975). After 5 hr. pretreatment a reduction in plasma free TP, liver TP, brain TP, 5-HT and 5-HIAA levels was reported (Green et al 1975). It was therefore possible that cortisol had a direct effect on the binding of TP to albumin. However it was found that a concentration of cortisol up to 1 mg/ml had no effect on the binding of TP to albumin in the *in vitro* system, and it is therefore unlikely that the effects of cortisol on brain TP and 5-HT levels occurred via a change in TP binding. The combinations of cortisol with aspirin and with oleic acid were designed to demonstrate any possible binding-stabilising effect, since this would be difficult to observe directly due

to very low levels of free TP. However, there appeared to be no increase in binding after cortisol and aspirin or oleic acid compared with aspirin or oleic acid alone. In fact, in combination with oleic acid cortisol tended to cause a slight increase in the free TP, though there were only a small number of determinations. Since both cortisol and oleic acid are normally present in plasma an interaction between these which affected TP binding could have had physiological significance.

Norethisterone at a concentration of 50 ug/ml was found to significantly increase the % free in this system. It is unlikely that this observation has any physiological significance since these concentrations are well outside even the therapeutic range. It has been reported previously that progesterone increased brain TP and 5-HT synthesis (Greengrass and Tonge 1975, Leonard and Hamburger 1974, Ladisich 1977) and norethisterone may be expected to have similar effects. It was possible that these effects may have been due to an influence on plasma TP binding. This seems unlikely, due to the high plasma levels needed to affect binding.

Oestrogens generally have the opposite effects decreasing brain TP and 5-HT (Greengrass and Tonge 1975, Leonard and Hamburger 1974) and could possibly have had a direct effect on TP binding. The oestrogenic hormones oestradiol and mestranol were tested in the in vitro system but were found to have no effect on binding at concentrations of 50 ug/ml.

The tricyclic antidepressant amitriptyline was found to displace TP from its binding to albumin. We found that decreased plasma free TP was significantly correlated with negative affect in a small group of post-partum women (Handley et al 1977, Chapter 1) and the % free has previously been found to be lower in depressed patients (Coppen et al 1972, 1974),

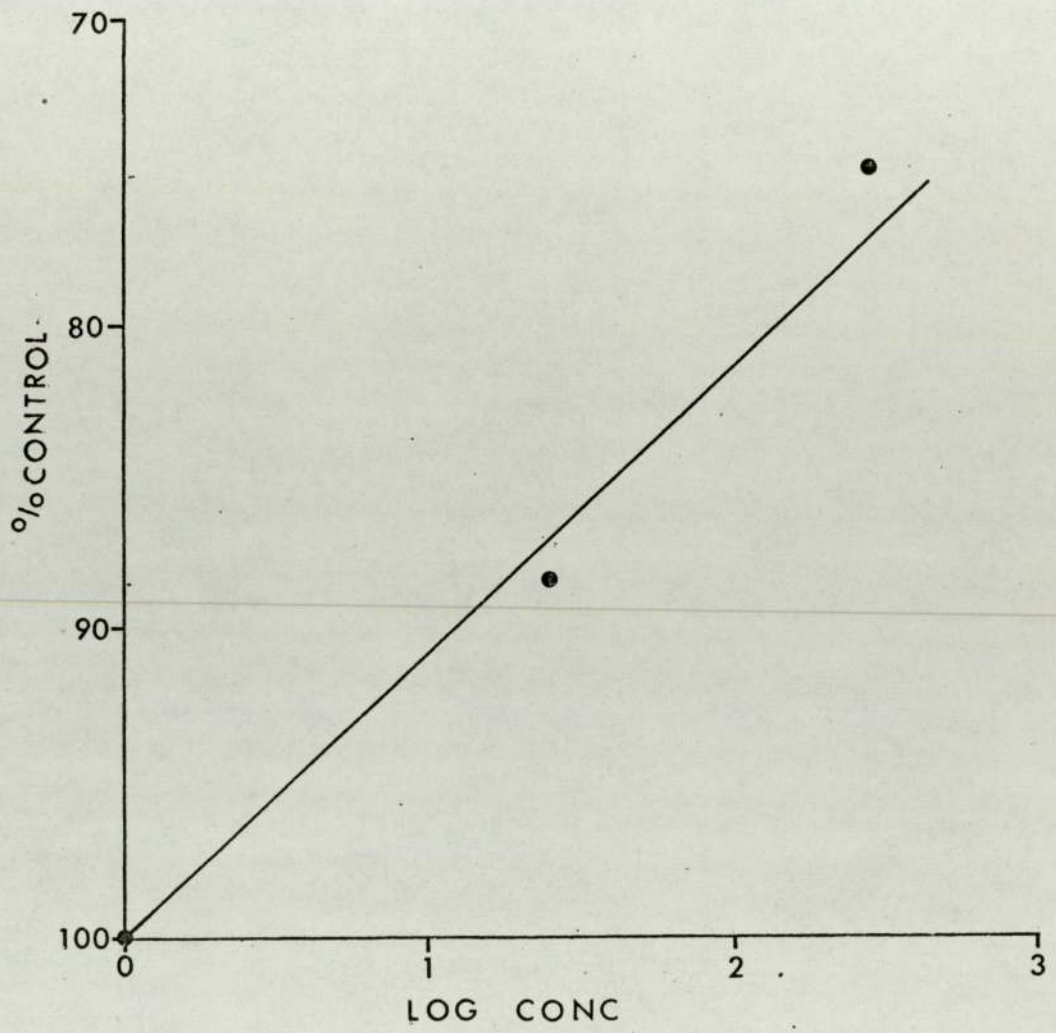
though not all authors agree with this finding (Niskanen et al 1976, Peet et al 1976). The proposed mechanism of action of amitriptyline as an antidepressant is by inhibition of the uptake of NA and 5-HT (Iversen et al 1975). However it is possible that if an increase in % free TP also occurs *in vivo* this may contribute to the therapeutic effect. However, after a dose of 150 mg per day of amitriptyline plasma levels of 55 - 75 ng/ml have been reported (Braithwaite et al 1972). This is very much lower than the concentration of amitriptyline used *in vitro* so this effect is unlikely to occur after a therapeutic dose.

The effect of the tetracyclic antidepressant maprotiline on TP binding was tested, but it was found to have no effect on binding.

Fig. 17

Log concentration/response curve for aspirin

(response is the reduction in the binding of trytophan to albumin after aspirin)

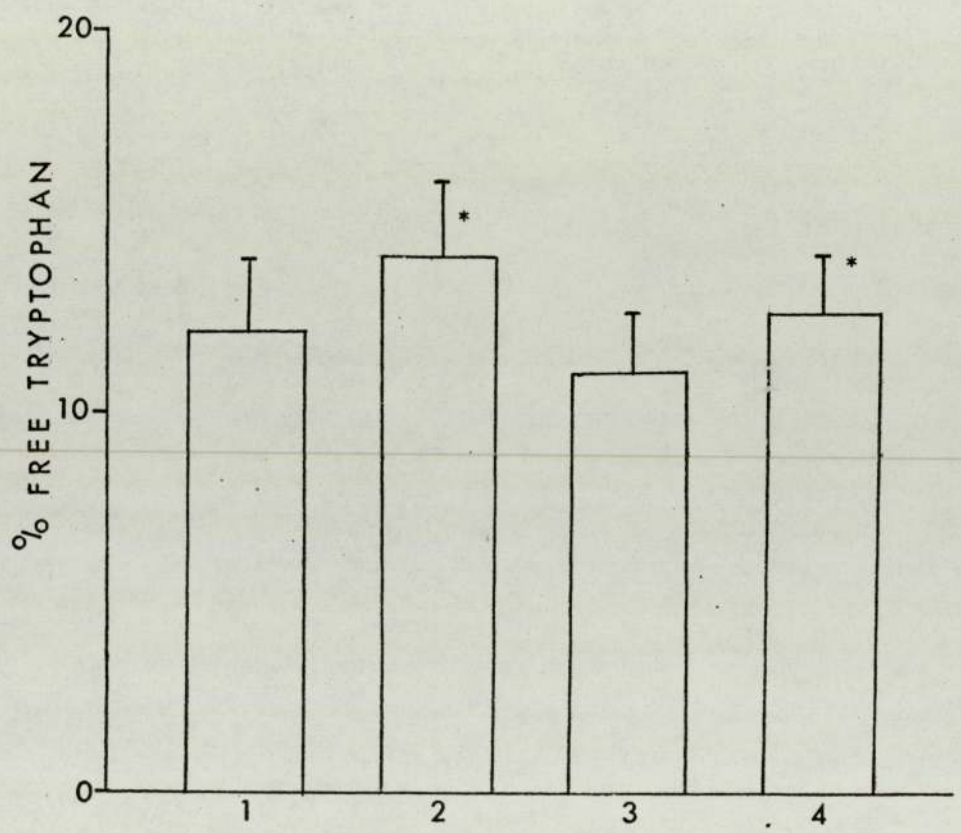


Linear regression coefficient r = 0.998

Fig. 18

Effects of some psychotropic compounds on % free tryptophan as determined by the isotopic method

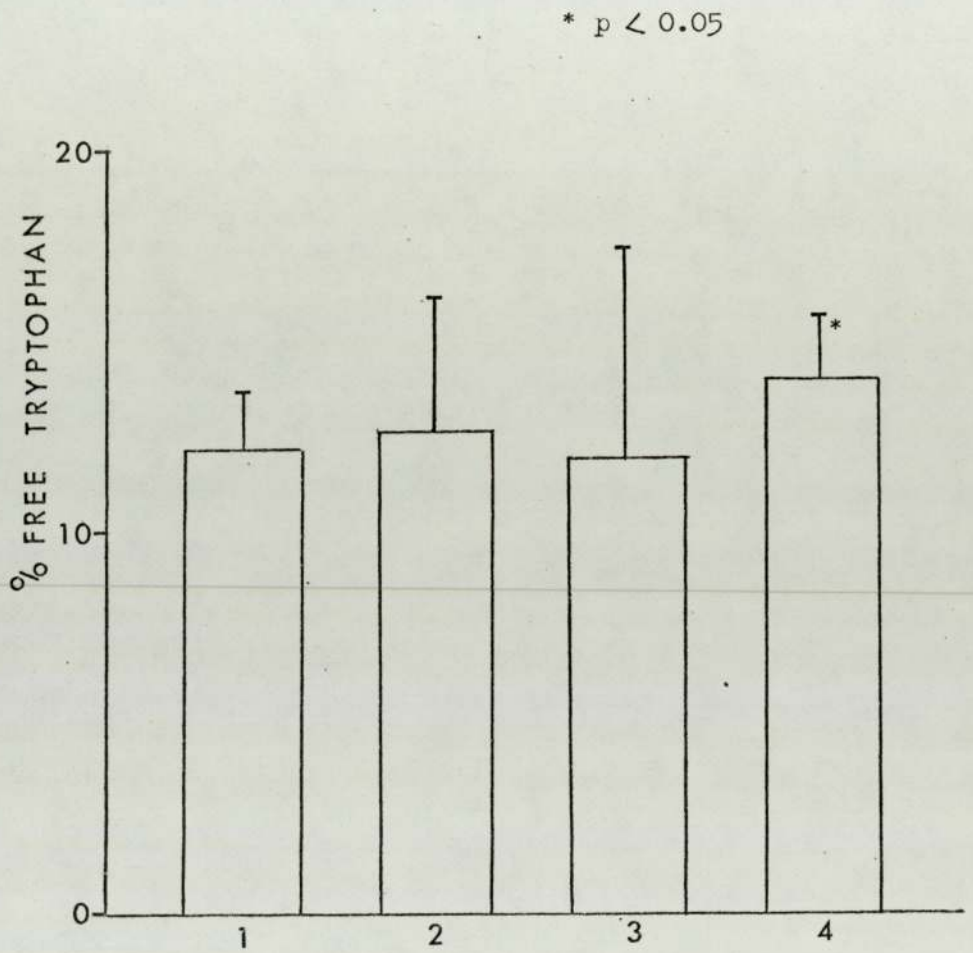
* $p < 0.05$ of relevant control



1 = control for amitriptyline (n = 11)
2 = amitriptyline 100 ug/ml (n = 10)
3 = control for nitrazepam (n = 11)
4 = nitrazepam 100 ug/ml (n = 9)

The results are expressed on the mean $\pm$ standard deviation.

Effect of female hormones on tryptophan binding as determined by the isotopic method



1 = control (n = 11)
2 = oestradiol 50 ug/ml (n = 9)
3 = mestranol 50 ug/ml (n = 8)
4 = norethisterone 50 ug/ml (n = 9)

The results are expressed as the mean $\pm$ standard deviation.

Table 3

Effect of nitrazepam and cortisol on tryptophan binding: a comparison of results from two methods

Treatment	Mean % free	Change from control
(i) non-isotopic method		
control	17.6	
nitrazepam 100 ug/ml	26.6	9% increase $p < 0.05$
cortisol 1 mg/100 ml	14.3	3,3% decrease not sig.
(ii) radioassay method		
control	12.2	
nitrazepam 100 ug/ml	15.3	3.1% increase $p < 0.01$
cortisol 1 mg/100 ml	12.74	0.54% increase not sig.

Table 4

Effect of aspirin on tryptophan binding

(non-isotopic method)

Treatment	Concentration in bottom compartment (ug/ml)	Binding as % control	Significance
control	9.62 ± 0.36 (11)		
aspirin 25 ug/ml	8.81 ± 0.54 (7)	91.6%	$p < 0.05$*
aspirin 250 ug/ml	7.21 ± 0.23 (12)	74.9%	$p < 0.001$*
aspirin 250 ug/ml + cortisol 1 mg/100 ml	6.88 ± 0.52 (12)	95.4%	not sig cf aspirin alone

The results are expressed as mean ± standard error with the number of determinations in brackets.

* one-tailed "t" test as it was expected that the free tryptophan would be increased

Table 5

Effect of psychoactive compounds on tryptophan binding

(non-isotopic method)

Treatment	ug/ml in bottom compartment	Binding as % control	Significance
control	9.94 ± 0.10 (18)		
amitriptyline 100 ug/ml	8.48 ± 0.08 (16)	85.1%	$p = 0.01$
maprotiline 100 ug/ml	9.51 ± 0.09 (16)	95.5%	not sig.
nitrazepam 100 ug/ml	8.73 ± 0.10 (16)	87.6%	$p < 0.05$

The results are expressed as the mean ± standard error with the number of observations in brackets.

Table 6

Effect of oleic acid on tryptophan binding

(non-isotopic method)

Treatment	Concentration (ug/ml) in bottom compartment	Binding as % control	Significance
1. albumin solution			
control	11.28 ± 0.37 (4)		
oleic acid 1.5 ug/ml	8.63 ± 0.20 (4)	77.1%	$p < 0.001$ *
oleic acid 1.5 ug/ml +cortisol 1 mg/ml	7.35 ± 0.16 (4)	66.1%	$p < 0.01$ * cf oleic acid alone
2. human plasma			
control	9.92 ± 1.30 (5)		
oleic acid 1.5 ug/ml	6.32 ± 0.54 (5)	63.7%	$p < 0.05$ *
3. mouse plasma			
control	13.27 ± 0.48 (3)		
oleic acid	11.70 ± 0.38 (3)	88.2%	$p < 0.05$ *

The results are expressed as mean ± SEM with the number of observations in brackets.

* indicates use of one tailed "t" test as oleic acid was expected to displace tryptophan from its binding.

Table 7

Effect of cortisol and female hormones on tryptophan binding

(non-isotopic method)

Treatment	Concentration in bottom compartment (ug/ml)	Binding as % control	Significance
1. cortisol			
control	9.67 ± 0.23 (17)		
cortisol 10 ug/100 ml	10.2 ± 0.36 (12)	105%	not sig.
cortisol 100 ug/100 ml	10.0 ± 0.29 (12)	103%	not sig.
cortisol 1 mg/100 ml	10.2 ± 0.44 (13)	105%	not sig.
2. female hormones			
control	9.81 ± 0.34 (8)		
oestradiol 50 ug/ml	9.83 ± 0.46 (8)	100%	not sig.
mestranol 50 ug/ml	9.27 ± 0.27 (8)	94.5%	not sig.
norethisterone 50 ug/ml	8.95 ± 0.53 (8)	91.2%	not sig.

The results are expressed as mean ± SEM with the number of observations in brackets.

CHAPTER 3

INFLUENCE OF CORTISOL AND CONTRACEPTIVE STEROIDS ON BRAIN 5-HT TURNOVER

3. Work in this laboratory has indicated that a change in central 5-HT function occurs after 24 hr. pretreatment with cortisol. Cortisol 75 ug/kg caused a marked increase in the head-twitch response to 5-HTP in mice after 24 hr. pretreatment (Handley and Miskin 1972). After five daily injections of cortisol the peak 5-HTP response was below that of controls (ibid.). Cortisol has often been shown to affect brain 5-HT levels and 5-HIAA levels (e.g. Curzon and Green 1968) but 5-HT turnover has not been measured directly. The purpose of this work was to investigate whether the effects of cortisol on the head-twitch response were reflected by a change in 5-HT turnover.

Changes in TP and 5-HT levels in mouse brain have been found in different stages of the oestrus cycle, brain 5-HT levels being maximal in the forebrain and midbrain at dioestrus (Greengrass and Tonge 1971). The possibility that these changes were accompanied by a change in whole brain 5-HT turnover in different stages of the cycle was investigated.

Norethisterone 20 ug/kg and oestradiol 1 ug/kg in combination had previously been found to increase brain 5-HT levels in female mice after 43 day treatment compared with dioestrus controls (Bond, 1979). The effect of a combination of norethisterone and oestradiol on brain 5-HT turnover was determined.

3.1 Preliminary experiments

3.1.1 Comparison of the turnover rate measured by two different methods

The brain 5-HT turnover rate was measured in a group of saline-treated male mice (25 $\pm$ 2 g) by two methods. Firstly 5-HT turnover was determined by the increase in brain 5-HT after

monoamine oxidase inhibition by pargyline 75 mg/kg. Secondly turnover was measured by the increase in brain 5-HIAA after inhibition of egress of this metabolite by probenecid 200 mg/kg (see experimental methods section). The results obtained by the two methods were in good agreement. The pargyline method estimated the 5-HT turnover as 1.706 $\pm$ 0.266 nmol/g/hr (Fig. 20) compared with 1.588 $\pm$ 0.234 nmol/g/hr by the probenecid method (Fig. 21) in saline-treated animals. The error is expressed as the 95% confidence interval as determined in experimental methods section 13.

3.1.2 Influence of vehicle of injection

In initial experiments arachis oil was used as the vehicle for injection since previous workers in this laboratory (Handley and Miskin 1972) had used this vehicle for behavioural experiments. Brain 5-HT turnover was measured in mice after subcutaneous pretreatment with cortisol in arachis oil 75 ug/kg, or vehicle only, or normal saline. Pargyline 75 mg/kg was used to inhibit monoamine oxidase and the increase in 5-HT was measured. A linear increase in brain 5-HT was observed after saline injection ($r = 0.918$, $p < 0.001$). After both arachis oil alone and after cortisol in arachis oil a slight deviation from linearity was seen ($r = 0.812$, $p < 0.01$, $r = 0.786$, $p < 0.01$ respectively) (Fig. 22). Both oil alone and cortisol in oil appeared to decrease 5-HT turnover rate as determined from changes in 5-HT levels 20 - 90 min. after pargyline. However this was largely due to high values of 5-HT levels 20 min. after pargyline (Fig. 22)

after oil pretreatment. At the other time intervals the mean 5-HT levels were very close to those found for saline treated controls. Arachis oil did not appear to be a satisfactory vehicle for injection.

Cortisol is insoluble in water but dissolves in dilute ethanol. 20 mg of cortisol was dissolved in 4 ml absolute ethanol and this stock solution was diluted with saline to a suitable concentration. Cortisol 75 ug/kg (0.1% $^{v}/_{v}$ ethanol) in ethanol and saline gave a satisfactory linear increase in brain 5-HT levels ($r = 0.822$, $p < 0.01$). There was no significant effect on 5-HT turnover after this dose of cortisol (Fig. 23). A dose of 5 mg/kg of cortisol in ethanol and saline (10% $^{v}/_{v}$ ethanol) also had no effect on turnover (Fig. 24). As the dose of cortisol increased the concentration of ethanol was increasing and this was thought to be unsatisfactory.

Cortisol-21-phosphate 5 mg/kg (base equivalent) in normal saline gave a linear increase in 5-HT levels after pargyline ($r = 0.839$, $p < 0.01$)(Fig. 25). No change in 5-HT turnover rate was found, in agreement with previous findings using ethanol and saline as vehicle. However this was considered to be the most suitable method of administering cortisol and all subsequent experiments were performed using cortisol phosphate in saline.

3.2 Effect of cortisol on brain 5-HT levels and turnover

Acute treatment

Male mice (25 ± 2 g) were treated with cortisol (as phosphate) 5 mg/kg or 20 mg/kg (base equivalent) 90 min. or 24 hr. previously.

Brain 5-HT levels were not significantly different from saline-treated controls 24 hr. after cortisol 5 mg/kg or 20 mg/kg or 90 min. after cortisol 5 mg/kg.

Brain 5-HT turnover was measured by the pargyline method described previously. In preliminary experiments a greater accumulation of brain 5-HT after pargyline was found 90 min. after cortisol 5 mg/kg than after saline alone (table 8). After 24 hr. pretreatment with cortisol 5 mg/kg the turnover rate of 5-HT was slightly reduced but this change was not statistically significant (Fig. 25). After 24 hr. pretreatment with cortisol 20 mg/kg the turnover rate was reduced by 30% ($p < 0.05$)(Fig. 26).

Chronic treatment

Male mice (25 ± 2 g) were pretreated daily for 7 days with cortisol (as phosphate) in saline 5 mg/kg and killed 24 hr. after the last dose. There was no significant difference in the brain 5-HT levels of these animals compared with animals treated with saline only for 7 days (table 9).

After 7 days pretreatment with cortisol 5 mg/kg the turnover rate was slightly reduced (Fig. 27) but this change was not statistically significant.

3.3 Effect of cortisol on plasma and brain tryptophan levels

Cortisol (as phosphate) 20 mg/kg (base equivalent) was injected subcutaneously into male mice (25 - 30 g). This dose was chosen as it had been found to cause a 30% reduction in 5-HT turnover rate. Animals were killed 24 hr. after cortisol and plasma and brain tryptophan were assayed as described previously.

3.3.1 plasma tryptophan

24 hr. pretreatment with cortisol 20 mg/kg caused a slight increase in plasma total tryptophan compared with saline treated controls (table 10). This change was not statistically significant. There was a large variation in plasma tryptophan between animals, presumably due to the ad libitum feeding.

3.3.2 brain tryptophan

There was no significant difference in brain tryptophan levels after cortisol 20 mg/kg compared with saline-treated controls (table 10). Unlike plasma tryptophan levels, brain tryptophan showed only very small variations between animals.

3.4 Effect of stage of oestrous cycle on brain 5-HT

Mice have a four day oestrous cycle characterised by four stages i.e. proestrus, oestrus, metoestrus and dioestrus (see experimental methods section).

Female mice (25 ± 3 g) were subjected to vaginal smear and grouped according to the stage of the oestrous cycle. Since brain 5-HT may be expected to be minimal at oestrus and maximal at dioestrus (Greengrass and Tonge 1971), brain 5-HT levels and turnover was measured in groups of female mice in oestrus and in dioestrus. The pargyline method described previously (see experimental methods section) was used to measure the turnover rate.

3.4.1 Brain 5-HT levels

Brain 5-HT levels were found to be higher in animals in dioestrus (n = 6) than those in oestrus (n = 6) but this change

was not statistically significant (table 11).

3.4.2 Brain 5-HT turnover was reduced in animals in dioestrus (n = 18) compared with those in oestrus (n = 20) but this change was not statistically significant (Fig. 28).

3.5 Effect of norethisterone and oestradiol on brain 5-HT

Female mice (15 $\pm$ 3 g) were subjected to vaginal smear and all animals used for measurement of brain 5-HT levels and turnover were in metoestrus stage 1 or 2. The animals were weighed and injected subcutaneously on each of 14 days with norethisterone 200 ug/kg and oestradiol 100 ug/kg (base equivalent) or vehicle alone. Norethisterone acetate and oestradiol benzoate were dissolved in saline and ethanol in the ratio 50:1. The site of injection was altered periodically to avoid changes in absorption from an inflamed injection site. Animals received food and water ad libitum. All animals had a mean initial weight of 25 g at the start of the experiment. This increased slightly during the experiment, but there was no significant difference in the weight gain for the test and control groups (table 12).

3.5.1 Brain 5-HT levels

Brain 5-HT was measured as described under experimental methods section. There was no significant difference between the brain 5-HT levels of animals treated with norethisterone 200 ug/kg and oestradiol 100 ug/kg or vehicle alone (table 11).

3.5.2 Brain 5-HT turnover

Brain 5-HT turnover was measured by the pargyline method

described previously (experimental methods section). There was no significant difference in the brain 5-HT turnover rate between animals pretreated for 14 days with norethisterone 200 ug/kg and oestradiol 100 ug/kg or vehicle alone (Fig. 29).

Discussion

1. Measurement of turnover rate

There are several methods of measuring the turnover rate of 5-HT in brain, including the isotopic and non-isotopic techniques described in the Experimental Methods section 8.1. Each of the methods available at present has disadvantages associated with it.

The isotopic methods cause less interference with the normal situation, but still have limitations. It is doubtful whether the estimation of the specific activity of ^{3}H-5-HT or ^{3}H-TP in the brain tissue measures the conversion of TP to 5-HT in serotoninergic neurons. Recent results suggest the existence of various pools of TP with different kinetic characteristics (Shields and Eccleston 1972). The ^{3}H label is labile and small amounts may be lost in the conversion of ^{3}H-TP to ^{3}H-5-HT and ^{3}H-5-HIAA.

The non-isotopic methods are simpler to perform but have other disadvantages. The inhibition of mono-amine oxidase, for example may affect other processes and other transmitters such as noradrenaline and dopamine. Inhibition of 5-HT reuptake and stimulation of release have been reported after mono-amine oxidase inhibition (MAOI)(Knoll and Magyar 1972). There may also be an increase in plasma corticoid levels after MAOI (Morot-Gaudry et al 1974) which may indirectly affect turnover. There also appears to be an increase in the apparent 5-HT turnover in the first 10 min. after pargyline (ibid.).

The inhibition of efflux of 5-HIAA after probenecid also has disadvantages in measuring 5-HT turnover. Probenecid was found to increase brain TP levels after a dose of 400 mg/kg, (Morot-Gaudry et al 1974) but

no significant effect was seen after 200 mg/kg (ibid.). It has also been reported that 5-HIAA efflux may not be completely inhibited by probenecid and that only a part of the 5-HIAA formed in brain is eliminated by the active transport process (Sharman 1968).

Despite these limitations there is little doubt that all the methods estimate changes in turnover but there is some doubt whether they reliably estimate the absolute rate of formation of 5-HT.

Two non-isotopic methods were used to estimate turnover rate, the pargyline method and the probenecid method, described in Experimental Methods section 8. Linear increases in 5-HT levels after pargyline and in 5-HIAA levels after probenecid were usually achieved.

The estimates of turnover rate in control animals made by the two methods were in good agreement - 1.588 nmol/g/hr. by the probenecid and 1.706 nmol/g/hr. by the pargyline method.

Morot-Gaudry et al (1974) found that the estimates of turnover of 5-HT varied according to the method used, the pargyline method gave the largest estimate, the isotopic method an intermediate value and the probenecid method gave the lowest estimate of turnover. The high values obtained by these authors for the turnover rate after pargyline was probably due to the fact that the increase in 5-HT was measured up to 10 min. after pargyline. Turnover has been reported to be stimulated temporarily during the first 10 min. after pargyline.

The values obtained for the turnover after probenecid (1.588 nmol/g/hr.) were of the same order as published results of 1.51 - 2.05 nmol/g/hr (Morot-Gaudry et al 1974) using this method. Tozer et al (1966) observed slightly higher values for the turnover of 5-HT up to 90 min. after pargyline of

2.32 nmol/g/hr. compared with present values of 1.706 nmol/g/hr.

2. Influence of vehicle of injection

The vehicle of injection appears to affect the linearity of the increase of 5-HT after pargyline and it was difficult to choose a vehicle which would be inert. Arachis oil was initially used as vehicle for injection of cortisol since Handley and Miskin (1972) had used this vehicle in their behavioural experiments and it was hoped to explain their behavioural effects. These authors desired to achieve a depot effect (Handley, personal communication) but this vehicle did not give a linear increase in 5-HT after pargyline. Also, arachis oil may contain free fatty acids which have been shown to displace TP from its binding to albumin (Knott and Curzon 1972, and Chapter 2). An increase in plasma % free may be expected to increase brain TP and stimulate 5-HT synthesis (Tagliamonte et al 1971).

Cortisol dissolves adequately in dilute ethanol but this was not thought to be an inert vehicle. Ethanol (25% $^{v}/_{v}$ in saline) has been reported to increase brain 5-HT levels up to 6 hr. after injection, followed by a reduction in levels 6 - 10 hr. after injection (Badawy and Evans 1977). 5-HT turnover has been reported to be increased after 8 to 14 day pretreatment whereas acute administration did not affect turnover (Kuriyama et al 1971). When high doses of cortisol were used the concentration of ethanol present may have been sufficient to have some effect on brain 5-HT (40% $^{v}/_{v}$ ethanol for 20 mg/kg dose of cortisol).

Cortisol-21-phosphate was water soluble and this salt was found to be the most satisfactory method of administration of cortisol.

3. Effect of cortisol

Preliminary experiments showed a greater accumulation of 5-HT 90 min. after pargyline and cortisol 5 mg/kg compared with controls. This indicated increased 5-HT synthesis after 90 min. pretreatment with cortisol 5 mg/kg and is in agreement with previous findings (Neckers and Sze 1975).

Brain 5-HT levels were found to be unaffected by 24 hr. pretreatment with cortisol. Brain 5-HT has previously been reduced after 5 hr. pretreatment in the rat (e.g. Curzon and Green 1968) but had returned to normal after 10 hr. treatment (ibid.). Chronic dosing with cortisol 5 mg/kg for 7 days was found to have no effect on brain 5-HT in agreement with previous findings (Curzon and Green 1968).

Brain 5-HT turnover rate was significantly reduced after 24 hr. pretreatment with a very high dose of cortisol (20 mg/kg) but no effect on turnover was observed after 24 hr. pretreatment or after 7 day pretreatment with cortisol 5 mg/kg. Cortisol 5 mg/kg was shown to reduce brain 5-HT and 5-HIAA levels in rats (Curzon and Green 1971) after 5 hr. pretreatment. This was suggested to indicate a reduction of 5-HT synthesis after this time and the changes were accompanied with an increased TP pyrrolase activity. Increased pyrrolase activity may be expected to increase plasma kynurenine levels via increased kynurenine production and this possibility was investigated in the following chapter. Low doses of cortisol (75 ug/kg) had previously been found to affect animal behaviour. The head-twitch response to 5-HTP and 5-HT was markedly increased after 24 hr. pretreatment with cortisol (Handley and Miskin 1972). However the twitch rate fell below normal after 5 day chronic treatment with cortisol. The head twitch response in mice is thought to give an

indication of "free" 5-HT in the brain (Corne et al 1963) and it was expected that this change in behaviour after cortisol may have been reflected by a change in 5-HT turnover. However a change in turnover rate was only demonstrated after a very high dose of cortisol (20 mg/kg) though a slight reduction in turnover was seen after a dose of 5 mg/kg. It is possible that this behaviour is more sensitive to subtle changes in brain 5-HT function than measurement of turnover. Turnover rate may be affected in some areas of mouse brain which is not detected by measurement of whole brain turnover rate.

Brain tryptophan levels were found to be unaffected by cortisol at any dose even though the 5-HT turnover rate had been reduced. Brain tryptophan has often been measured after cortisol and has been found to be unchanged (Azmitia and McEwen 1974, Thierry et al 1968), increased after 90 min. treatment (Neckers and Sze 1975) or decreased after 5 hr. pretreatment (Green and Curzon 1968, Green et al 1975). However, brain TP has not previously been measured after 24 hr. pretreatment with cortisol and it is possible that the brain TP is normalised more quickly than the 5-HT turnover.

Plasma TP was also little affected by cortisol 20 mg/kg. Liver TP and plasma free TP have previously been reported to be reduced but plasma TP was unaffected (Green et al 1975).

4. Effects of female hormones

(i) Effect of stage of the oestrous cycle

Brain 5-HT levels were found to be higher in animals in dioestrus than those in oestrus in agreement with previous findings (Greengrass and Tonge 1971, Bond 1979). Brain 5-HT turnover was also reduced

by 30% in animals in dioestrus compared with those in oestrus. However these were only preliminary experiments with a small number of animals. The turnover rate of 5-HT in animals in oestrus was similar to that found in male mice in Chapter 3 section 1 and by other workers (Morot-Gaudry et al 1974). Whole brain TP had been found to increase in oestrus compared with dioestrus (Bond 1979) and these results would be consistent with an increase in turnover rate at oestrus compared with dioestrus.

However there were procedural difficulties in measuring turnover in the oestrus cycle, by this method. In all turnover experiments a large number of observations is needed to produce statistical significance. To minimise variation in brain 5-HT (Hardeland and Rensing 1968) experiments have to be performed at the same time of day. Vaginal smear was therefore performed in the morning on 40 to 50 female mice, which were then grouped according to the stage of the oestrus cycle. Turnover rate was determined by the pargyline method between 1 and 4 p.m. There was an unavoidable delay between vaginal smear and the removal of brains which could be up to 4 hours for some animals. This delay may have been sufficient for the stage of the oestrus cycle to have changed and this possibility reduces the reliance which could be placed on the results obtained.

(ii) Effect of a combination of norethisterone and oestradiol

Weight gain is sometimes reported as a side-effect of oral contraceptive usage in Man. On administration of the contraceptive combination of norethisterone 200 ug/kg and oestradiol 100 ug/kg to mice, no significant difference in weight gain was found between the

treated and control groups.

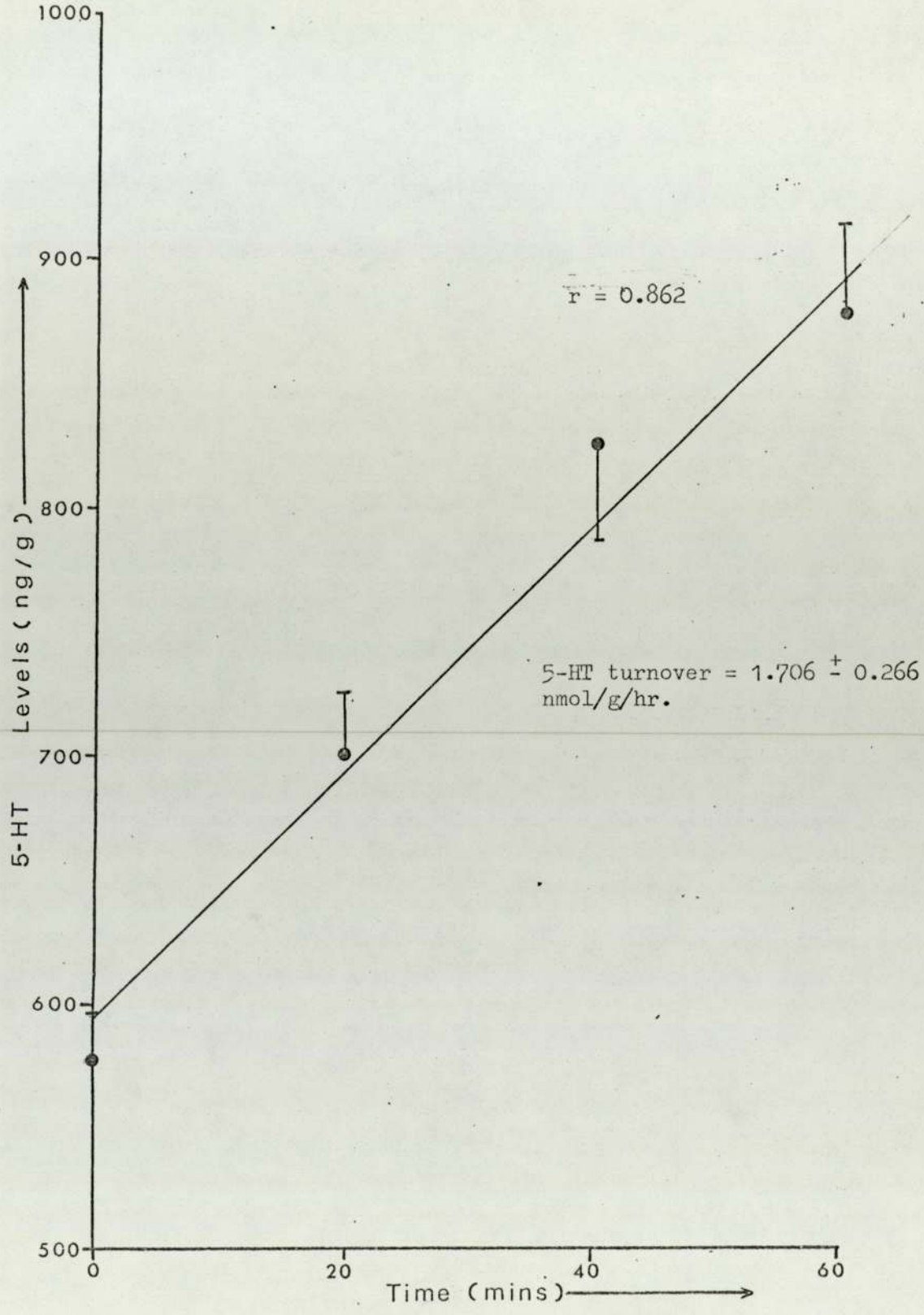
14 day pretreatment with norethisterone and oestradiol had no significant effect on brain 5-HT levels compared with saline/ethanol controls. The brain 5-HT levels after saline/ethanol were not significantly different from dioestrus values in female mice (table 11). However dioestrus values in female mice were slightly higher than levels in male mice (table 11). Brain 5-HT has previously been found to be increased after 43 day pretreatment with norethisterone 20 ug/kg and oestradiol 1 ug/kg (Bond 1979).

A combination of norethisterone/oestradiol had no effect on 5-HT turnover compared with vehicle alone. This observation is in agreement with previous findings after similar low doses of a contraceptive combination. Thus a combination of oestradiol 40 ug/100 g and progesterone 4 mg/100 g in female rats failed to affect 5-HT synthesis (Hyyppa 1973). However many authors have reported effects on brain 5-HT turnover when high doses of these steroids have been used. Progesterone at a dose of 10 to 20 mg/kg (Ladisch 1977) or 10 mg/animal (Glowinski et al 1973) was found to increase brain 5-HT synthesis. Oestradiol generally had the opposite effect (Leonard and Hamburger 1974). These contraceptive steroids have been used in sequence and turnover was decreased by oestrogen treatment and restored by progesterone (Fuxe et al 1974).

The main purpose of the experiments using the contraceptive combination was to confirm whether there was any effect on 5-HT turnover with relatively low doses. Several authors have reported changes after treatment with contraceptive steroids but the doses were

very large, up to 50 mg/kg of progesterone may have been used in one study (Glowinski et al 1973). The possible relevance of these findings to the administration of oral contraceptive combinations in Man was examined. It was found, however, that 200 ug/kg of norethisterone and 100 ug/kg of oestradiol did not affect 5-HT turnover, and since such doses are still larger than those used in oral contraception, it is unlikely that turnover is affected. These results suggested that a more detailed study of the actions of these steroids on 5-HT turnover would provide limited additional information. It was therefore decided that the effects of these steroids on TP metabolism would be further investigated (see Chapter 4).

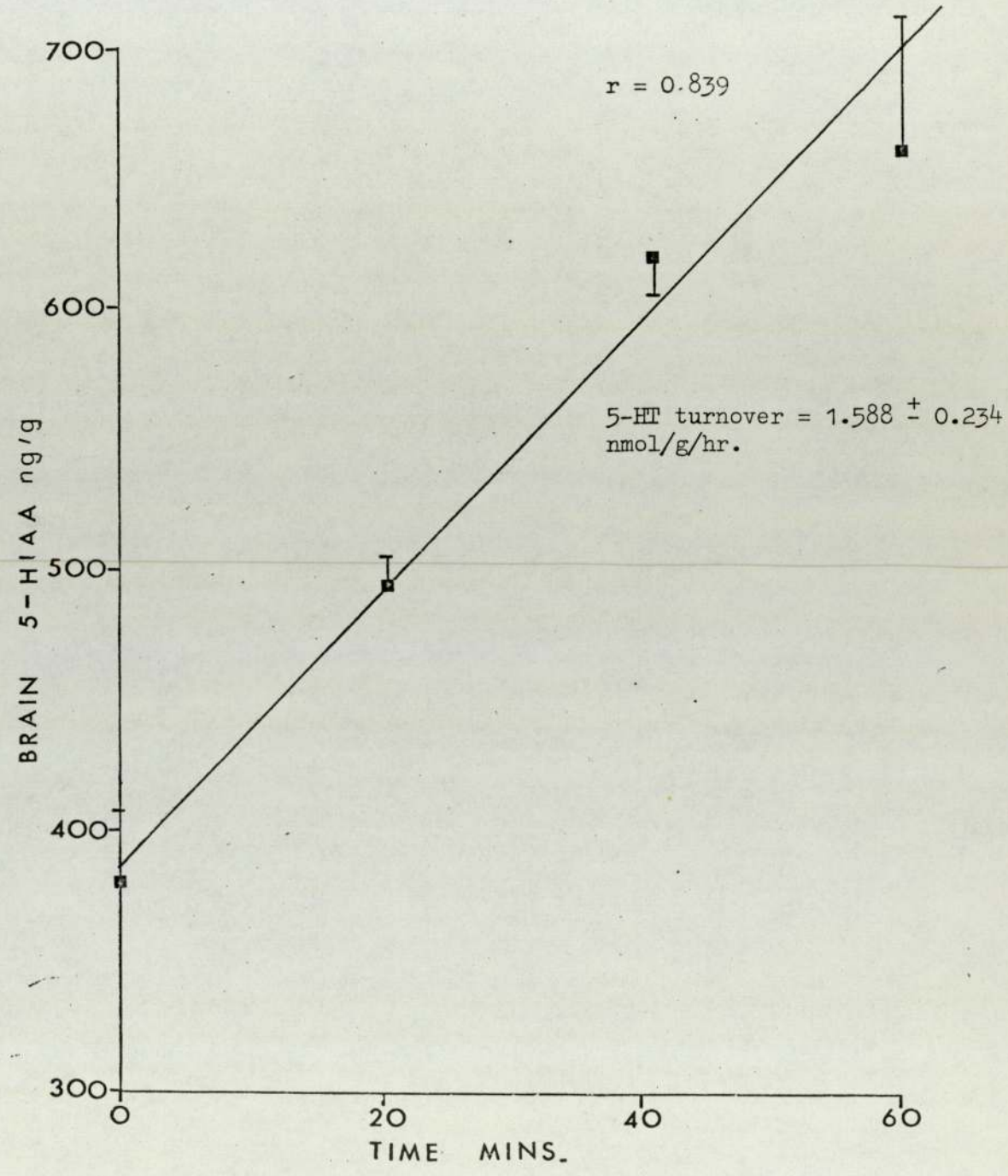
5-HT turnover in saline-treated controls determined by the pargyline method



The results are expressed as the mean of 6 determinations, and the bars represent the standard error.

r = linear regression coefficient

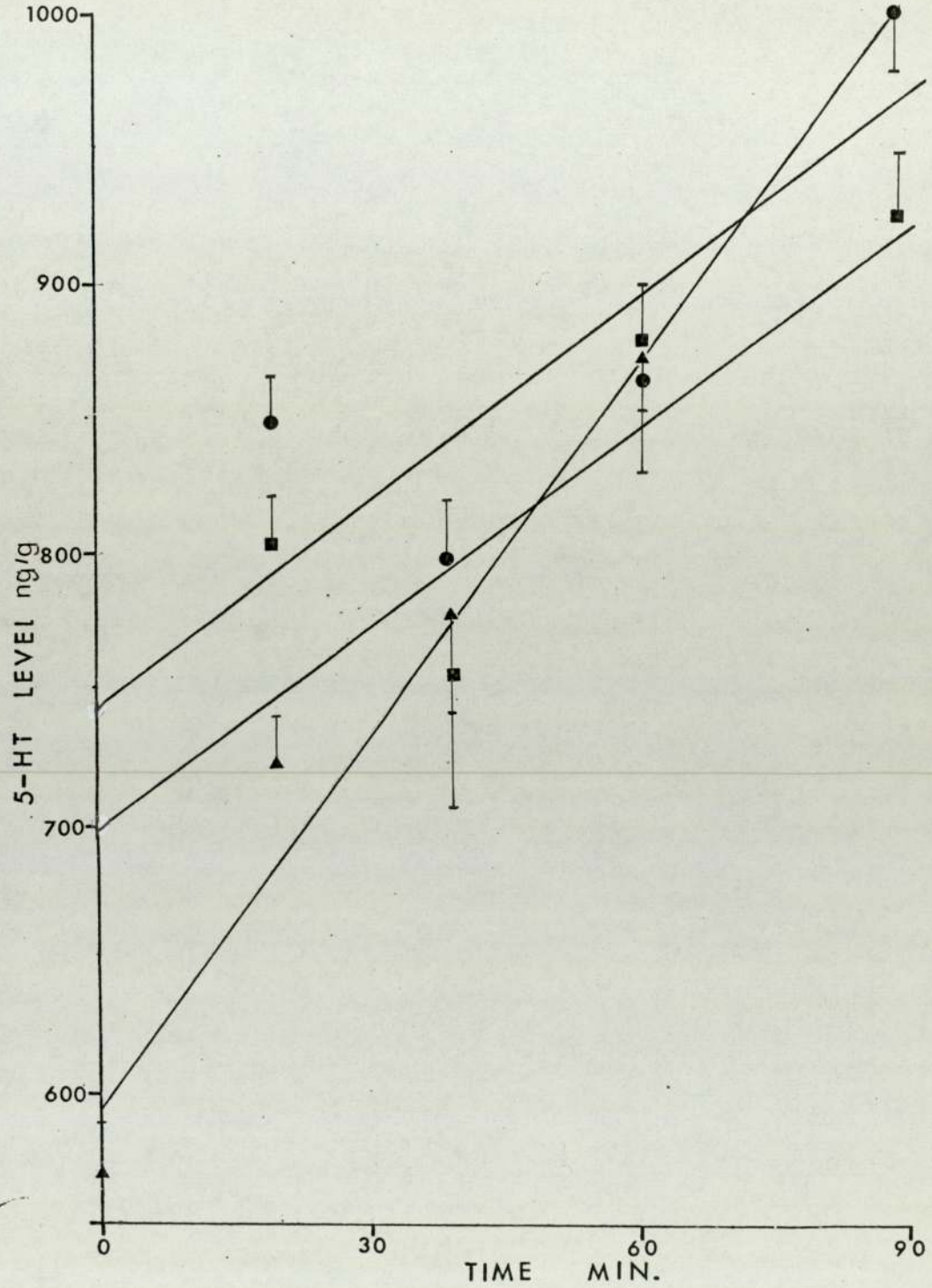
5-HT turnover in saline-treated controls determined by the probenecid method



The results are expressed as the mean of 4 - 6 determinations and the bars represent the standard error.

r = linear regression coefficient

Effect of cortisol 75 ug/kg in arachis oil on brain 5-HT turnover after 24 hr. pretreatment



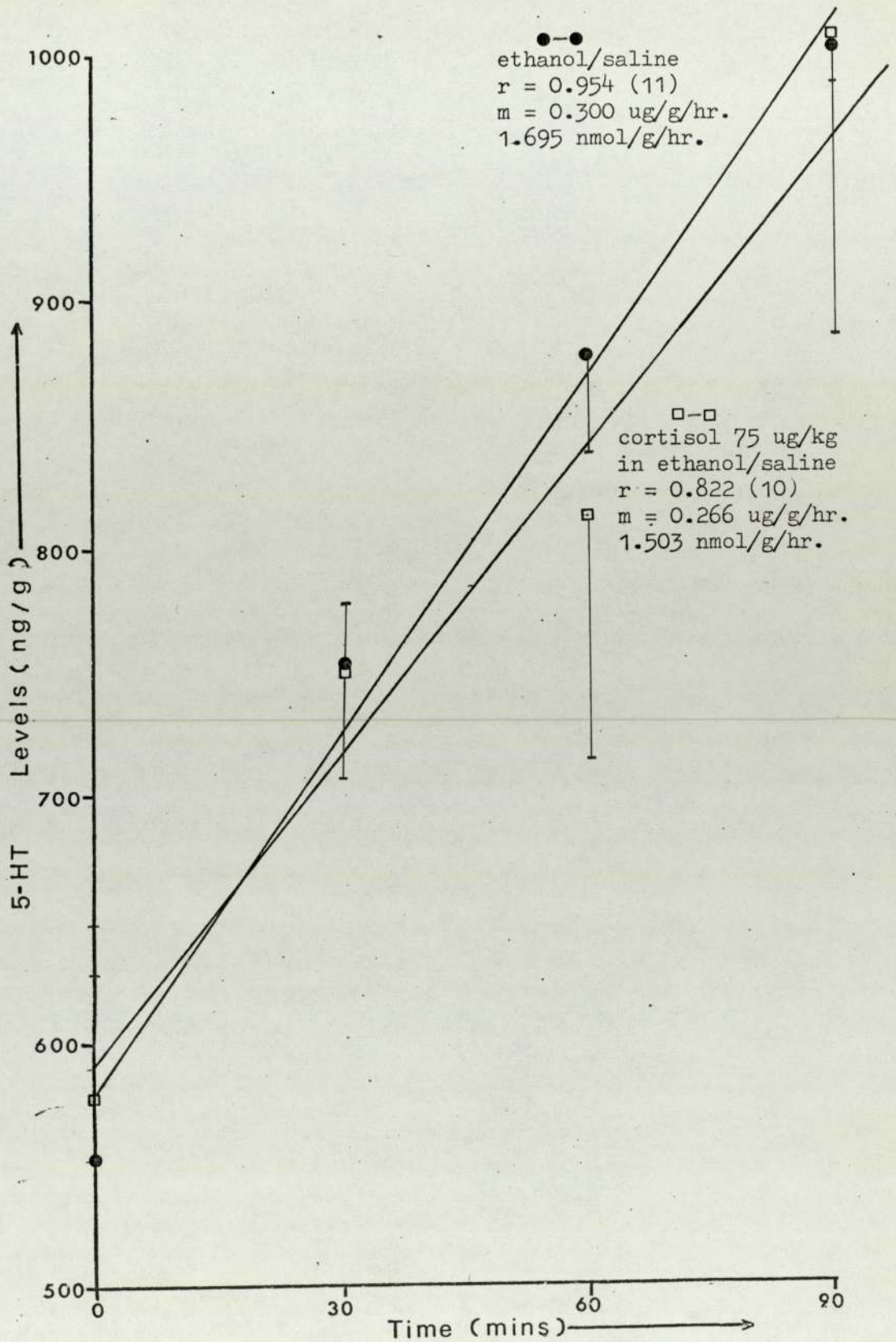
▲—▲ saline only after 24 hr. r = 0.918 (n = 11)
m = 0.276 ug/g/hr, 1.559 nmol/g/hr

●—● arachis oil after 24 hr. r = 0.864 (n = 10)
m = 0.156 ug/g/hr, 0.881 nmol/g/hr.

■—■ cortisol 75 ug/kg in arachis oil after 24 hr. (n = 11)
r = 0.690, m = 0.152 ug/g/hr., 0.858 nmol/g/hr.

r = linear regression coefficient, m = turnover rate,
n = number of determinations

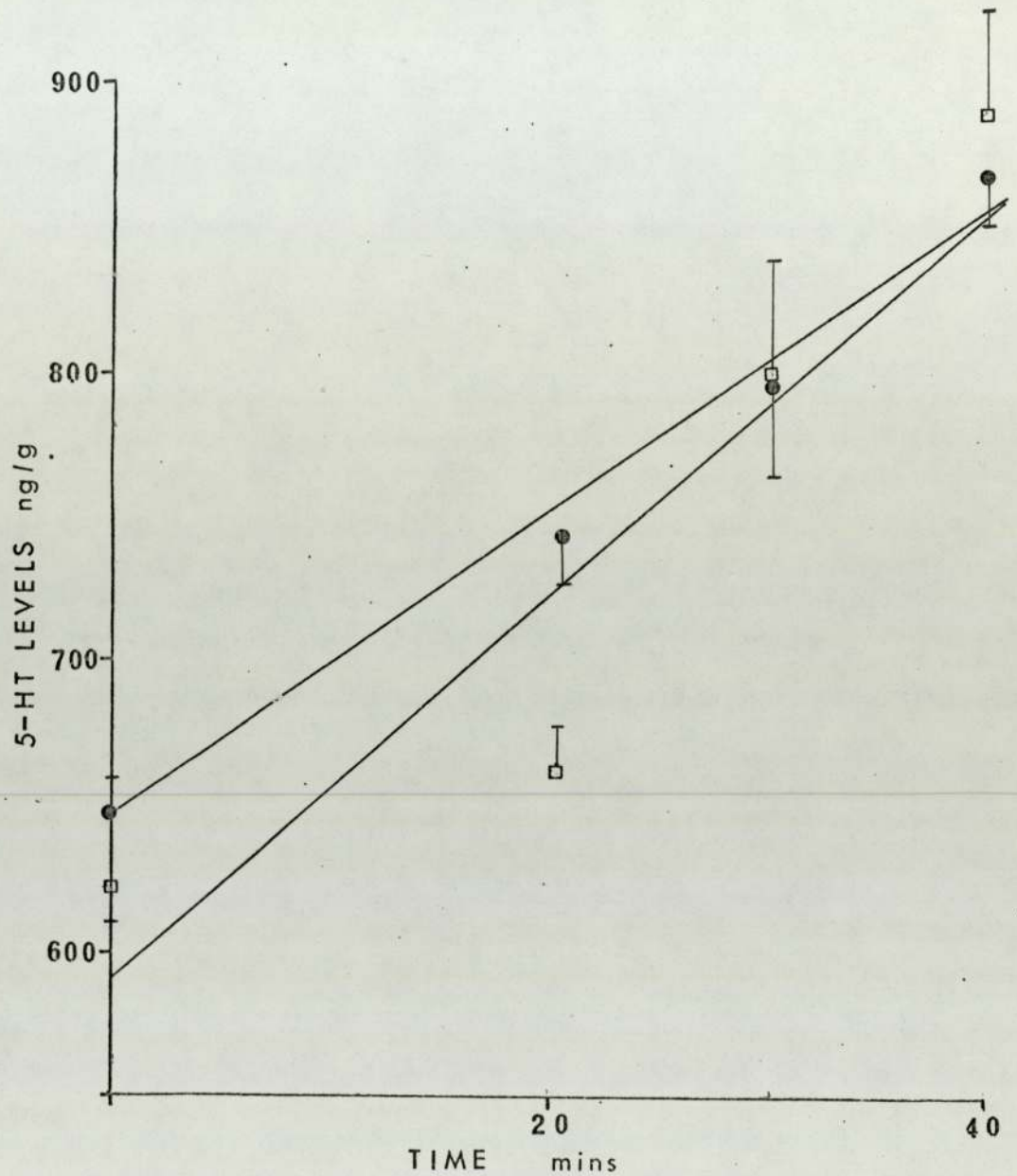
Effect of cortisol 75 ug/kg in ethanol/saline on brain 5-HT turnover after 24 hr. pretreatment



r = linear regression coefficient, m = turnover rate

The number of determinations are in brackets

Effect of cortisol 5 mg/kg in ethanol (10% v/v) and saline on brain 5-HT turnover after 24 hr. pretreatment



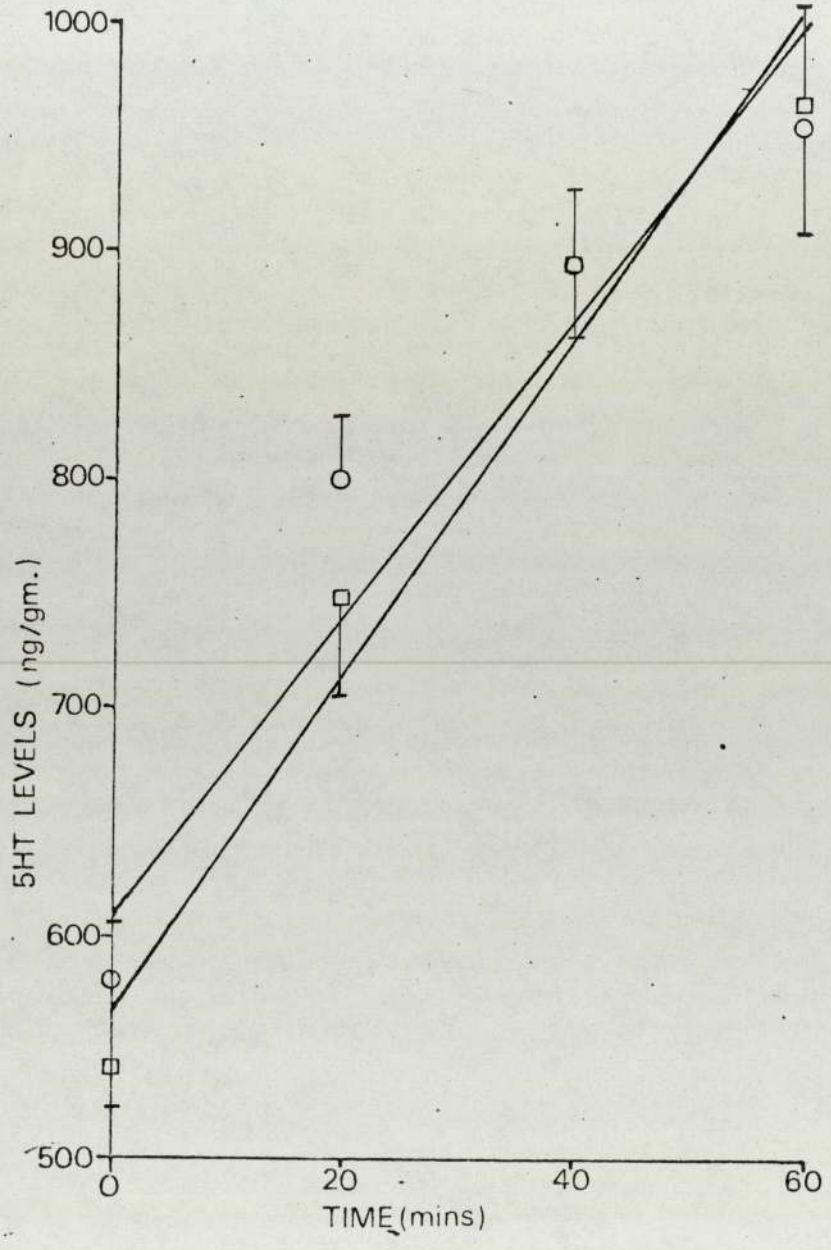
●—● 24 hr. pretreatment with ethanol (10% v/v) and saline (n = 10) r = 0.915, m = 0.326 ug/g/hr. (1.841 nmol/g/hr.)

□-□ 24 hr. pretreatment with cortisol 5 mg/kg in ethanol/ saline (n = 11) r = 0.875, m = 0.390 ug/g/hr. (2.203 nmol/g/hr.

r = linear regression coefficient

m = turnover rate

Effect of cortisol (as phosphate) 5 mg/kg (base equivalent) on brain 5-HT turnover

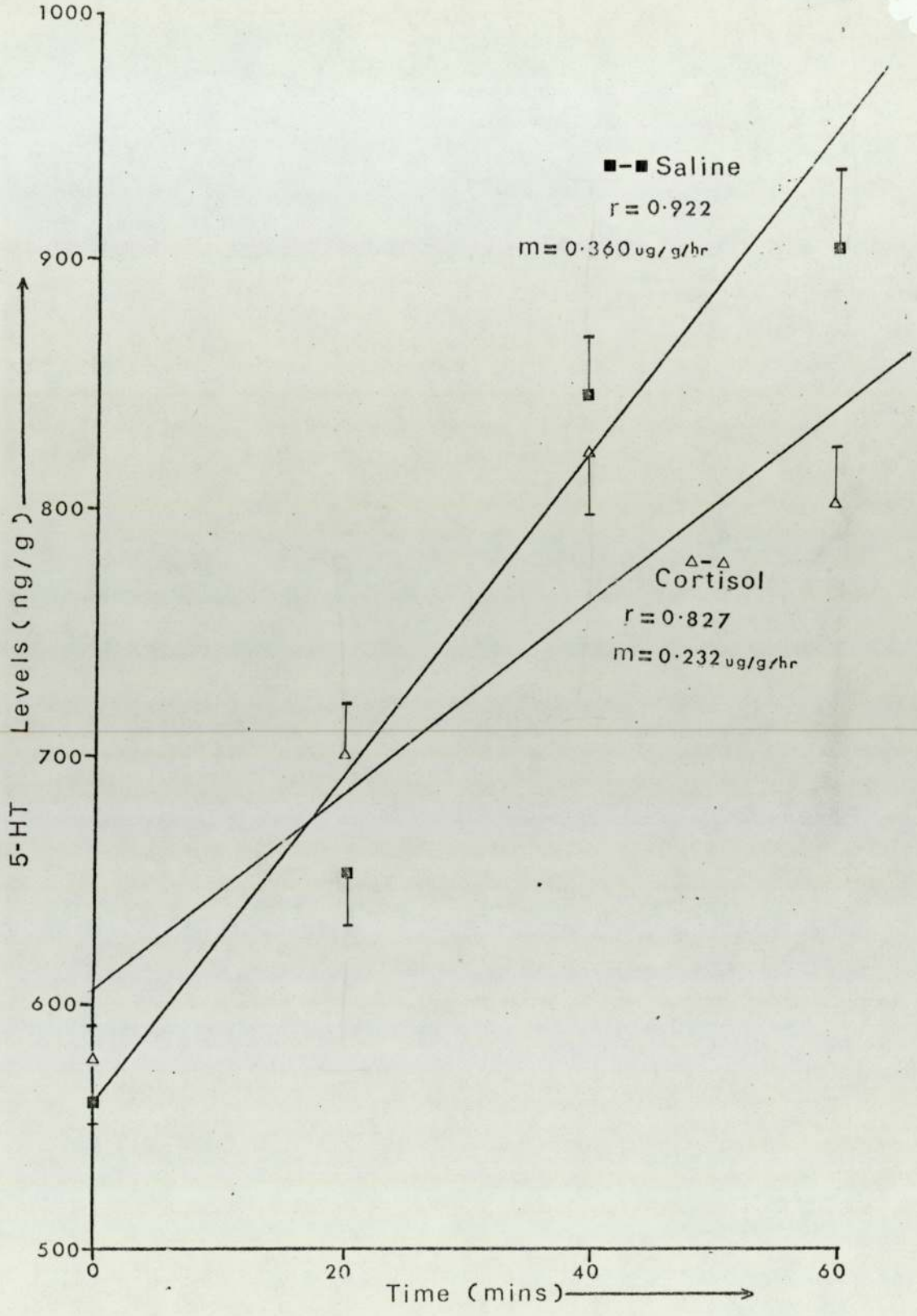


□—□ saline control r = 0.888, m = 0.426 ug/g/hr.(2.406 nmol/g/hr.)

△—△ cortisol (as phosphate) 5 mg/kg (base equivalent) r = 0.837, m = 0.372 ug/g/hr. (2.101 nmol/g/hr)

Each symbol is the mean of at least 6 determinations.

Effect of 24 hr. pretreatment with cortisol 20 mg/kg on 5-HT turnover (pargyline method)



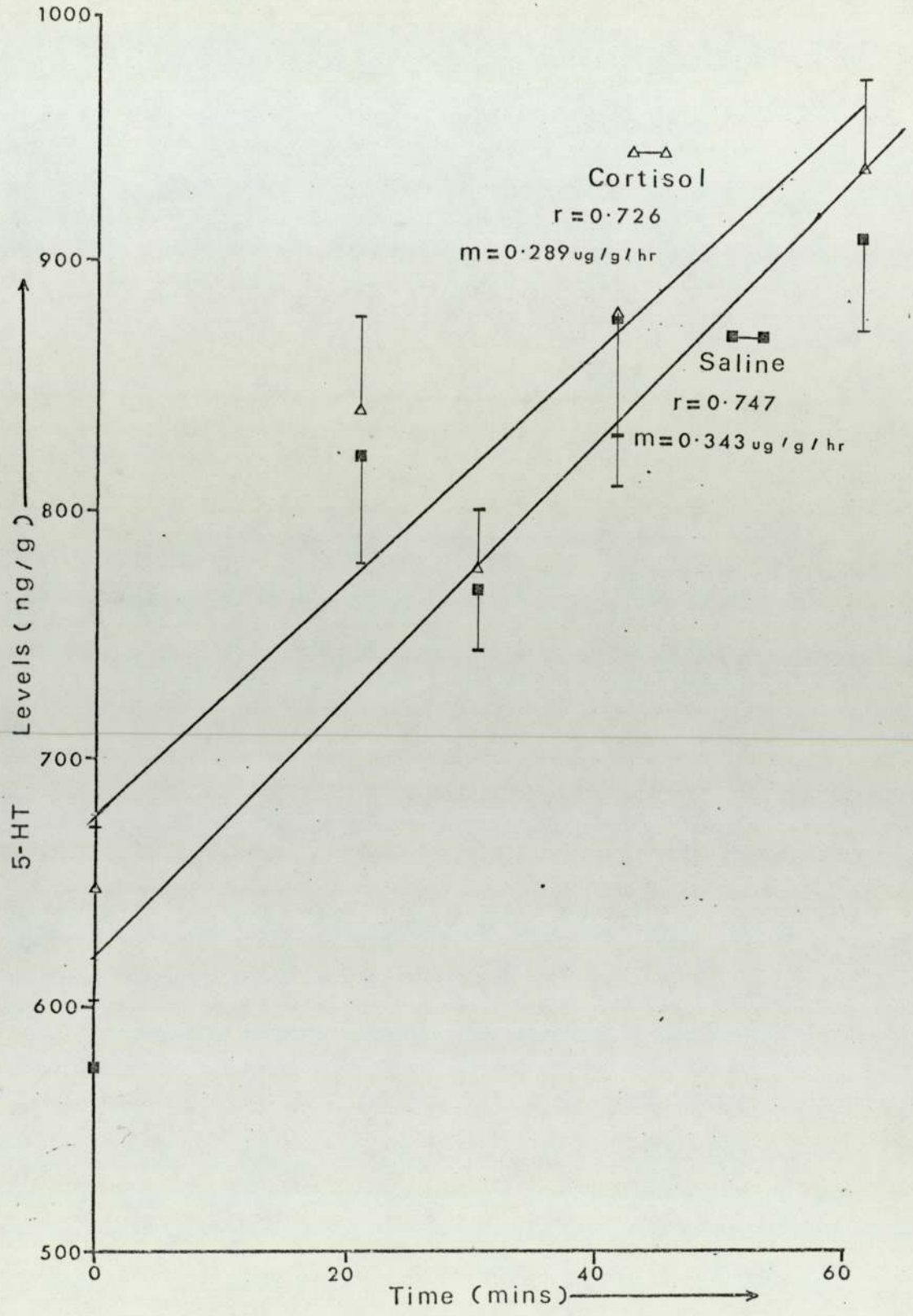
Each symbol represents the mean of at least 6 determinations.

Turnover rate saline = 2.034 $\pm$ 0.192 nmol/g/hr.

cortisol = 1.311 $\pm$ 0.089 nmol/g/hr.

r = linear regression coefficient, m = turnover ug/g/hr.

Effect of 7 day pretreatment with cortisol 5 mg/kg on 5-HT turnover (pargyline method)



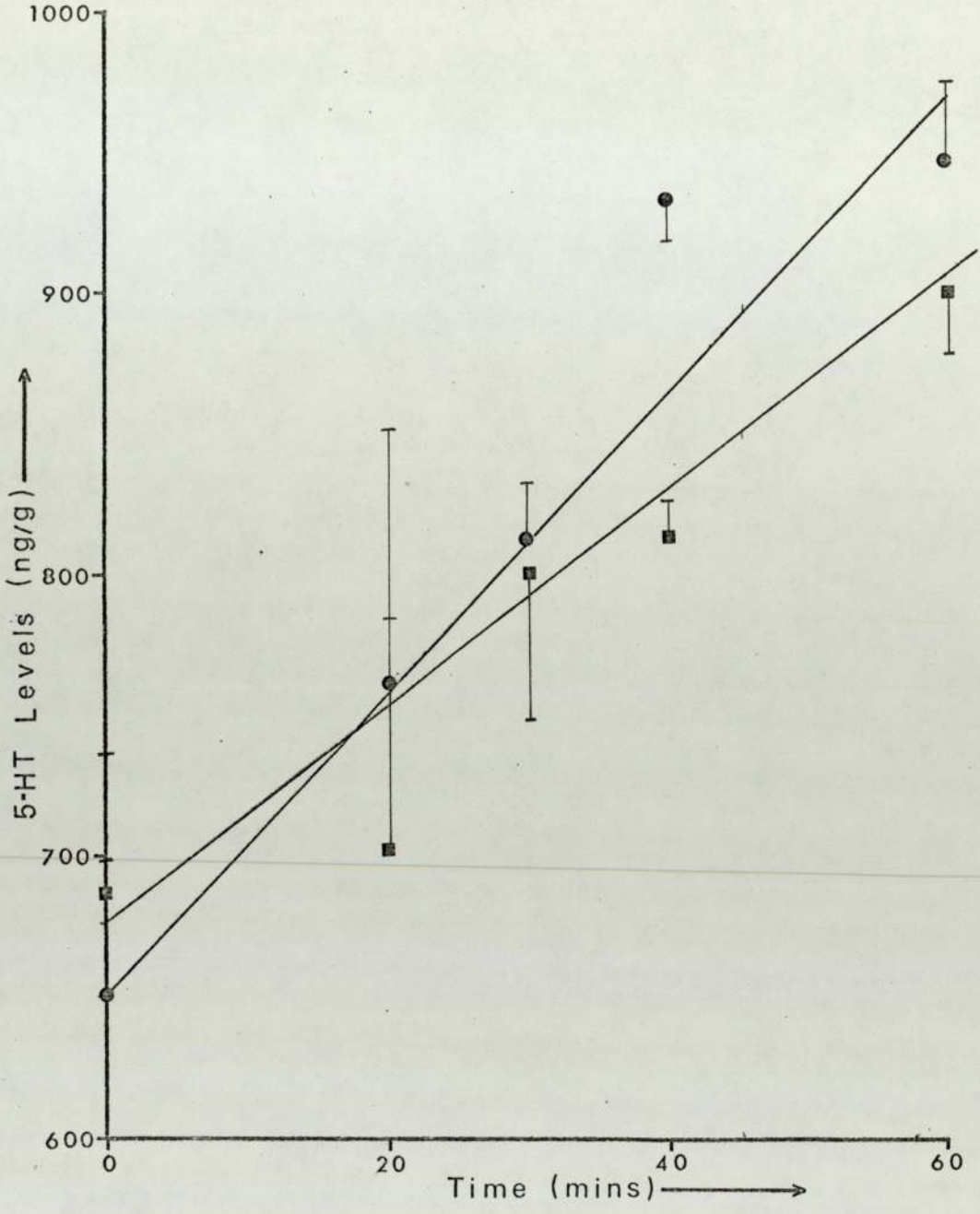
Each symbol represents the mean of 3 - 10 determinations.

Turnover rate saline = 1.938 $\pm$ 0.802 nmol/g/hr

cortisol = 1.633 $\pm$ 0.706 nmol/g/hr

r = linear regression coefficient, m = turnover ug/g/hr.

Effect of stage of the oestrus cycle on brain 5-HT turnover in female mice



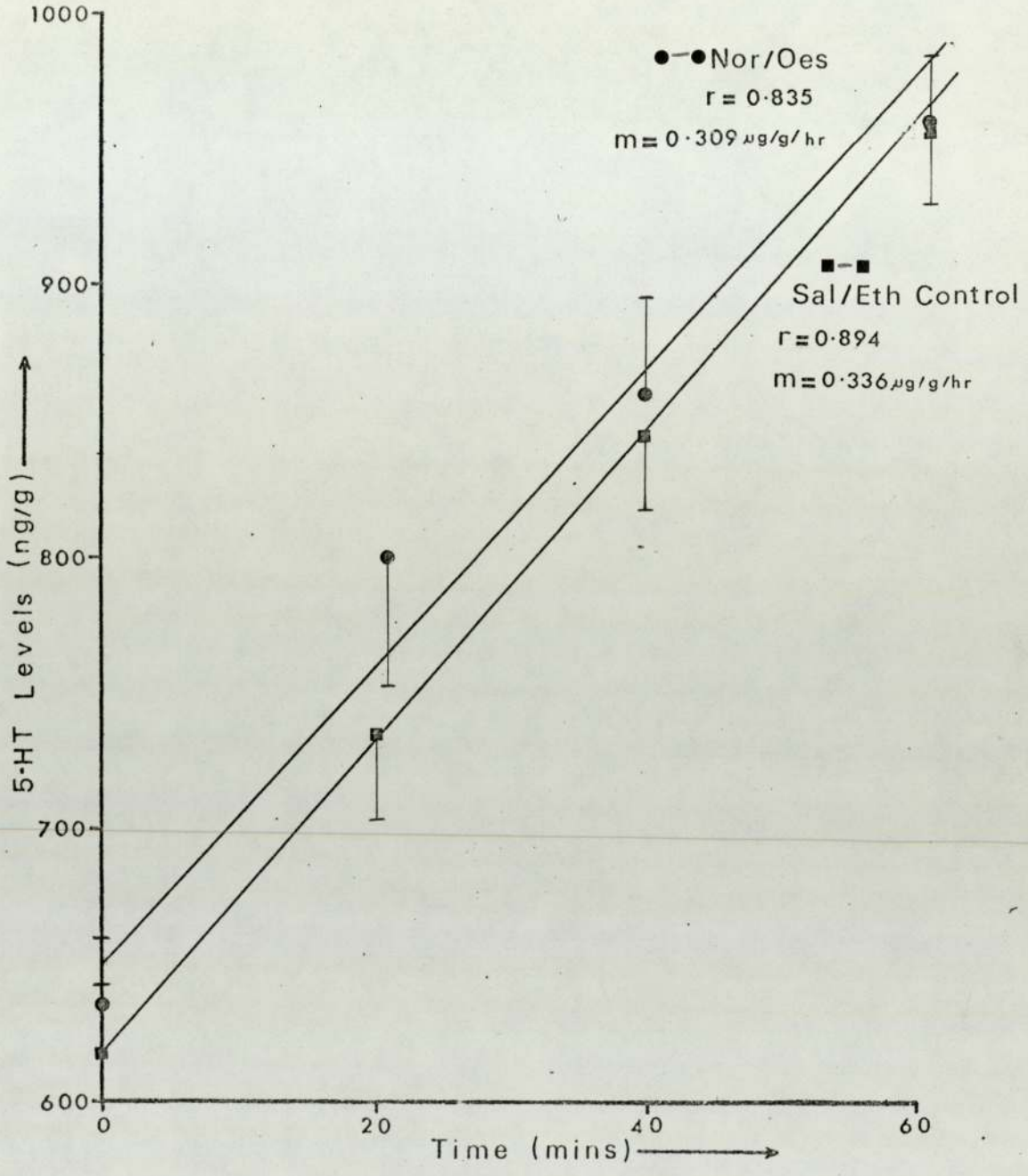
●—● female mice in oestrus (n = 20)
r = 0.845, m = 0.317 ug/g/hr., 1.790 nmol/g/hr.

■—■ female mice in dioestrus (n = 18)
r = 0.730, m = 0.206, 1.163 nmol/g/hr.

r = linear regression coefficient, m = turnover rate

n = number of determinations

Effect of a combination of 14 day pretreatment with norethisterone 200 ug/kg and oestradiol 100 ug/kg on 5-HT turnover (pargyline method)



Each symbol represents the mean of 6 determinations.

Turnover rate after 14 day pretreatment with norethisterone and oestradiol = 1.746 $\pm$ 0.328 nmol/g/hr.

Turnover rate 14 day treatment with vehicle (saline/ethanol 50:1) only = 1.890 $\pm$ 0.119 nmol/g/hr.

r = linear regression coefficient, m = turnover ug/g/hr.

Table 8

Effect of 90 min. pretreatment with cortisol 5 mg/kg on the accumulation of 5-HT after pargyline

Treatment	Brain 5-HT (ug/g)	Apparent turnover rate
saline only	0.614 ± 0.039 (6)	
saline + pargyline 75 mg/kg 90 min.	0.894 ± 0.039 (7)	0.280 ug/g/hr.
cortisol 5 mg/kg after 90 min.	0.622 ± 0.051 (4)	
cortisol 5 mg/kg + pargyline 75 mg/kg 90 min.	0.998 ± 0.044 (4)	0.377 ug/g/hr.

The results are expressed as mean±standard error with the number of determinations in brackets.

Table 9

Effect of cortisol on brain 5-HT levels

Treatment	Brain 5-HT (ug/g)
saline only 24 hr.	0.554 ± 0.009 (13)
cortisol 5 mg/kg 24 hr.	0.580 ± 0.022 (11)
cortisol 20 mg/kg 24 hr.	0.574 ± 0.013 (8)
saline only 7 day	0.574 ± 0.027 (10)
cortisol 5 mg/kg 7 day	0.643 ± 0.027 (11)†

The results are expressed as mean ± standard error with the number of observations in brackets.

† indicates a trend towards increased brain 5-HT ($p = 0.05 - 0.1$) Cortisol is as phosphate (base equivalent)

Table 10

Effect of 24 hr. pretreatment with cortisol 20 mg/kg on plasma and brain tryptophan

1. Plasma

Treatment	Tryptophan (ug/ml)
saline injected controls	17.65 ± 2.99 (6)
cortisol 20 mg/kg 24 hr.	18.55 ± 2.55 (6)

2. Brain

Treatment	Tryptophan (ug/g)
saline injected controls	2.99 ± 0.13 (6)
cortisol 20 mg/kg 24 hr.	2.93 ± 0.16 (6)

The results are expressed as the mean ± standard error with the number of determinations in brackets. Cortisol was given as the phosphate (base equivalent) in saline.

Table 11

Brain 5-HT levels in the oestrus cycle and after norethisterone 200 ug/kg and oestradiol 100 ug/kg (female mice)

Treatment	Brain 5-HT ± SEM (ug/g)
saline only to female mice in oestrus	0.645 ± 0.032 (6)
saline only to female mice in dioestrus	0.686 ± 0.038 (6)
14 day treatment with saline/ethanol 1:50 (females)	0.617 ± 0.032 (6)
14 day treatment with norethisterone 200 ug/kg/oestradiol 100 ug/kg in saline/ethanol 1:50 (females)	0.632 ± 0.024 (6)
male mice, saline treated	0.554 ± 0.009 (13)

The number of determinations are in brackets.

Table 12

Weights of female mice after 14 day treatment with norethisterone 200 ug/kg and oestradiol 100 ug/kg or vehicle only

Mean weight of both groups at start of experiment was 25.0 g.

Treatment	Mean body weight (g)
vehicle only 14 day	25.50 ± 1.20 (9)
norethisterone 200 ug/kg and oestradiol 100 ug/kg for 14 days	25.44 ± 0.69 (9)

The results are expressed as the mean body weight ± standard error with the number of animals in brackets.

CHAPTER 4

INFLUENCE OF CORTISOL AND CONTRACEPTIVE STEROIDS ON PLASMA AND BRAIN KYNURENINE LEVELS

Cortisol has been shown to induce TP pyrrolase in many animal species (e.g. Knox and Auerbach 1954, Monroe 1968, Altman and Greengard 1966(a)): and this results in increased kynurenine production (Green et al 1976). Pyrrolase induction was accompanied by a reduction in brain 5-HT levels in the rat (Curzon and Green 1968).

Kynurenine also reduced brain 5-HT levels (Green and Curzon 1970) and reduced the uptake of TP into brain slices (ibid.). It was suggested that the effects of cortisol on central 5-HT was via elevated kynurenine levels (Green and Curzon 1970) which tended to reduce brain TP and 5-HT synthesis. This suggestion was supported by the fact that the fall in brain 5-HT occurred more slowly after cortisol than after kynurenine itself.

Contraceptive steroids have also been reported to influence TP metabolism to kynurenine though the changes have been attributed to an induction of pyrrolase by some authors (Adams et al 1973, Rose 1966) whereas others dispute this (Green et al 1978).

The purpose of this work was to determine whether cortisol affected plasma or brain kynurenine levels and whether this was related to changes in 5-HT turnover observed previously (Chapter 3). The action of a contraceptive combination of norethisterone and oestradiol on TP metabolism to kynurenine was further investigated.

4.1 Effect of cortisol on plasma and brain kynurenine levels

Male mice (25 - 30 g) were injected subcutaneously with cortisol (as phosphate) in saline. Doses are expressed as base equivalents. The plasma and brain kynurenine were determined as described previously.

4.1.1 Plasma kynurenine

24 hr. pretreatment with cortisol 5 mg/kg significantly decreased plasma kynurenine levels compared with saline-injected controls ($p < 0.05$)(table 13). Plasma kynurenine was further reduced after 24 hr. pretreatment with cortisol 20 mg/kg ($p < 0.01$)(table 13). After 7 day pretreatment with cortisol 5 mg/kg the plasma kynurenine levels were not significantly different from those in saline-treated controls (table 13).

4.1.2 Brain kynurenine

The brain kynurenine levels were not significantly altered after 24 hr. pretreatment with cortisol 5 mg/kg compared with saline-treated controls (table 14). After 7 day pretreatment with cortisol 5 mg/kg there was nc significant difference in the brain kynurenine levels when compared with saline-treated controls (table 14).

4.2 Effect of norethisterone/oestradiol on plasma and brain kynurenine levels

Kynurenine levels were measured in plasma and brain by the method of Joseph et al (1978)(experimental method sections 6 and 7).

4.2.1 14 day pretreatment

Female mice (25 $\pm$ 3 g) were injected subcutaneously daily for 14 days with norethisterone 200 ug/kg and oestradiol 100 ug/kg. The vehicle for injection was ethanol in saline 1 in 50 and control groups were injected with vehicle or saline only. The injection site was varied to avoid possible changes in absorption from an inflamed injection site, and animals were killed on the 15th day.

4.2.1.1 Plasma kynurenine

The saline/ethanol controls gave significantly higher plasma kynurenine levels than the saline controls (see table 15)($p < 0.01$). However the plasma kynurenine levels after norethisterone/oestradiol were significantly greater than those after saline alone ($p < 0.001$) or after saline/ethanol 50:1 ($p < 0.02$)(table 15).

4.2.1.2 Brain kynurenine

A combination of norethisterone/oestradiol had no effect on brain kynurenine levels compared with saline controls or saline/ethanol controls. There was no significant difference between brain kynurenine levels in the saline controls and the saline/ethanol controls (table 16).

4.2.2 2 hr. pretreatment

Female mice (25 ± 3 g) were injected subcutaneously 2 hr. previously with norethisterone 200 ug/kg and oestradiol 100 ug/k vehicle or saline only as before.

4.2.2.1 Plasma kynurenine

After 2 hr. pretreatment the contraceptive combination caused a 25% increase in plasma kynurenine levels when compared with saline/ethanol controls (table 15).

4.2.2.2 Brain kynurenine

2 hr. pretreatment with norethisterone/oestradiol had no effect on brain kynurenine levels (table 16).

Discussion

Plasma kynurenine levels were significantly reduced after 24 hr. pretreatment with cortisol (5 mg/kg and 20 mg/kg) but levels returned to normal on repeated dosing with cortisol (5 mg/kg) for 7 days. These observations indicated that cortisol had caused a change in the metabolism of TP to kynurenine which appeared to have returned to normal on repeated dosing with cortisol.

Cortisol has been shown to induce TP pyrrolase in many animal species, including the mouse (Monroe 1968), rat (e.g. Knox and Auerbach 1954) and Man (Altman and Greengard 1966(a)). Pyrrolase has been found to be induced after 3 to 11 hr. pretreatment with cortisol 5 mg/kg in the rat (Green and Curzon 1968). Since induction of pyrrolase increases kynurenine production in the liver (Green, Woods and Joseph 1976) this would be expected to increase plasma kynurenine levels. However 24 hr. after cortisol treatment plasma kynurenine levels were reduced. It is likely that pyrrolase is no longer affected by cortisol after 24 hr. pretreatment. It is possible that several hours after pyrrolase induction the liver TP levels decline, producing a functional deficiency of TP. Thus, when the enzyme activity returns to normal little kynurenine is formed from TP, and as kynurenine is further metabolised by the liver plasma levels decline.

Pyrrolase activity is positively correlated with kynurenine excretion (Altman and Greengard 1966(b)) and during enzyme induction, kynurenine excretion is also increased. However this may be expected to return to normal as plasma levels become normal.

Repeated dosing with cortisol had no effect on plasma kynurenine levels after 7 days. This points to the existence of mechanisms which control plasma kynurenine levels which compensate for the presence of

cortisol so that plasma kynurenine levels are no longer affected by cortisol.

Brain kynurenine levels were unaffected 24 hr. after cortisol treatment even though plasma levels were markedly reduced. Brain kynurenine levels are controlled by the rate of uptake of kynurenine from the plasma and the rate of synthesis of kynurenine in brain. Kynurenine appears to be taken up into brain by two mechanisms, one of which appears to be active (see chapter 6). At high plasma levels kynurenine is taken up along a concentration gradient whereas kynurenine can be taken up actively at normal plasma levels. Cortisol would not be expected to directly influence kynurenine production in the brain since cerebral pyrrolase is not inducible by cortisol (Gal 1974).

24 hr. after cortisol 20 mg/kg a significant reduction in brain 5-HT turnover had previously been found (chapter 3). However at this time there was no increase in kynurenine production which might have been expected to accompany a reduction in turnover if the latter were mediated by increased pyrrolase activity (Green and Curzon 1970). Cortisol increases pyrrolase activity and decreases brain 5-HT levels (Green and Curzon 1968) and 5-HIAA levels indicating reduced synthesis (ibid.). However a reduction in brain 5-HT turnover can occur in the absence of pyrrolase induction and it is therefore unlikely that 5-HT synthesis is directly related to increased kynurenine production after cortisol.

The increase in plasma kynurenine levels after 14 day treatment with saline/ethanol may be due to an increase in TP pyrrolase activity, which has been reported to occur after acute ethanol intake in some strains of mice (Badawy and Evans 1977). There have been contradictory reports

on the actions of oral contraceptives on pyrrolase activity. Some workers (e.g. Adams et al 1973, Rose 1966) report an induction of pyrrolase (as determined by an increased excretion of kynurenine) after oral contraceptives, whereas other workers report that pyrrolase is not induced (Green et al 1978).

The highest plasma levels of kynurenine were obtained after 14 day treatment with norethisterone and oestradiol and this combination had begun to affect plasma levels after 2 hr. This observation would be consistent with an induction of pyrrolase by this combination. Induction may be expected to occur after 2 - 10 hr., with maximal effect after 4 - 6 hr. (Curzon and Green 1968). We found that a single intraperitoneal injection of dl-kynurenine 5 mg/kg gave similar plasma kynurenine levels (after 30 min. the levels were 1.412 ug/ml) to those found after 14 day treatment with a combination of norethisterone and oestradiol (1.510 ug/ml). However the injected kynurenine increased brain kynurenine after 2 hr. to 0.308 ug/g, an increase of 77% over controls, but there was no such increase after 14 day treatment with norethisterone/oestradiol. Chronic administration of the contraceptive combination probably causes a gradual increase in plasma kynurenine levels, which would not be comparable in pharmacokinetics terms with injecting a single large dose of kynurenine. The norethisterone/oestradiol combination could also have other effects including a possible interference with brain kynurenine uptake. It is unlikely that norethisterone/oestradiol influences the rate of synthesis of kynurenine in brain since the cerebral enzyme is not inducible by cortisol (Gal 1974) and is therefore less likely to be influenced by other steroid hormones.

Thus the metabolism of TP to kynurenine was altered by norethisterone/

oestradiol but this change was not accompanied by a change in 5-HT turnover (see previous chapter). It appears that brain 5-HT turnover rate is unrelated to plasma or brain kynurenine levels under these conditions.

Table 13

Effect of cortisol on plasma kynurenine levels

Treatment	Plasma kynurenine levels (ug/ml)
Saline control 24 hr.	0.548 ± 0.025 (17)
cortisol 5 mg/kg 24 hr.	*0.456 ± 0.021 (9)
cortisol 20 mg/kg 24 hr.	**0.404 ± 0.038 (15)
saline control 7 days	0.546 ± 0.075 (10)
cortisol 5 mg/kg 7 days	0.588 ± 0.063 (14)

Results are expressed as mean ± standard error with the number of determinations in brackets.

*$p < 0.05$

**$p < 0.01$

Table 14

Effect of cortisol on brain kynurenine levels

Treatment	Brain kynurenine levels (ug/g)
saline control 24 hr.	0.158 ± 0.013 (9)
cortisol 5 mg/kg 24 hr.	0.170 ± 0.031 (10)
cortisol 20 mg/kg 24 hr.	0.163 ± 0.021 (8)
saline control 7 days	0.209 ± 0.026 (8)
cortisol 5 mg/kg 7 days	0.208 ± 0.031 (8)

Results are expressed as mean ± standard error with the number of determinations in brackets.

Table 15

Effect of norethisterone 200 ug/kg and oestradiol 100 ug/kg on plasma kynurenine levels

Treatment	Kynurenine (ug/ml)
saline control 2 hr.	0.666 ± 0.045 (8)
saline/ethanol 50:1 control 2 hr.	0.599 ± 0.050 (9)
norethisterone 200 ug/kg and oestradiol 100 ug/kg in saline/ethanol 2 hr.	0.750 ± 0.049 (7)
saline control 14 day	0.729 ± 0.050 (12)
saline/ethanol 50:1 control 14 day	*1.052 ± 0.101 (11)
norethisterone 200 ug/kg and oestradiol 100 ug/kg in saline/ethanol 14 day	**1.510 ± 0.130 (10)

The results are expressed as the mean ± standard error with the number of determinations in brackets.

*$p < 0.01$ compared with saline control

**($p < 0.001$ compared with saline control
($p < 0.02$ compared with saline/ethanol control

Table 16

Effect of norethisterone 200 ug/kg and oestradiol 100 ug/kg on brain kynurenine levels

Treatment	Kynurenine (ug/g)
saline control 2 hr.	0.174 ± 0.008 (7)
saline/ethanol 50:1 control 2 hr.	0.184 ± 0.016 (8)
norethisterone 200 ug/kg and oestradiol 100 ug/kg in saline/ethanol 2 hr.	0.175 ± 0.013 (8)
saline control 14 day	0.196 ± 0.021 (10)
saline/ethanol 50:1 control 14 day	0.187 ± 0.026 (11)
norethisterone 200 ug/kg and oestradiol 100 ug/kg in saline/ethanol 14 day	0.209 ± 0.024 (14)

The results are expressed as the mean ± standard error with the number of determinations in brackets.

CHAPTER 5

EFFECT OF KYNURENINE ON PLASMA AND BRAIN TRYPTOPHAN AND KYNURENINE AND ON BRAIN 5-HT TURNOVER

5. A low dose of dl-kynurenine (0.5 mg/kg) was found to potentiate the head-twitch response to 5-hydroxytryptophan (5-HTP) and 5-HT in the mouse (Handley and Miskin 1977). A higher dose of dl-kynurenine (5 mg/kg) caused a complete antagonism of the behaviour. The 5-HTP or 5-HT head-twitch is thought to be an indicator of "free" 5-HT in the brain (Corne et al 1963), so these results may indicate a dual effect of kynurenine on brain 5-HT. This was unexpected since a reduction in TP uptake by kynurenine (Green and Curzon 1970) would be expected to cause a dose-dependant decrease in brain 5-HT synthesis.

L-kynurenine 5 mg/kg has been found to reduce brain 5-HT levels and 5-HIAA levels indicating decreased 5-HT synthesis in the rat (Green and Curzon 1970). However the biphasic dose-response curve of kynurenine on the head-twitch response was thought to warrant further investigation. The effects of various doses of dl-kynurenine on brain 5-HT turnover were therefore determined.

Plasma and brain kynurenine levels were also measured to determine whether these had any relation to brain 5-HT turnover.

It had previously been reported that there was a negative correlation between plasma TP and kynurenine levels after TP loading in the rat (Joseph, 1972). This relation between tryptophan and kynurenine levels was further investigated.

5.1 Effect of kynurenine on brain 5-HT

Male mice (25 $\pm$ 2 g) were injected intraperitoneally 2 hr. previously with dl-kynurenine (as sulphate) in saline or with saline only. Doses are expressed as the base equivalent. A 2 hr. pretreatment interval was chosen since it had previously been found to cause a maximal reduction of brain 5-HT levels in the rat (Green

and Curzon 1970).

5.1.1 Brain 5-HT levels

Dl-kynurenine at a dose of 0.5 mg/kg and 5 mg/kg had no significant effect on brain 5-HT levels (table 18). Brain 5-HT levels were significantly increased 2 hr. after kynurenine 20 mg/kg ($p < 0.001$)(table 17).

5.1.2 Brain 5-HT turnover

Brain 5-HT turnover rate was measured by the pargyline method and the probenecid method described previously.

After kynurenine 0.5 mg/kg the increase in brain 5-HT after pargyline showed deviation from linearity with a very low regression coefficient ($r = 0.365$, $p < 0.05$)(Fig. 30). The probenecid method gave a linear increase in 5-HIAA after kynurenine 0.5 mg/kg. The regression coefficient for a straight line was highly significant ($r = 0.778$, $p < 0.001$) and kynurenine 0.5 mg/kg caused a 50% reduction in brain 5-HT turnover measured by this method (Fig. 31) ($p = 0.02$). Though the pargyline method did not give a good line fit the values obtained for the turnover rates were in good agreement with those obtained by the probenecid method (Figs 30 and 31).

No significant change in brain 5-HT turnover was seen by either method after kynurenine 5 mg/kg (Figs 32 and 33). Similarly there was no change in turnover rate after kynurenine 20 mg/kg measured by the pargyline method (Fig. 34).

5.2 Effect of kynurenine on plasma and brain kynurenine levels

Male mice were pretreated with dl-kynurenine intraperitoneally 2 hr. previously and brain and plasma kynurenine levels were measured

as described in Experimental Methods sections 6 and 7.

5.2.1 Plasma

Plasma kynurenine was not significantly different from control levels 2 hr. after kynurenine 0.5 mg/kg or kynurenine 5 mg/kg (table 18). Kynurenine 20 mg/kg significantly increased plasma kynurenine after 2 hr. ($p < 0.05$) (table 18).

5.2.2 Brain

Brain kynurenine was markedly increased 2 hr. after kynurenine 0.5 mg/kg so that levels were 89% above control ($p < 0.01$). Kynurenine 5 mg/kg markedly increased brain kynurenine levels to 77% above controls after 2 hr. ($p < 0.001$). Kynurenine 20 mg/kg significantly increased brain kynurenine so that levels were 74% above control after 2 hr. ($p < 0.01$) (table 18)

Thus each dose of kynurenine produced brain kynurenine levels which were significantly above control values. This rise in kynurenine levels was not dose related and the brain levels after each dose did not differ significantly from each other.

5.3 Influence of kynurenine on brain and plasma tryptophan levels

Male mice (25 - 30 g) were pretreated with dl-kynurenine intraperitoneally 30 min. or 2 hr. previously and plasma tryptophan levels measured as described previously.

5.3.1 Plasma tryptophan

Plasma tryptophan levels were increased 30 min. after kynurenine 0.5 mg/kg by 16% ($p = 0.1$ to 0.05) but fell to 12.5% below controls after 2 hr. ($p = 0.1$ to 0.05). Neither

of these changes was statistically significant (Fig. 35, table 19). Kynurenine 5 mg/kg had no significant effect on plasma TP after 30 min., but significantly decreased plasma tryptophan after 2 hr. ($p < 0.001$).

5.3.2 Brain tryptophan

Dl-kynurenine 0.5 mg/kg increased brain tryptophan levels after 30 min. ($p < 0.001$)(Fig. 36 table 20). Levels had fallen toward normal after 2 hr. ($p = 0.1$ to 0.05, trend only). A dose of 5 mg/kg significantly increased brain TP levels ($p < 0.02$)(Fig. 36 table 20). Levels declined toward normal after 2 hr. when there was no significant difference from controls.

5.3.3 Correlation with kynurenine levels

There was no correlation between plasma TP and kynurenine levels (table 21). Brain kynurenine was also not correlated with brain TP after either dose (table 21)(see also tables 19 and 20).

Discussion

Dl-kynurenine 0.5 mg/kg had no significant effect on brain 5-HT levels but caused a marked reduction in brain 5-HT turnover rate in the mouse. A dose of 5 mg/kg did not affect brain 5-HT levels or turnover, whereas dl-kynurenine 20 mg/kg increased brain 5-HT levels without affecting turnover rate. It had previously been shown that dl-kynurenine 0.5 mg/kg caused a marked potentiation of the head-twitch response to 5-HTP or 5-HT in the mouse (Handley and Miskin 1977). This effect was reversed by a higher dose (5 mg/kg) of kynurenine, which completely suppressed the behaviour. These results were matched by dual effects on 5-HT turnover rate, a low dose of kynurenine reducing turnover whereas a higher dose

had no effect. A reduction in brain 5-HT turnover after kynurenine may occur by direct stimulation of post-synaptic 5-HT receptors, which would activate a neuronal feedback loop. This would tend to reduce the firing rate of 5-HT neurons and reduce 5-HT turnover rate. This is unlikely to be the case since it would be expected that kynurenine alone would have increased the spontaneous head-twitch rate, which was not found to occur (Handley and Miskin 1977).

Another possible mechanism of action of kynurenine in reducing turnover rate is via stimulation of presynaptic 5-HT receptors. However this would reduce 5-HT release which could not explain the potentiation of the head twitch response accompanying the reduction in turnover. The effects of kynurenine on plasma and brain TP was investigated to try to clarify these anomalous results.

Dl-kynurenine had little effect on plasma TP after 30 min. pre-treatment with a dose of 0.5 mg/kg or 5 mg/kg. After 2 hr. treatment with kynurenine there was a dose-dependant fall in plasma TP. Plasma TP had previously been found to be reduced after l-kynurenine 5 mg/kg in the rat, though the change was not statistically significant (Joseph and Kadam 1979).

Brain TP was markedly increased 30 min. after dl-kynurenine 0.5 mg/kg. Levels of TP had fallen after 2 hr. pretreatment with kynurenine, but were still greater than controls. Similarly brain TP was increased 30 min. after kynurenine 5 mg/kg but to a lesser extent than after kynurenine 0.5 mg/kg. Brain TP levels were normal 2 hr. after kynurenine 5 mg/kg, in agreement with Joseph (1972) who found normal brain TP levels 2 hr. after l-kynurenine 5 mg/kg.

However no increase in brain TP was found after 30 min. and 60 min.

pretreatment with l-kynurenine 5 mg/kg in the rat (Joseph and Kadam, 1979). In fact a slight though not statistically significant reduction was reported at these times.

These results are also contradictory to those found by Curzon and Green (1971) who reported a reduction of tryptophan uptake into brain slices by l-kynurenine. However this work was done in vitro and may not be paralleled by the in vivo situation.

Though the results reported in this work were unusual the experiments were repeated and the results were found to be consistent.

The effects of kynurenine on the head-twitch response and on turnover rate could possibly be explained by an inhibition of 5-HT uptake by kynurenine, masked at higher doses by 5-HT receptor blockade. The possibility that kynurenine affected 5-HT uptake was investigated in chapter 7.

L-kynurenine 5 mg/kg had previously been reported to decrease brain 5-HT after 2hr. pretreatment both in whole rat brain (Green and Curzon, 1970) and in regions of rat brain (Curzon and Green, 1971). These authors also reported a reduction in brain 5-HIAA levels (Green and Curzon 1970) suggesting a reduction in 5-HT synthesis. However, the reduction in 5-HIAA levels was less marked than the reduction in 5-HT levels and a significant fall in 5-HIAA levels only occurred in two regions of rat brain, the midbrain and striatum (Curzon and Green 1971). It is possible that there may have been a reduction in turnover rate in these areas which was not reflected by a reduction in whole brain 5-HT turnover. It has recently been reported that l-kynurenine 5 mg/kg had no significant effect on brain 5-HT levels after 2 hr. treatment in the rat (Joseph and Kadam 1979). Similarly no changes in brain TP or 5-HIAA were found.

These results are in agreement with present findings in the mouse when 2 hr. after dl-kynurenine 5 mg/kg there was no change in brain TP, 5-HT or 5-HT turnover.

It has been shown that kynurenine is bound to plasma albumin (Hankes and Schmaeler 1978 and Joseph and Kadam 1979). Therefore kynurenine may displace TP from its binding and increase plasma free TP and hence brain TP. However, brain TP was increased to a lesser extent after kynurenine 5 mg/kg than 0.5 mg/kg. It is surprising that a reduction in 5-HT turnover rate 2 hr. after kynurenine 0.5 mg/kg was preceded by markedly increased brain TP levels at 30 min. The higher dose of kynurenine (5 mg/kg) which had less effect on brain TP had no effect on turnover rate. The mechanism by which kynurenine has a dual effect on turnover rate remains unclear.

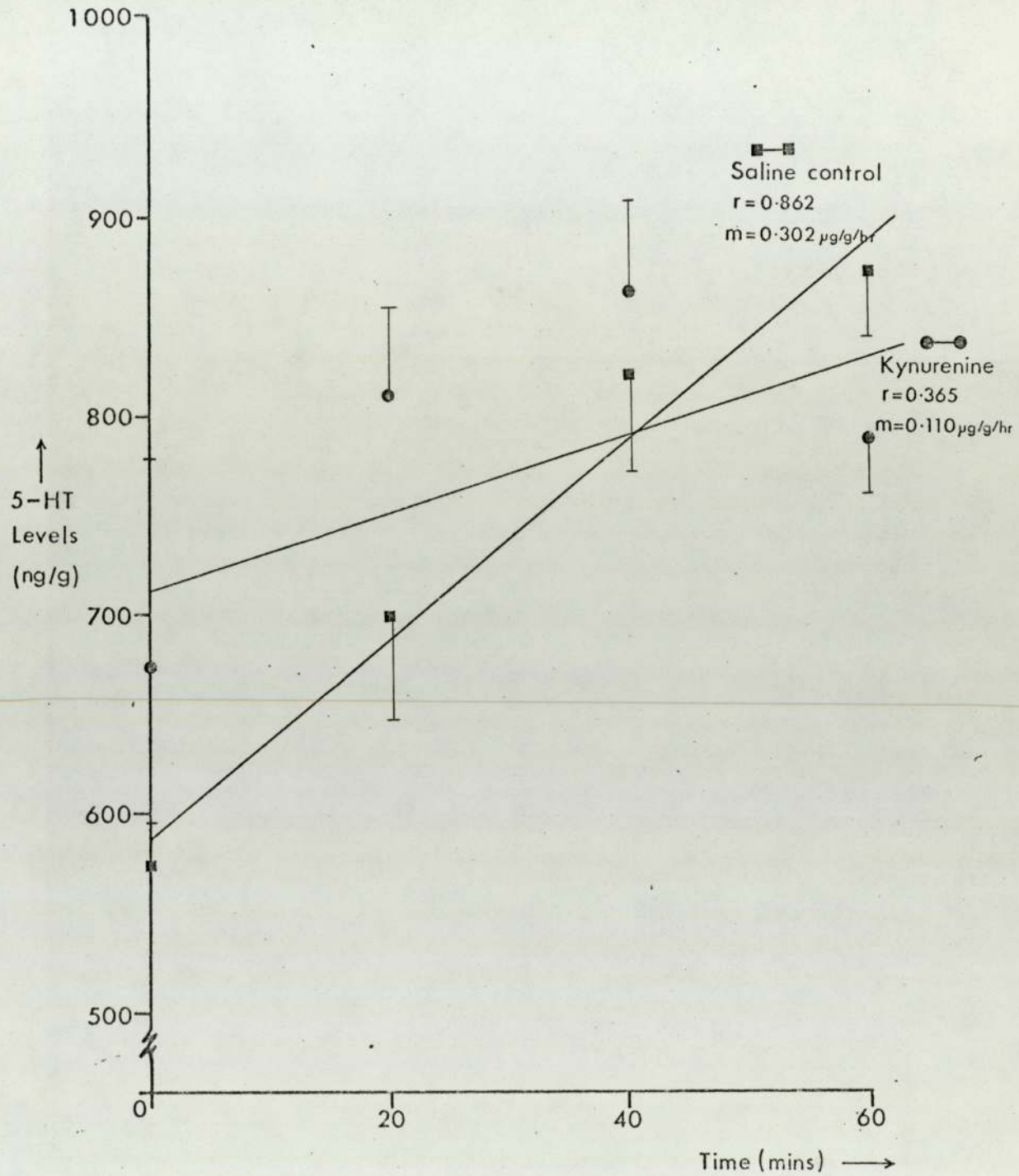
The changes in brain TP were accompanied by a reduction in plasma TP after kynurenine 5 mg/kg. It is possible that this reduction in plasma total TP, which would also reduce the concentration of free TP, lowers brain TP uptake and hence brain TP levels after kynurenine 5 mg/kg compared with those after 0.5 mg/kg. The mechanism by which kynurenine could lower plasma TP after kynurenine 5 mg/kg is unclear and measurement of brain and plasma kynurenine after kynurenine 20 mg/kg may have given more information of the mechanisms involved.

No correlation was found between plasma TP and plasma kynurenine levels. It has previously been reported that there is a negative correlation between plasma TP and kynurenine levels after TP loading in the rat (Joseph, thesis 1972). Plasma TP was markedly increased after TP loading and as plasma TP fell towards normal plasma kynurenine tended to rise. This rise in plasma kynurenine may be due to increased kynurenine

production after induction of pyrrolase by a TP load (Green, Woods and Joseph 1976).

Fig. 30

Effect of dl-kynurenine 0.5 mg/kg on 5-HT turnover by the pargyline method



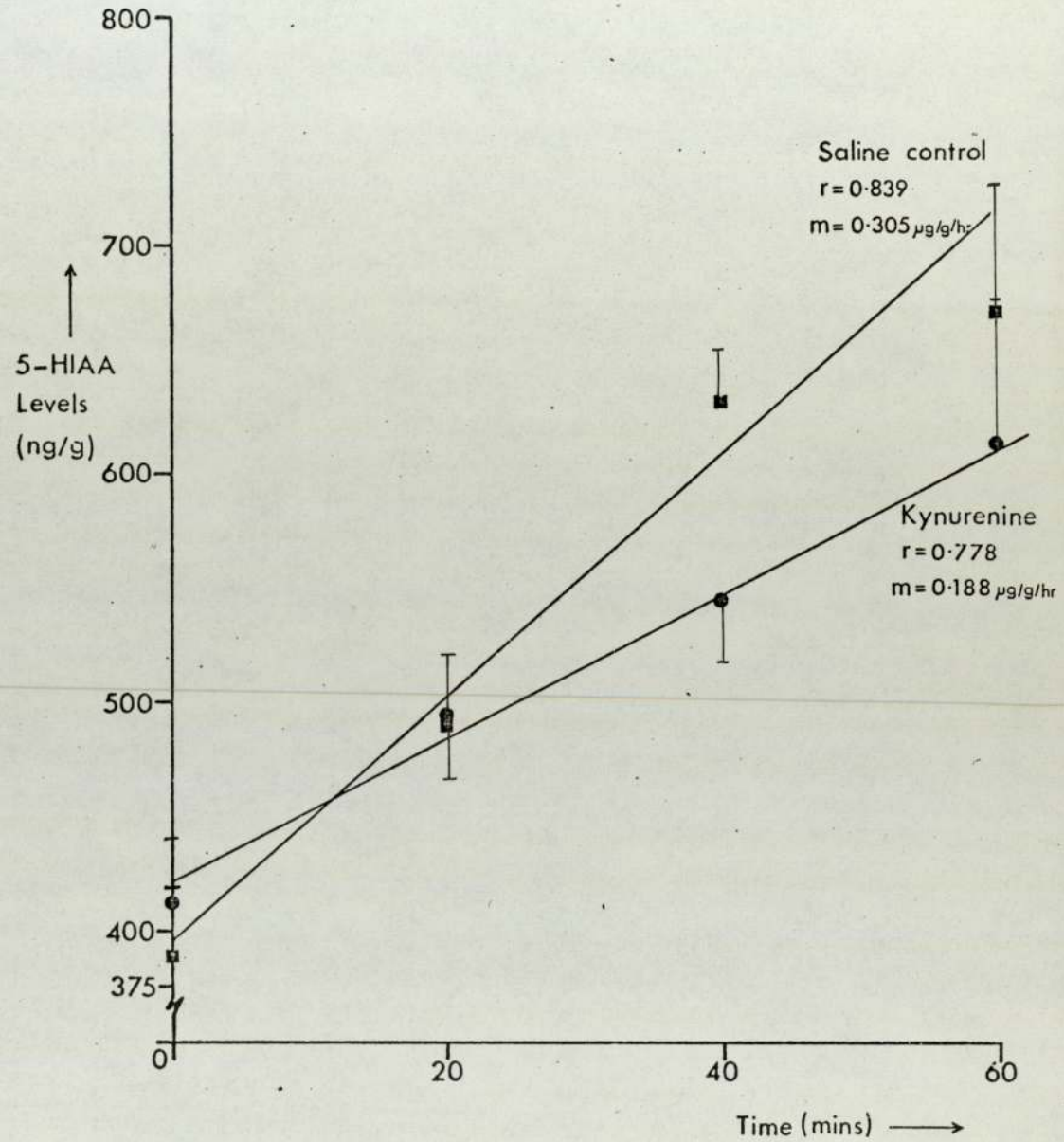
Each symbol represents the mean of 4 - 6 determinations.

Turnover rate control = 1.706 $\pm$ 0.266 nmol/g/hr.

kynurenine = 0.621 $\pm$ 0.661 nmol/g/hr.

r = linear regression coefficient, m = turnover in ug/g/hr.

Effect of dl-kynurenine 0.5 mg/kg on 5-HT turnover by the probenecid method



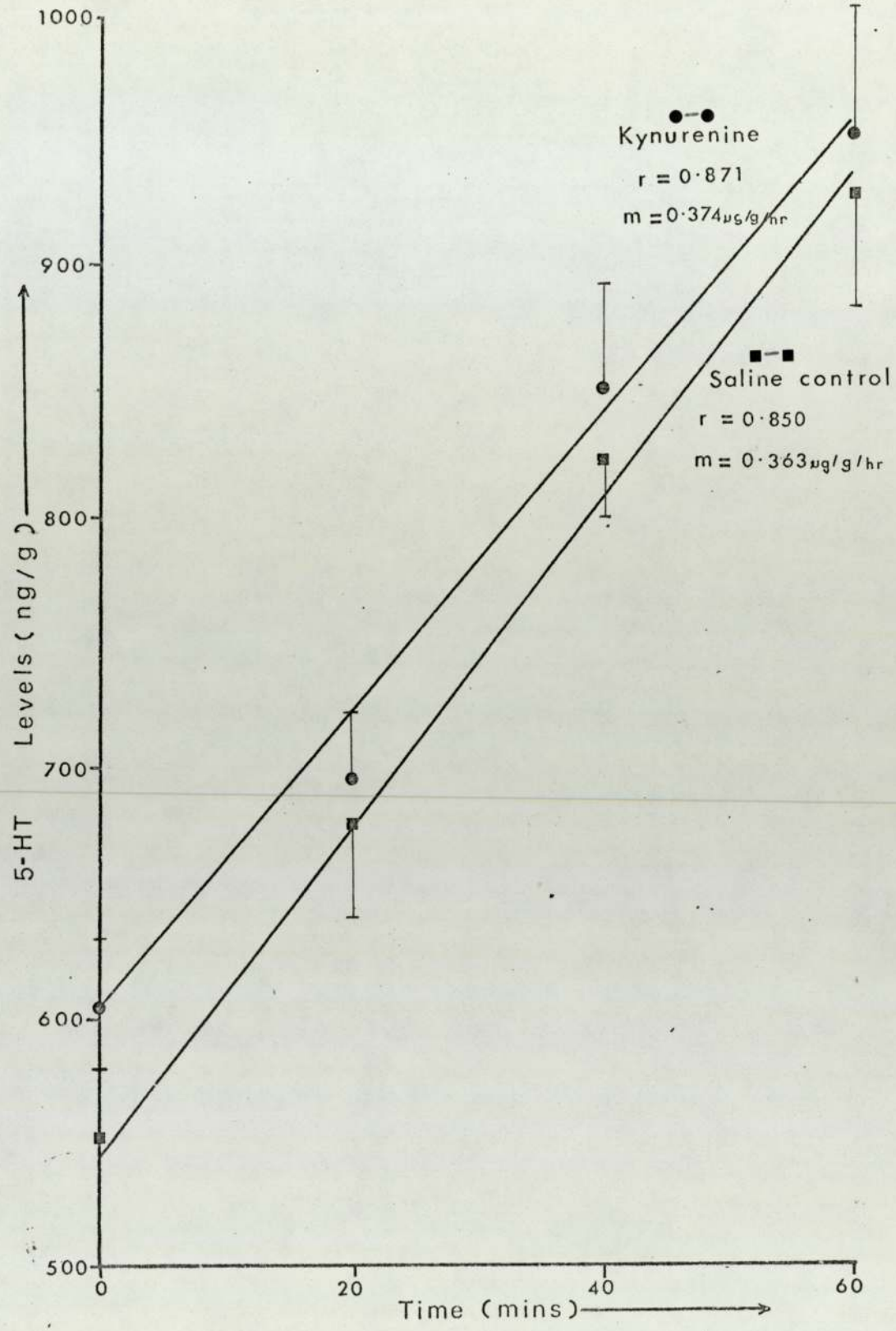
Each symbol represents the mean of 4 - 6 determinations.

Turnover rate control = 1.588 ± 0.234 nmol/g/hr.

kynurenine = 0.974 ± 0.391 nmol/g/hr.

r = linear regression coefficient, m = turnover in ug/g/hr.

Effect of dl-kynurenine 5 mg/kg on 5-HT turnover by the pargyline method



Each symbol represents the mean of at least 5 determinations.

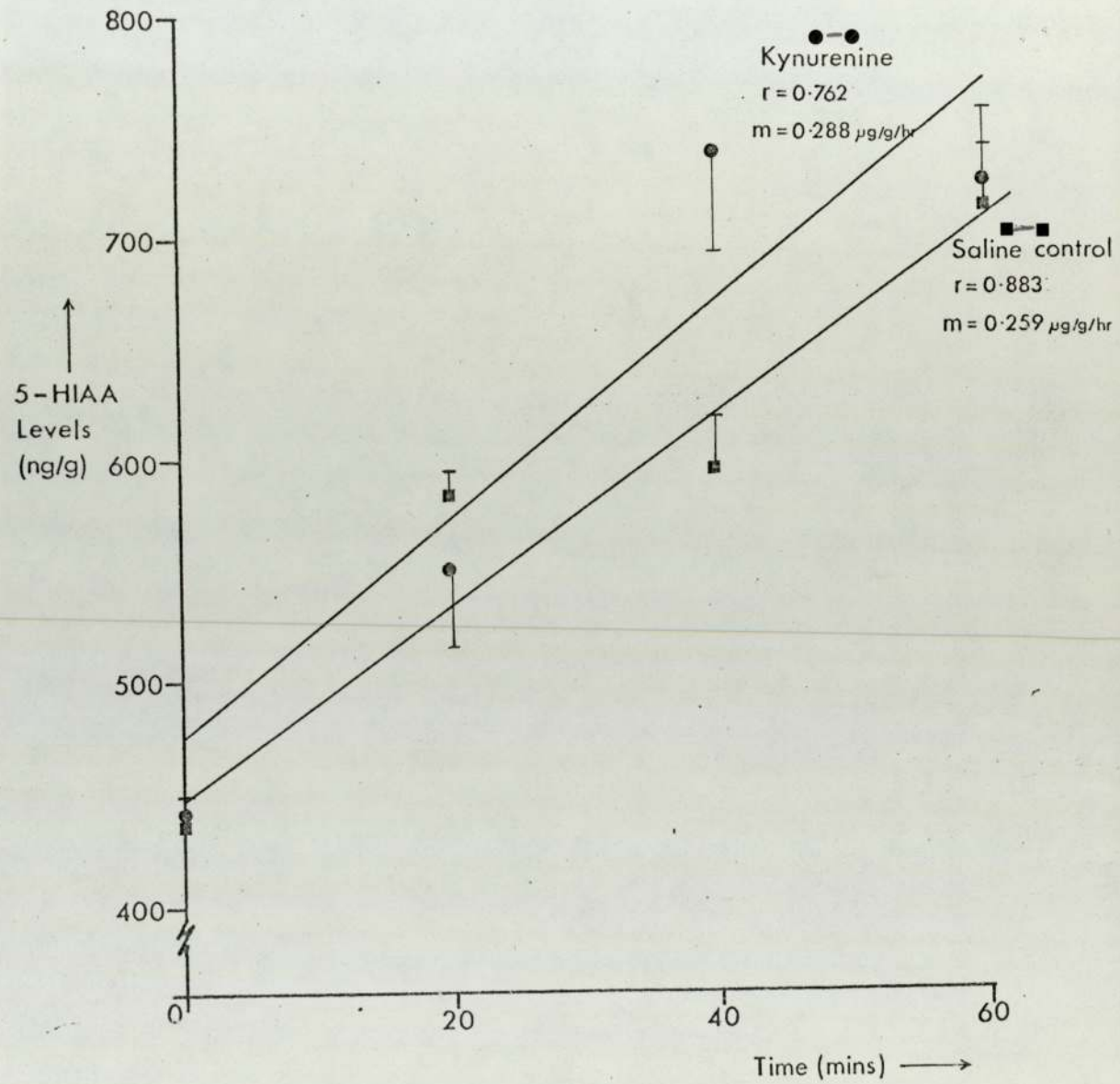
Turnover rate control = 2.113 $\pm$ 0.35 nmol/g/hr.

kynurenine = 2.050 $\pm$ 0.365 nmol/g/hr.

r = linear regression coefficient, m = turnover in ug/g/hr.

Fig. 33

Effect of dl-kynurenine 5 mg/kg on 5-HT turnover by the probenecid method



Each symbol represents the mean of 4 - 6 determinations.

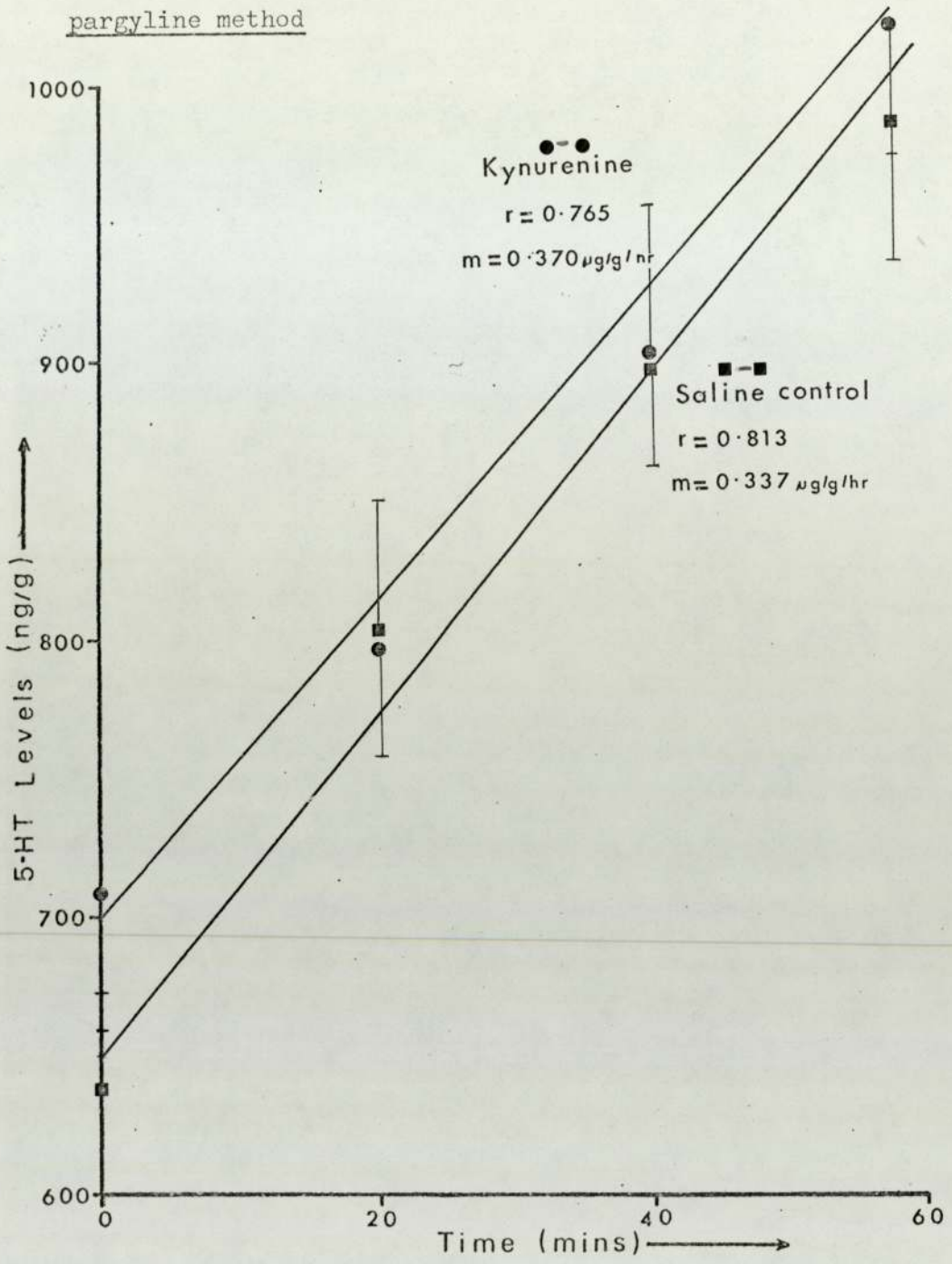
Turnover rate control = 1.349 $\pm$ 0.047 nmol/g/hr.

kynurenine = 1.500 $\pm$ 0.240 nmol/g/hr.

r = linear regression coefficient, m = turnover in ug/g/hr.

Fig. 34

Effect of dl-kynurenine 20 mg/kg on 5-HT turnover by the pargyline method

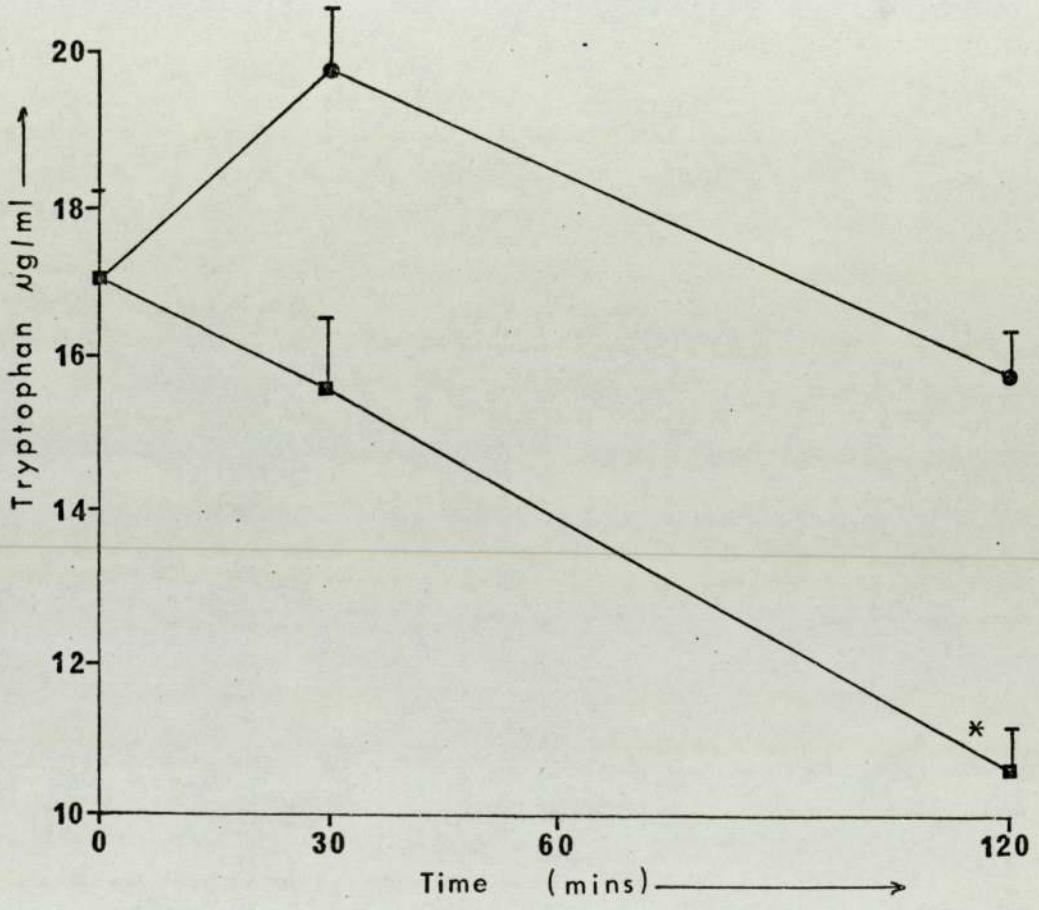


Each symbol represents the mean of at least 6 determinations.

Turnover rate control = 2.090 $\pm$ 0.559 nmol/g/hr.

kynurenine = 1.904 $\pm$ 0.644 nmol/g/hr.

Effect of kynurenine on plasma tryptophan



● - Represents treatment with dl-kynurenine 0.5 mg/kg

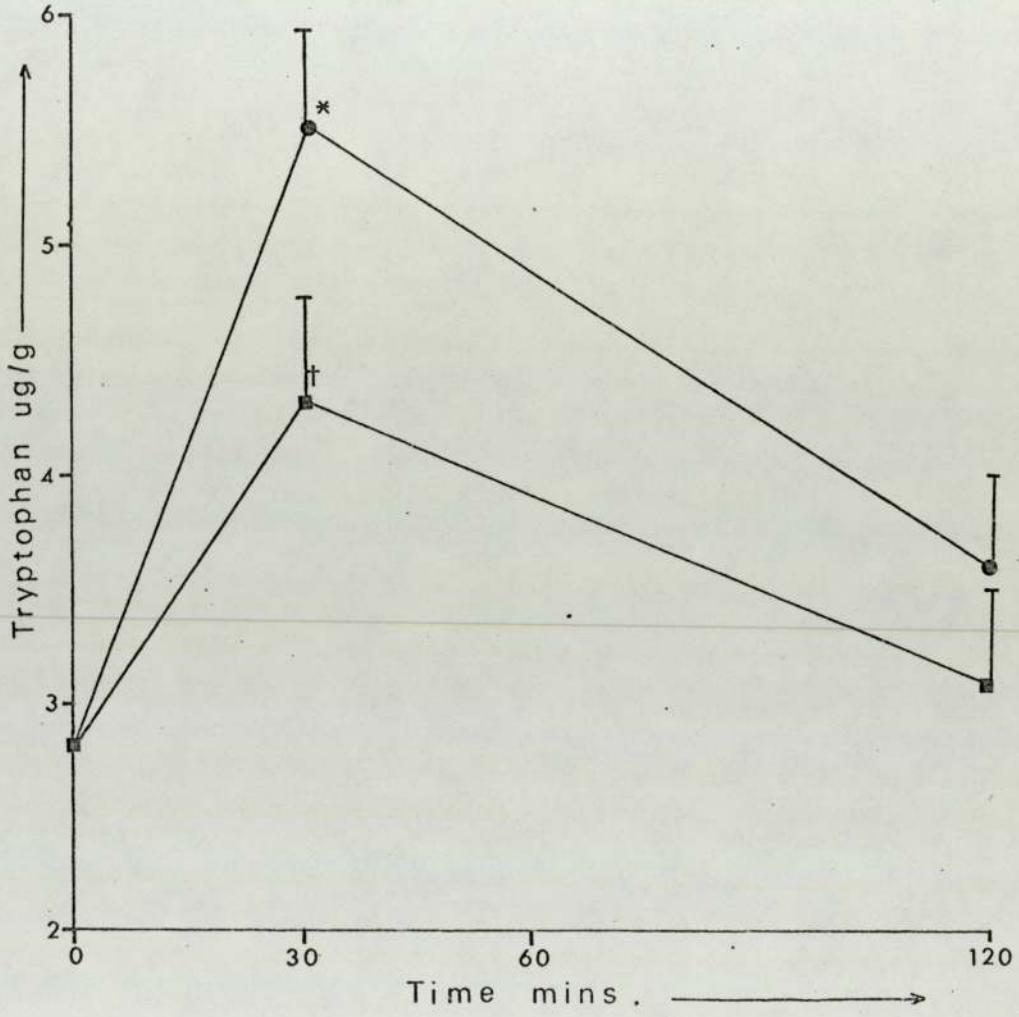
■ - Represents treatment with dl-kynurenine 5 mg/kg

The results are expressed as the mean ± SEM of at least six determinations.

*p < 0.001

Fig. 36

Effect of kynurenine on brain tryptophan



● - Represents treatment with dl-kynurenine 0.5 mg/kg

■ - Represents treatment with dl-kynurenine 5 mg/kg

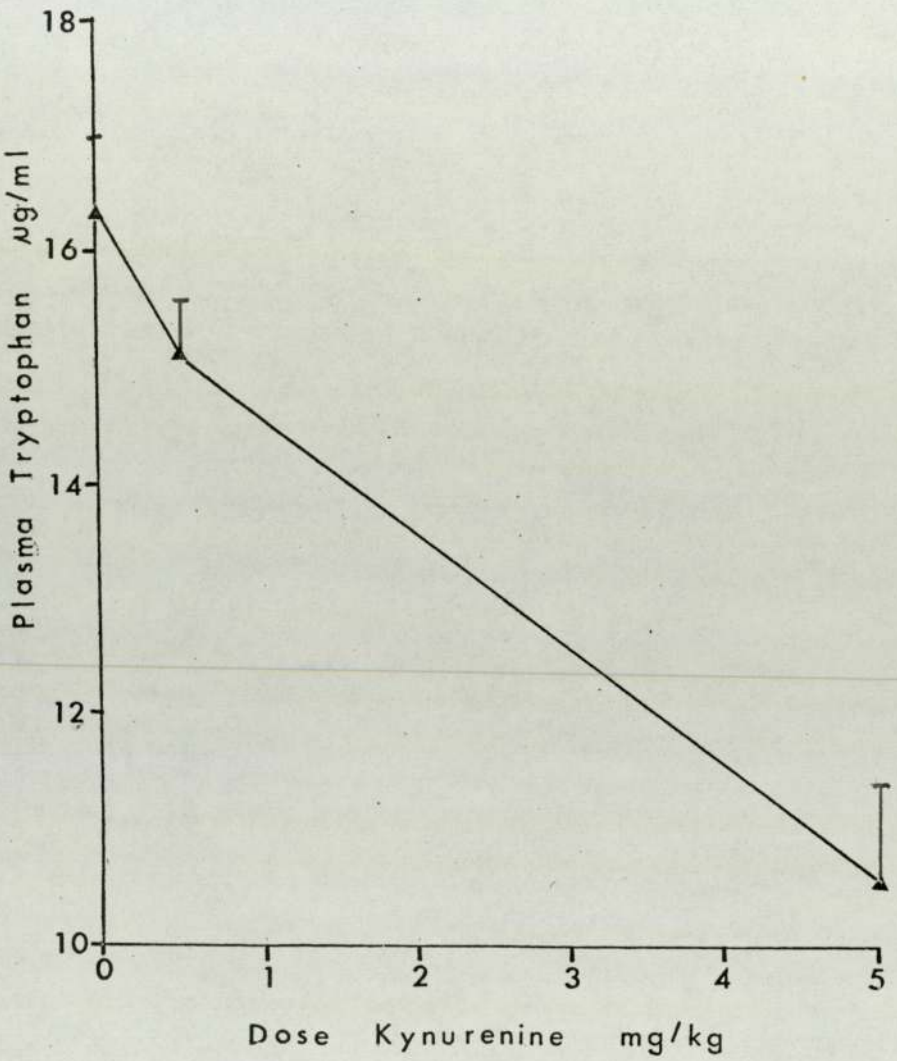
Results are expressed as the mean ± SEM of at least six determinations.

† $p < 0.02$

* $p < 0.001$

Effect of dl-kynurenine on plasma tryptophan after 2 hr. pretreatment.

I Dose/Response curve



Each symbol represents the mean of at least 9 determinations.

Fig. 38

Effect of kynurenine on plasma tryptophan after 2 hr. pretreatment

II Log/Dose Response curve

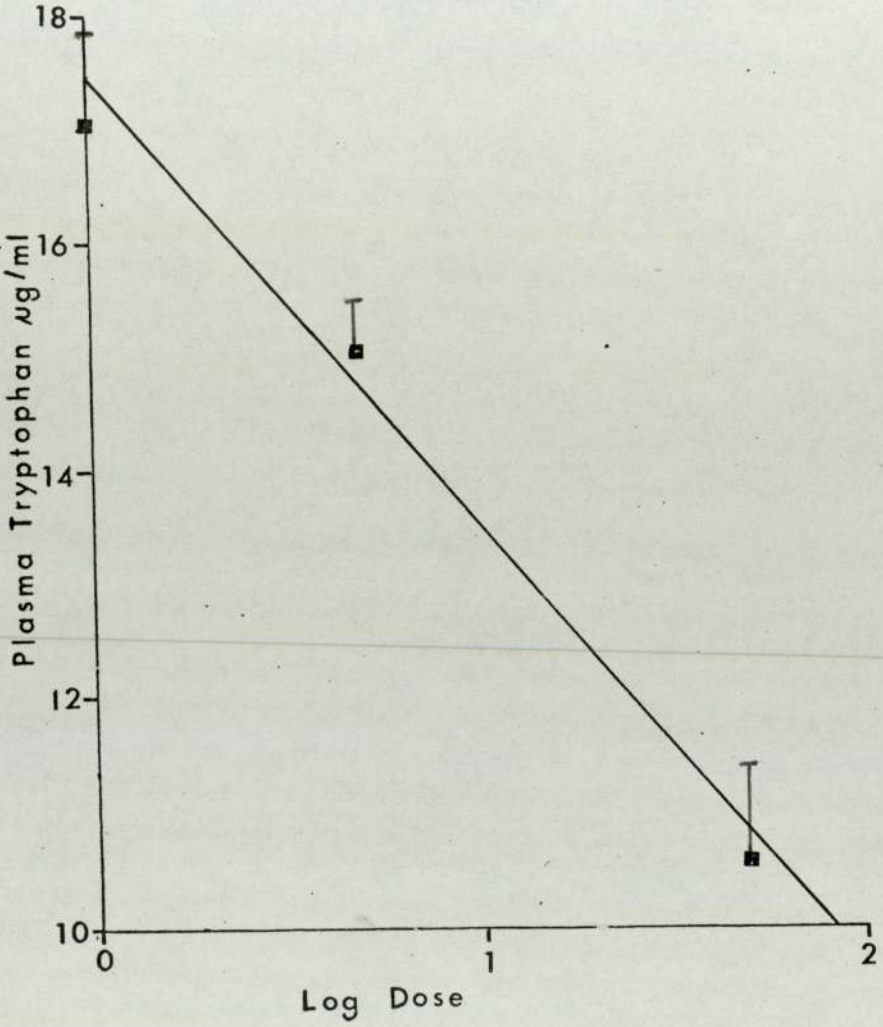


Table 17

Effect of DL-kynurenine on brain 5-HT levels

Treatment	Brain 5-HT (ug/g)
saline injected control	0.585 ± 0.014 (18)
DL-kynurenine 0.5 mg/kg after 2 hr.	0.579 ± 0.119 (6)
DL-kynurenine 5 mg/kg after 2 hr.	0.609 ± 0.025 (8)
DL-kynurenine 20 mg/kg after 2 hr.	*0.710 ± 0.036 (6)

* $p < 0.01$ compared with saline treated control

The results are expressed as mean ± standard error with the number of determinations in brackets.

Table 18

Plasma and Brain kynurenine levels 2 hr. after DL-kynurenine

Treatment	Plasma Kynurenine ug/ml	Brain Kynurenine ug/g
saline only	0.672 ± 0.020 (6)	0.166 ± 0.009 (22)
DL-kynurenine 0.5 mg/kg	0.754 ± 0.095 (7)	†0.329 ± 0.044 (10)
DL-kynurenine 5 mg/kg	0.641 ± 0.052 (10)	*0.308 ± 0.020 (11)
DL-kynurenine 20 mg/kg	**0.893 ± 0.013 (11)	0.289 ± 0.093 (11)

The results are expressed as the mean ± standard error of the mean with the number of determinations in brackets.

* $p < 0.001$)
† $p < 0.01$) compared with saline controls
** $p < 0.05$)

Table 19

Effect of kynurenine injection on plasma TP and kynurenine

Treatment	Tryptophan levels (ug/ml)	Kynurenine levels (ug/ml)
saline control	17.00 ± 1.31 (12)	0.674 ± 0.020 (6)
DL-kynurenine 0.5 mg/kg 30 min.	19.66 ± 1.09 (9)	*0.869 ± 0.110 (5)
DL-kynurenine 0.5 mg/kg 2 hr.	14.88 ± 0.49 (10)	0.754 ± 0.095 (7)
DL-kynurenine 5 mg/kg 30 min.	15.66 ± 0.91 (9)	**1.415 ± 0.131 (7)
DL-kynurenine 5 mg/kg 2 hr.	**10.47 ± 0.71 (9)	0.641 ± 0.052 (9)

The results are expressed as mean ± standard error with the number of determinations in brackets.

*$p < 0.05$) compared with saline controls, student "t" test
**$p < 0.001$) (two-tail)

Table 20

Effect of kynurenine injection on brain TP and kynurenine

Treatment	Tryptophan levels (ug/g)	Kynurenine levels (ug/g)
saline control	2.80 ± 0.310 (12)	0.166 ± 0.019 (22)
DL-kynurenine 0.5 mg/kg after 30 min.	*5.511 ± 0.397 (9)	† 0.217 ± 0.028 (7)
DL-kynurenine 0.5 mg/kg after 2 hr.	*3.620 ± 0.380 (12)	**0.314 ± 0.044 (10)
DL-kynurenine 5 mg/kg after 30 min.	*4.325 ± 0.430 (8)	# 0.278 ± 0.025 (9)
DL-kynurenine 5 mg/kg after 2 hr.	3.118 ± 0.469 (9)	# 0.308 ± 0.020 (11)

The results are expressed as the mean ± standard error with the number of observations in brackets.

# $p < 0.001$
† $p = 0.1 - 0.05$
* $p < 0.02$
** $p < 0.01$

compared with saline controls (two-tailed student t-test)

Table 21

Correlations between plasma tryptophan and kynurenine levels and brain tryptophan and kynurenine levels after an injected dose of kynurenine

Treatment	Correlation coefficient (r)	Significance
Plasma tryptophan levels vs plasma kynurenine levels after dl-kynurenine 0.5 mg/kg	0.638	not sig.
Plasma tryptophan levels vs plasma kynurenine levels after dl-kynurenine 5 mg/kg	0.358	not sig.
Brain tryptophan levels vs brain kynurenine levels after dl-kynurenine 0.5 mg/kg	0.121	not sig.
Brain tryptophan levels vs brain kynurenine levels after dl-kynurenine 5 mg/kg	0.498	not sig.

The correlation is between the mean TP levels and mean kynurenine levels in each case.

CHAPTER 6

UPTAKE OF KYNURENINE INTO BRAIN AFTER AN INJECTED DOSE

It was shown by Gal and Sherman (1978) that a tracer dose of ^{3}H-kynurenine injected peripherally produced labelled kynurenine in brain, 60% of the cerebral pool originating from the periphery. The remainder of the cerebral kynurenine levels were suggested to be formed in the brain. The enzyme TP pyrrolase has been identified in brain (Gal 1974) but unlike the peripheral enzyme it was not inducible by cortisol.

In the previous chapter (5) brain 5-HT turnover rate had been determined after 2 hr. pretreatment. Changes in plasma and brain kynurenine levels were measured at the same time to assess whether these could be related to changes in 5-HT turnover. Unfortunately the brain kynurenine levels, though elevated, were not dose-dependent and appeared to be independent of changes of brain 5-HT turnover rate.

Since little work had previously been done on the mechanism of uptake of kynurenine into the brain, this was further investigated.

6. Uptake of kynurenine into brain after an injected dose

A solution of dl-kynurenine (as sulphate) in saline was injected intraperitoneally into male mice (25 - 30 g) and the animals killed after 0, 30, 60 or 120 min. Kynurenine was determined by GLC as described in experimental methods sections 6 and 7.

6.1 Plasma kynurenine levels

A small dose of dl-kynurenine (0.5 mg/kg) significantly increased plasma kynurenine levels after 30 min. ($p < 0.05$) but levels declined by 60 min. so that they were no longer significantly greater than controls and were normal after 2 hr. (Fig. 39).

Kynurenine 5 mg/kg increased plasma kynurenine levels to more

than double normal levels after 30 min. ($p < 0.001$). Again levels declined after 60 min. but they were still significantly greater than controls ($p < 0.01$). Levels had fallen to normal after 2 hr. (Fig. 41).

Similarly a 20 mg/kg dose caused a very large increase in plasma kynurenine after 30 min. ($p < 0.001$) but levels declined after 60 min. though they were still greater than controls ($p < 0.01$). After 2 hr. levels were further reduced but were still significantly above control levels ($p < 0.05$)(Fig. 43).

6.2 Brain kynurenine levels

It was found that a proportion of the plasma kynurenine was taken up into the brain and in the first 30 min. after kynurenine 0.5 mg/kg brain levels were significantly increased ($p < 0.05$). By 60 min. there was a decline in brain levels such that they were not significantly different from controls. However brain kynurenine again increased and at 2 hr. levels were 198% of control ($p < 0.01$) (Fig. 40).

A dose of 5 mg/kg of kynurenine caused a significant increase in brain kynurenine after 30 min. ($p < 0.001$) but there was a similar decline after 60 min. though levels were still significantly increased over control levels ($p < 0.01$). However the brain levels rose again and were 185.5% of control levels after 2 hr. ($p < 0.001$) (Fig. 42). The levels of kynurenine found in brain 2 hr. after kynurenine 5 mg/kg were not significantly different from those found 2 hr. after kynurenine 0.5 mg/kg.

30 min. after kynurenine 20 mg/kg the brain levels were very

high, more than four times normal ($p < 0.001$). However by 60 min. levels declined, and continued to fall so that after 2 hr. the brain levels had returned to similar levels to those found 2 hr. after 0.5 mg/kg or 5 mg/kg. In this case there was no secondary rise in brain kynurenine levels from the 60 min. level as was found with the other doses. After 2 hr. the brain levels were 174.1% of control levels ($p < 0.01$)(Fig. 44).

Extremely high brain kynurenine levels were found 30 min. after kynurenine 20 mg/kg. As the plasma kynurenine levels were also very high at this time it was thought that the high plasma levels may have contributed to the high brain levels observed via the residual volume of blood in the brain. The residual volume of blood in the brain was therefore determined.

6.3 Determination of residual volume

^{3}H-dextran of very high molecular weight (MW 77,500 average) was injected into male mice (25 - 30 g) and the animals sacrificed after 30 min. The brain was removed and treated in exactly the same way as the brains used for the kynurenine determination. Since the dextran would still be present only in the blood, the residual volume of blood can be determined as described under experimental methods section 11.

The residual volume was found to be

25.1 ± 2.1 ul of blood per brain

(expressed as mean $\pm$ standard error n = 2). This value compares favourably with published results of 30 ul (Dittmer 1961) and is in good agreement with other results from this laboratory using

different methodology (Handley, 1970).

After 30 min. pretreatment with dl-kynurenine 20 mg/kg plasma levels were 3.416 ug/ml. If the plasma is assumed to be approximately 50% of the blood volume this would represent 1.708 ug/ml in the whole blood. Therefore 25 ul of blood contains approximately 0.043 ug of kynurenine. Since the brain kynurenine levels after this dose were observed to be 0.865 ug/g, a whole brain (approximately 0.4 g) contains approximately 0.346 ug. The residual volume of blood in brain therefore introduces an error in the measurement of brain kynurenine levels which may be as much as 12.4%.

Though the brain levels can be considered to be over-estimated by up to 12.4% the extremely high brain levels of kynurenine observed after dl-kynurenine 20 mg/kg can only be due to intracerebral kynurenine.

6.4 Correlations between brain and plasma kynurenine levels

There were highly significant correlations between the mean plasma and mean brain kynurenine levels after kynurenine 20 mg/kg at all times after injection (table 22, Fig. 47). Plasma and brain kynurenine were also significantly correlated after kynurenine 5 mg/kg after 0, 30 and 60 min. (table 22, Fig. 46). This correlation was markedly reduced if the 2 hr. value was included (table 22). There was also a significant correlation between brain and plasma levels after kynurenine 0.5 mg/kg after 0, 30 and 60 min. (table 22 and Fig. 45) but this correlation was markedly reduced if the 2 hr. value was included (table 22).

Though the brains and plasmas were taken from the same mice

the tissues had to be pooled for the determinations. Therefore no individual/individual correlations could be presented. All correlations are therefore between the mean values of plasma and brain levels at each time interval.

Discussion

Kynurenine readily enters the plasma from the peritoneal cavity producing high plasma kynurenine levels within 30 min. of intraperitoneal injection. It is rapidly distributed to the tissues and is metabolised by the liver (see Curzon and Knott 1977) and excreted by the kidney (Altman and Greengard 1966 (b)).

Kynurenine was taken up into brain producing increased levels after 30 min. It has previously been reported that kynurenine is taken up into brain from the periphery (Gal and Sherman 1978, Joseph and Kadam 1979). Brain kynurenine levels were found to follow the plasma levels closely at all times except for 2 hr. after dl-kynurenine 0.5 mg/kg and 5 mg/kg.

When plasma levels were high, kynurenine entered the brain along a concentration gradient. At all times except for 2 hr. after kynurenine 0.5 mg/kg and 5 mg/kg, plasma and brain kynurenine levels were strongly positively correlated with an approximately constant proportion of plasma levels entering the brain. However 2 hr. after dl-kynurenine 0.5 mg/kg and 5 mg/kg plasma kynurenine levels were normal but the brain levels were markedly increased. There was a secondary rise in the brain levels from 60 min. to 2 hr. after injection. 2 hr. after kynurenine 0.5 mg/kg and 5 mg/kg the uptake must be active as it occurs against a concentration gradient. A greater proportion of plasma

kynurenine was taken up at these times.

Thus, there appear to be two uptake systems for kynurenine. It is likely that active uptake occurs continually but is masked at high plasma kynurenine levels by passive diffusion. Active uptake is likely to be by a carrier mechanism. This may be the same mechanism as that which takes up TP, and kynurenine competes with TP for uptake (Curzon and Green 1970).

The profile of plasma kynurenine levels after dl-kynurenine 5 mg/kg was similar to that found in the rat (Joseph and Kadam 1979) after l-kynurenine 5 mg/kg with very similar % increases in kynurenine levels. However the brain kynurenine levels after dl-kynurenine 5 mg/kg in the mouse showed a smaller increase than those found after l-kynurenine 5 mg/kg in the rat. There was also a secondary rise in brain kynurenine levels 2 hr. after this dose in the mouse. The brain levels in the mouse were never more than 45% of the plasma levels. It appears that the d-isomer of kynurenine may not be taken up into brain at all. This would explain the lower brain levels after dl-kynurenine 5 mg/kg than after l-kynurenine 5 mg/kg. It may be expected that only the l-isomer is taken up since the carrier mechanism is likely to be specific for l-amino acids.

It has been shown that kynurenine is bound to plasma proteins, mainly albumin and α and β globulins (Hankes and Schmaeler 1978). About 17% of plasma kynurenine was bound to plasma protein at 37°C (ibid.). The binding of kynurenine to protein may also influence the uptake into brain. It has been suggested for TP that only the unbound portion enters the brain (Tagliamonte et al 1971) and this may apply to

kynurenine also. Kynurenine is about 83% free in the plasma but only 26 - 45% of plasma levels were taken up into brain. The d-isomer of kynurenine takes longer to bind to protein than the l-isomer (Hankes and Schmaeler 1978) so most of the d-isomer is likely to be in the "free" form. However, the d-isomer does not appear to be taken up into the brain.

It has been shown previously that kynurenine can also be synthesised in brain (Gal 1974). However, it is unlikely that the rate of synthesis of kynurenine would have altered significantly and the changes in brain levels are likely to be due completely to peripheral uptake. It is possible, however, that a large increase in brain kynurenine levels would tend to reduce pyrrolase activity and reduce kynurenine synthesis, lowering brain levels and giving a false impression of the extent of uptake into brain.

Fig. 39

Effect of DL-kynurenine 0.5 mg/kg on plasma kynurenine levels

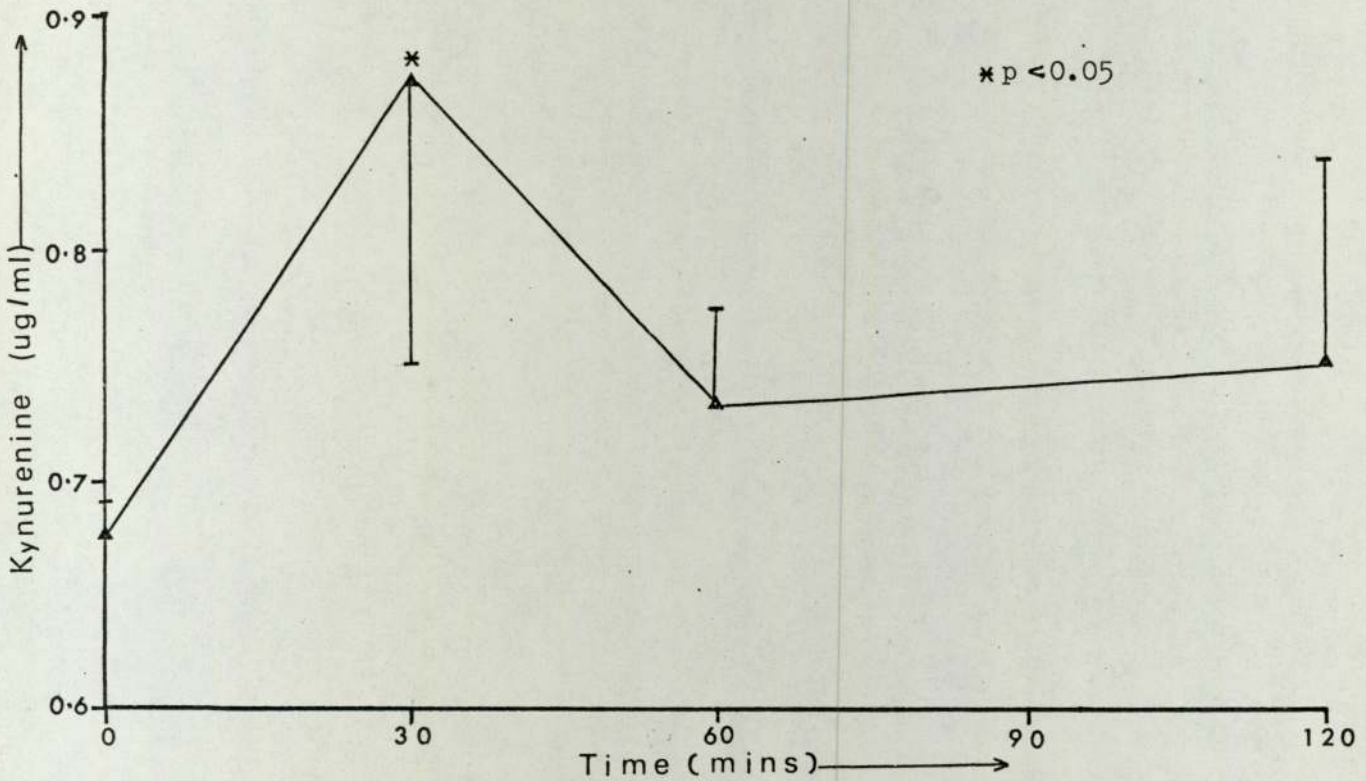


Fig. 40

Effect of DL-Kynurenine 0.5 mg/kg on brain kynurenine levels

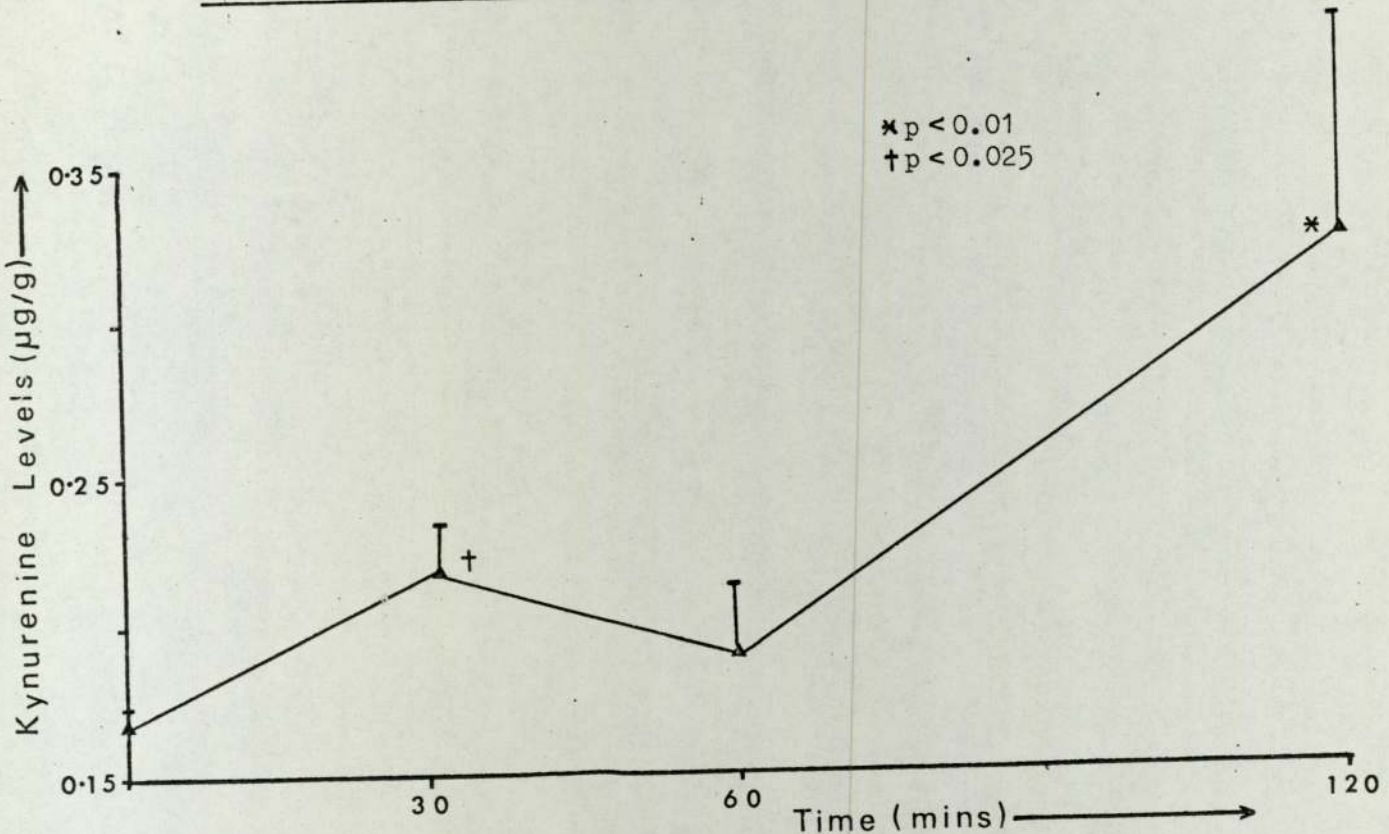


Fig. 41

Effect of DL-kynurenine 5 mg/kg on plasma kynurenine levels

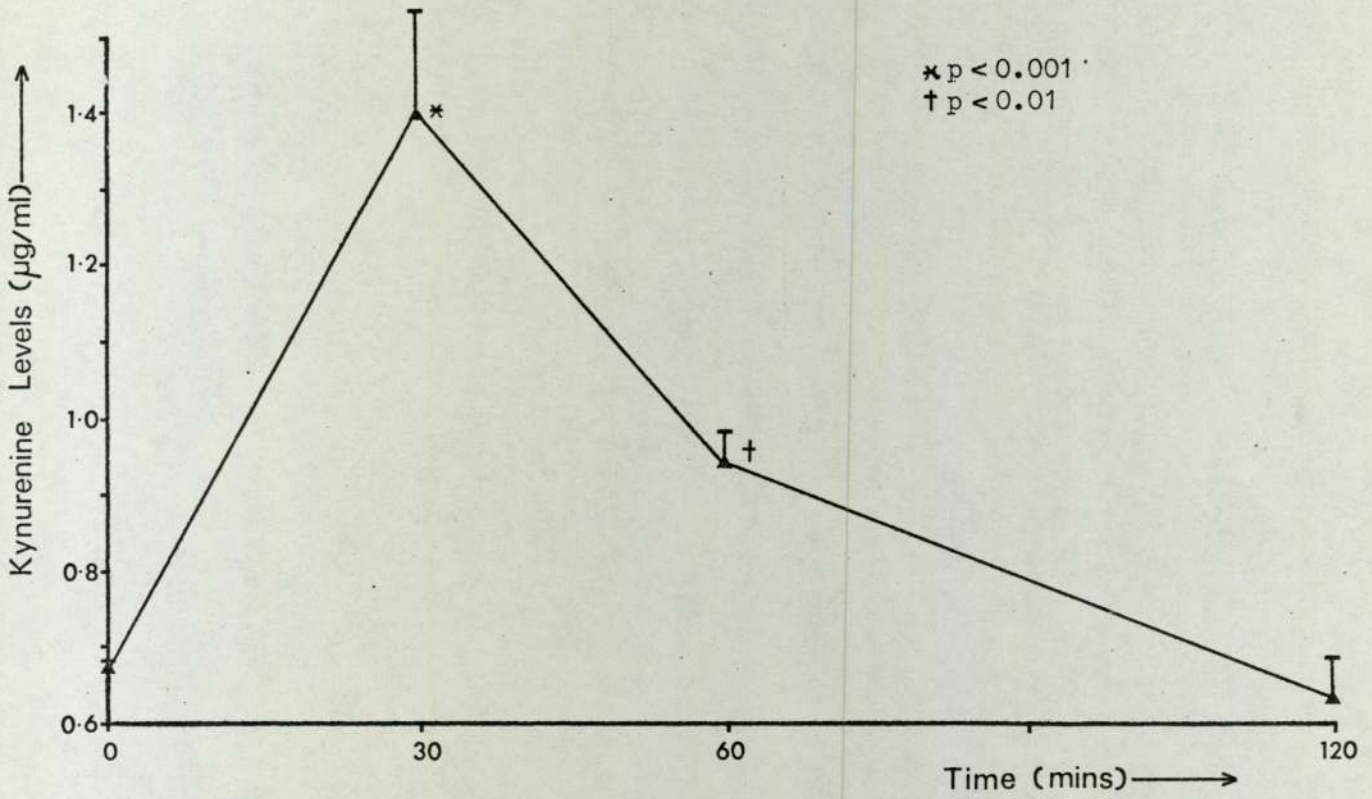


Fig. 42

Effect of DL-kynurenine 5 mg/kg on brain kynurenine levels

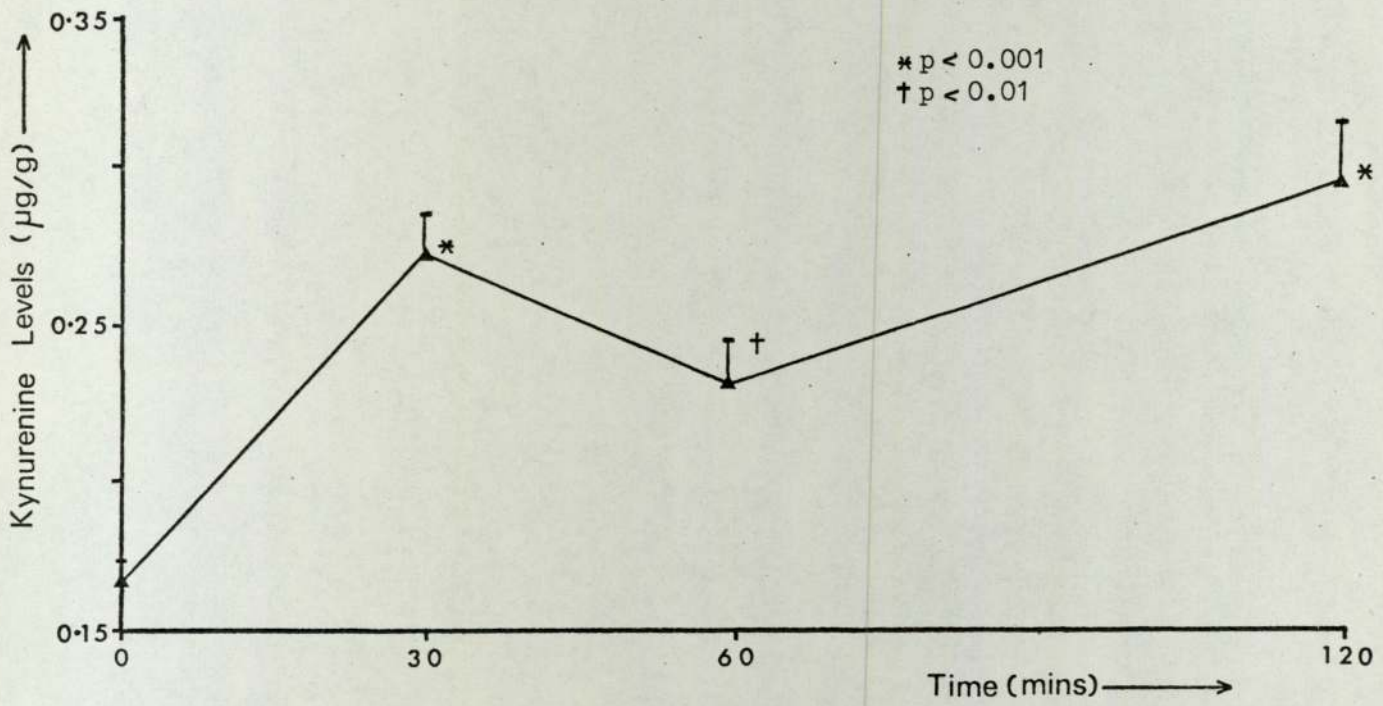


Fig. 43

Effect of DL-kynurenine 20 mg/kg on plasma kynurenine levels

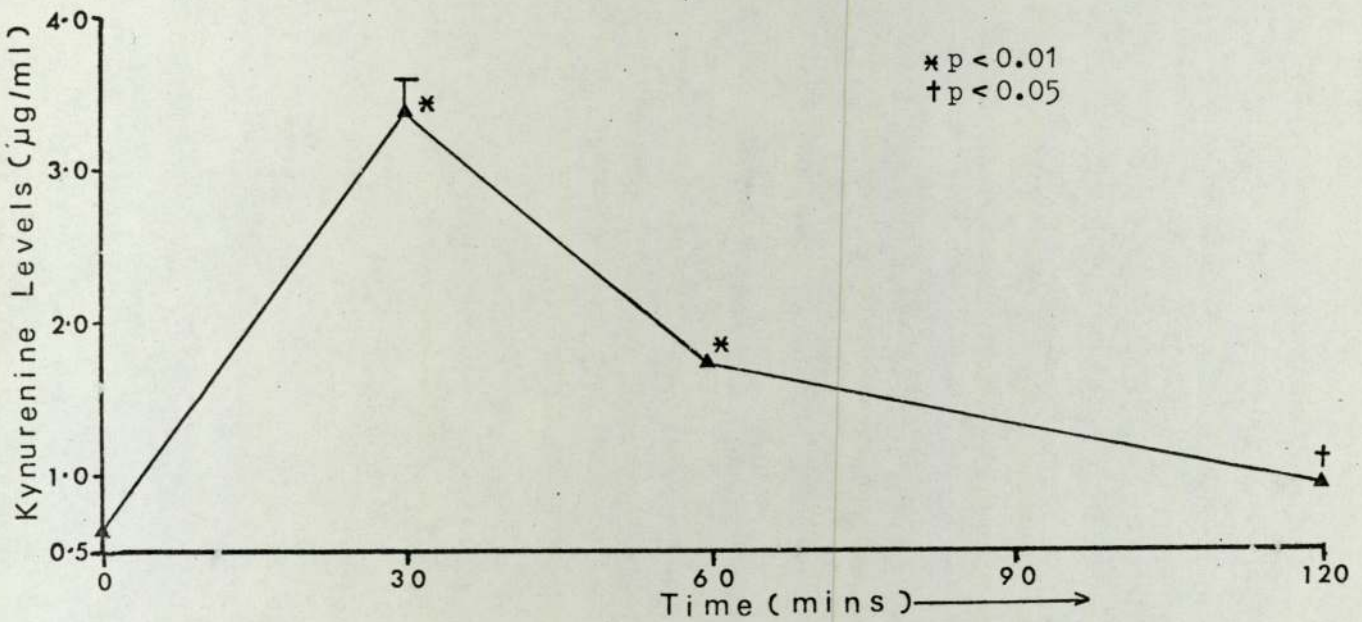


Fig. 44

Effect of DL-kynurenine 20 mg/kg on brain kynurenine levels

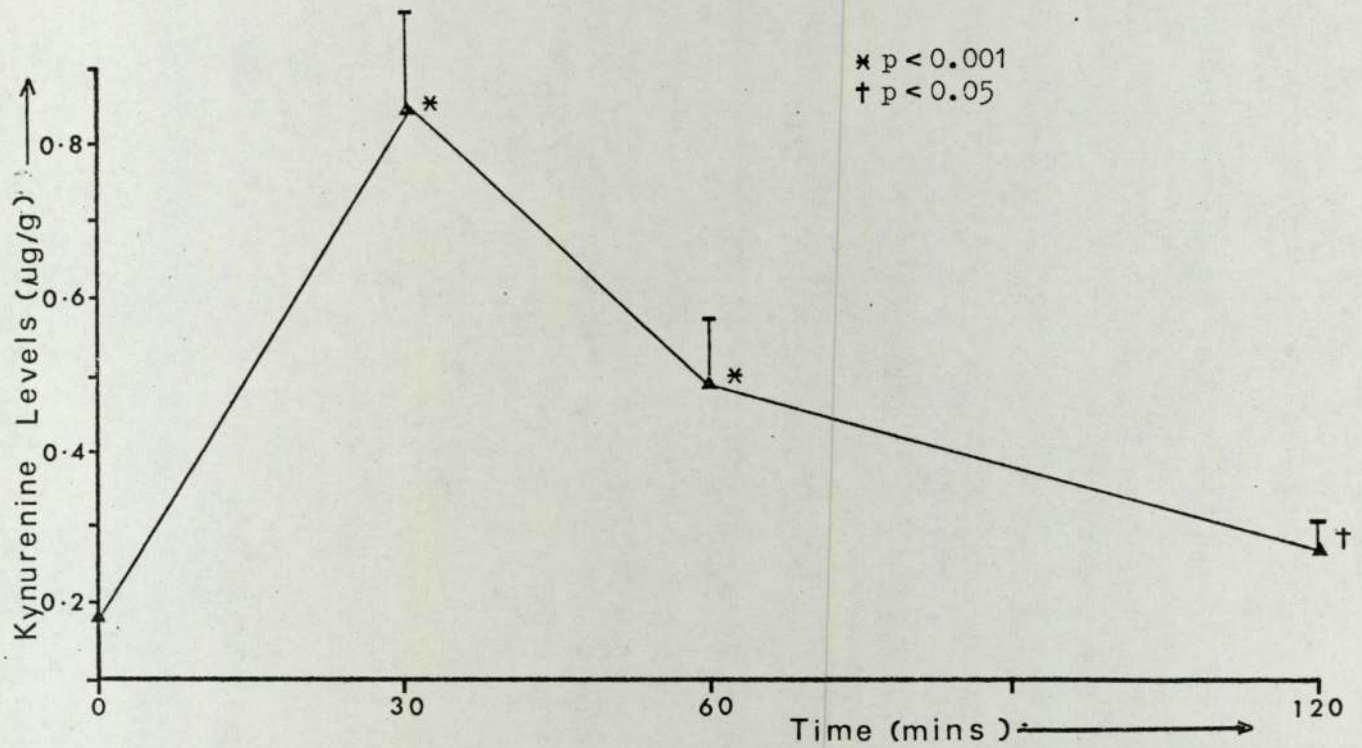
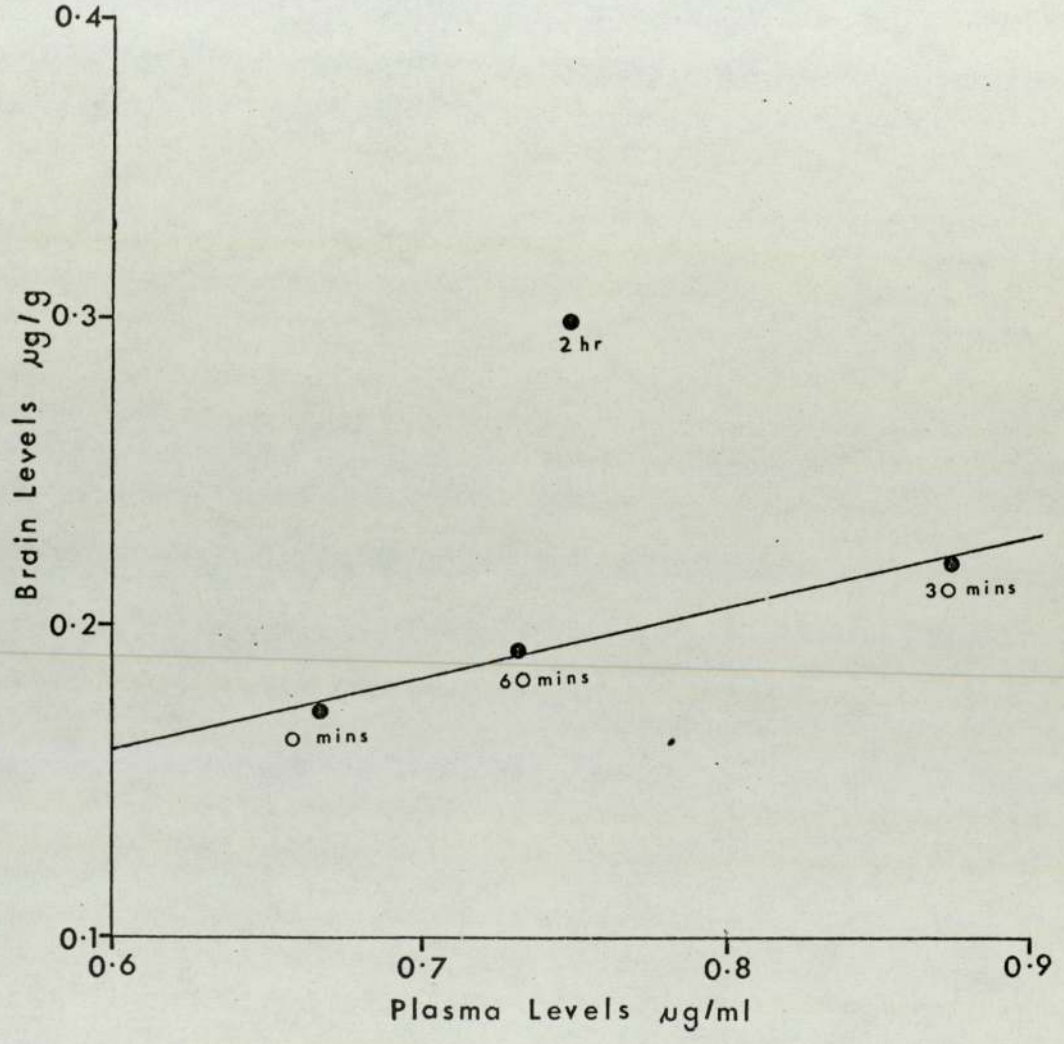
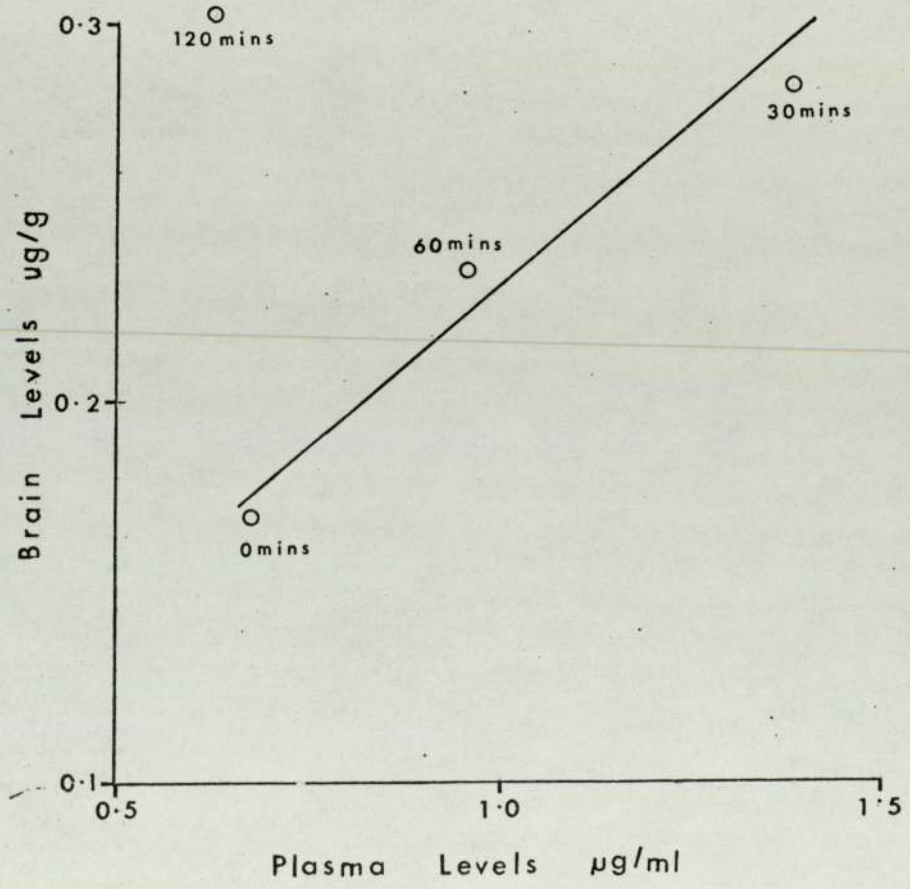


Fig. 45

Correlation between plasma and brain kynurenine levels after dl-kynurenine 0.5 mg/kg



Correlation between plasma and brain kynurenine levels after an injected dose of dl-kynurenine 5 mg/kg



Correlation between plasma and brain kynurenine levels after an injected dose of dl-kynurenine 20 mg/kg

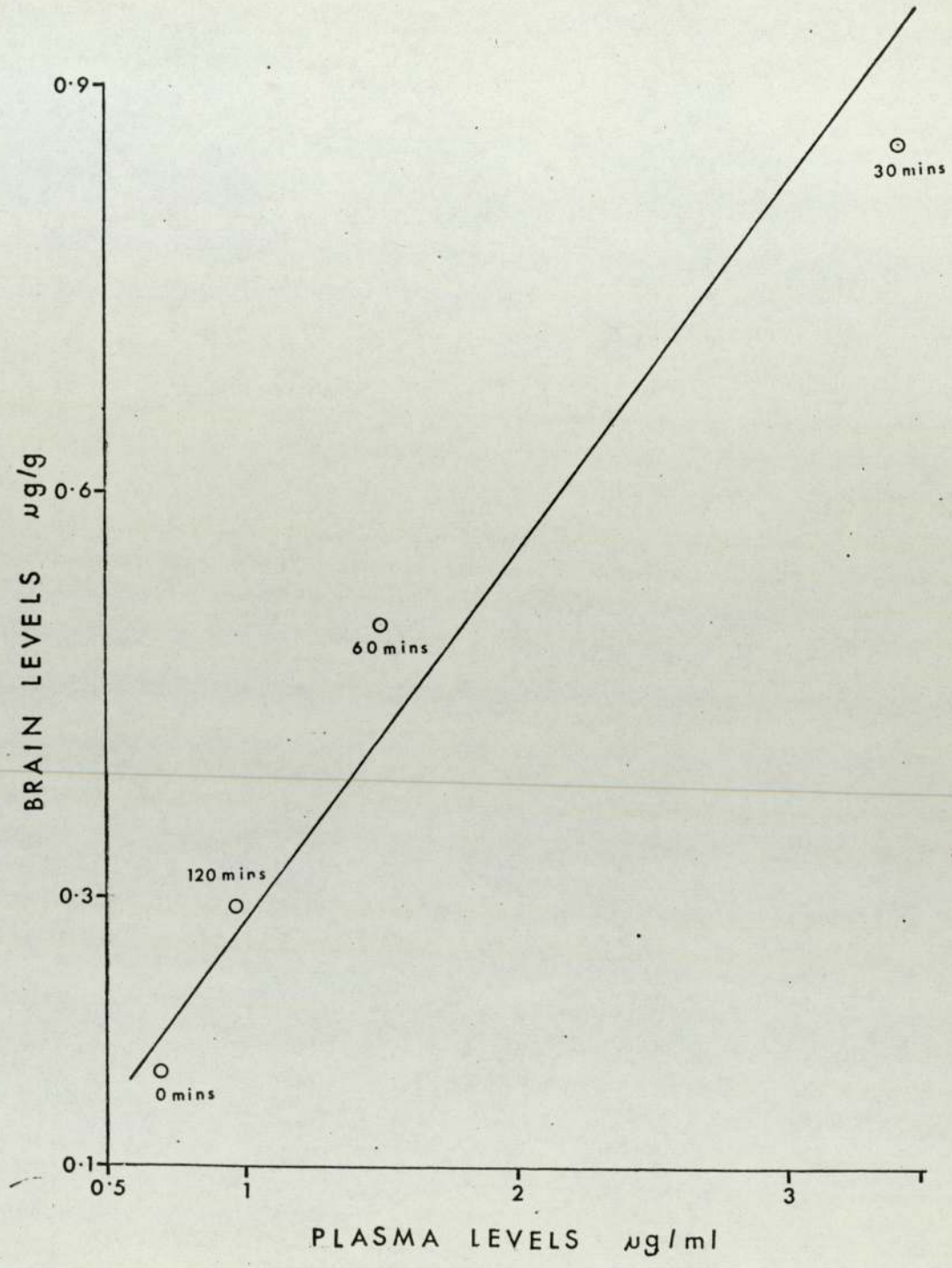


Table 22

Correlation between plasma and brain kynurenine levels after an injected dose

Treatment	Correlation coefficient (r)	Significance
dl-kynurenine 0.5 mg/kg 0 - 60 min.	0.989	$p < 0.01$
dl-kynurenine 0.5 mg/kg 0 - 120 min.	0.260	n.s.
dl-kynurenine 5 mg/kg 0 - 60 min.	0.961	$p < 0.01$
dl-kynurenine 5 mg/kg 0 - 120 min,	0.270	n.s.
dl-kynurenine 20 mg/kg 0 - 60 min.	0.979	$p < 0.01$
dl-kynurenine 20 mg/kg 10 - 120 min.	0.976	$p < 0.01$

The correlation is between the means of plasma and brain kynurenine after various times.

CHAPTER 7

EFFECT OF KYNURENINE ON 5-HT UPTAKE

7.1 Introduction

Isolated brain tissues such as brain slices or isolated nerve endings (synaptosomes) can be used to study mechanisms of uptake, storage and release of transmitters at the cellular level. Synaptosomes have the enzymes for amine synthesis, organelles for storage and a plasma membrane which is thought to maintain many of the properties of the presynaptic membranes *in situ* (Iversen 1975).

7.1.1 Uptake of 5-HT

It has been shown (Iversen 1967, Iversen et al 1975) that neurons have associated with them a specific transport system for the transmitter which they manufacture and release. Thus neurons which contain 5-HT have their own high-affinity uptake system for this amine. An uptake of ^{3}H-5-HT has also been described *in vitro* in brain slices or synaptosomes (Ross and Renyi 1967). The high-affinity uptake of ^{3}H-5-HT by synaptosomes in rat brain homogenates seems to be associated with 5-HT containing nerve-terminals since such uptake is severely reduced in homogenates prepared from the brains of animals in which such terminals were selectively destroyed (Kuhar et al 1972). The uptake of 5-HT is mediated by a saturable process with a Km of approximately 0.17 uM (Shaskan and Snyder 1970). The process is temperature-dependent and sodium-dependent and is inhibited by metabolic inhibitors and ouabain (Iversen 1975). 5-HT is also taken up into adrenergic neurons so that there is also a lower affinity uptake with a Km of 8.0 uM (Shaskan and Snyder 1970) into these neurons. The uptake process can be

inhibited by various drugs and the most potent inhibitors are the tertiary amine tricyclic antidepressants, particularly imipramine and amitriptyline (Iversen et al 1975). When synaptosomes are incubated with a low concentration of ^{3}H-5-HT high affinity uptake can be demonstrated. If a high concentration of ^{3}H-5-HT is used the high affinity uptake is masked by non-specific uptake.

7.1.2 Uptake of kynurenine

Kynurenine is taken up into brain probably by an active process (see chapter 6). It has been shown to compete with tryptophan for uptake into brain slices (Green and Curzon, 1970).

It was found that low doses of kynurenine (0.5 mg/kg) caused marked potentiation of the head twitch response to both 5-HTP and 5-HT in mice (Handley and Miskin 1977). The same dose of kynurenine was found to decrease 5-HT turnover (Gould and Handley 1978, chapter 5). A higher dose of kynurenine (5 mg/kg) antagonised the head twitch response and did not affect turnover rate. Thus different doses of kynurenine had a differential effect on brain 5-HT function. These observations could not be explained by a competition of kynurenine with tryptophan for uptake since this would tend to reduce brain TP and brain 5-HT turnover in a dose-dependent manner.

A possible explanation of the dual effect of kynurenine on the head twitch and on turnover rate (chapter 5) was via an inhibition of uptake of 5-HT at low doses of kynurenine masked

by 5-HT receptor blockade at higher doses. To investigate this possibility the effect of various concentrations of kynurenine on the in vitro uptake of ^{3}H-5-HT was studied.

7.2 Results

7.2.1 In vitro synaptosomal uptake of 5-HT

A crude synaptosomal fraction was prepared from the brains of male mice as described in experimental methods section 10. The uptake of ^{3}H-5-HT (0.2 uM SA 500 mCi/mmol) in a physiological buffer solution (see experimental methods section 10) at 37°C and pH 7.4 was studied.

7.2.1.1 Effect of amitriptyline

The presence of amitriptyline 1 uM reduced the uptake of ^{3}H-5-HT by more than 90% ($p < 0.05$)(Fig. 49). A higher concentration of amitriptyline (10 uM) abolished all active uptake ($p < 0.01$)(Fig. 48) and the uptake at 0°C (blank values) was also reduced.

7.2.1.2 Effect of kynurenine

The presence of l-kynurenine 0.1 uM, 1 uM or 1 mM had no significant effect on the uptake of ^{3}H-5-HT. Dl-kynurenine 1 mM caused a 30.5% reduction in the uptake of ^{3}H-5-HT, though this change was not statistically significant (Fig. 49).

7.2.2 In vivo effect of kynurenine on 5-HT uptake

Male mice were pretreated 2 hr. previously with dl-kynurenine 0.5 mg/kg or 20 mg/kg. Animals were killed, the brains removed and a crude synaptosomal fraction was prepared

immediately. The ability of synaptosomes from these animals to take up ^{3}H-5-HT (0.2 uM SA 500 mCi/mmol) was studied.

There was no significant difference in the uptake of ^{3}H-5-HT after pretreatment with dl-kynurenine 0.5 mg/kg or 5 mg/kg from saline-treated controls (Fig. 50).

Discussion

Amitriptyline 1 uM caused a marked reduction in the uptake of ^{3}H-5-HT into a crude synaptosomal fraction prepared from mouse brain. A concentration of 10 uM amitriptyline reduced the uptake of ^{3}H-5-HT to below that observed at 0 - 4°C. It is possible that some active uptake occurs when the preparation is on ice, and this is completely inhibited by this concentration of amitriptyline. It has previously been shown that amitriptyline is a potent inhibitor of 5-HT uptake with an inhibitory concentration (ID 50) of 0.8 uM (Iversen et al 1975). The purpose of these experiments was to demonstrate the validity of the technique as developed in these laboratories.

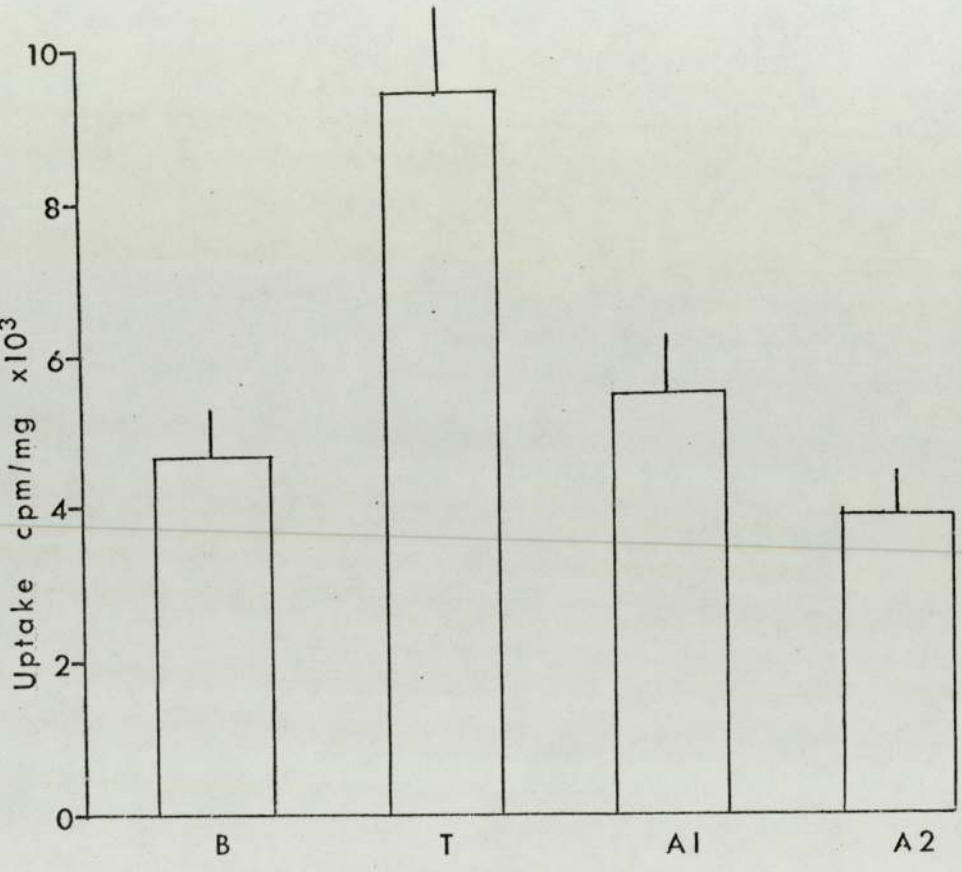
In the in vitro studies l-kynurenine was found to have no effect on the uptake of ^{3}H-5-HT at a concentration of up to 1 mM. Dl-kynurenine 1 mM caused a 30% reduction in 5-HT uptake. This observation may indicate a differential effect of the D-isomer of kynurenine on 5-HT uptake. However it is unlikely to be of physiological significance because of the very high concentration used.

The brains of animals pretreated with dl-kynurenine 0.5 mg/kg or 20 mg/kg had the same capacity to take up ^{3}H-5-HT as saline-treated controls.

It was unlikely, therefore, that the potentiation of the head-twitch response and reduction of 5-HT turnover after dl-kynurenine 0.5 mg/kg could be explained by an effect on brain 5-HT uptake.

Fig. 48

Effect of amitriptyline on the synaptosomal uptake of ^{3}H-5-HT



B = synaptosomes incubated at 0 - 4°C (Blank)(n = 8)

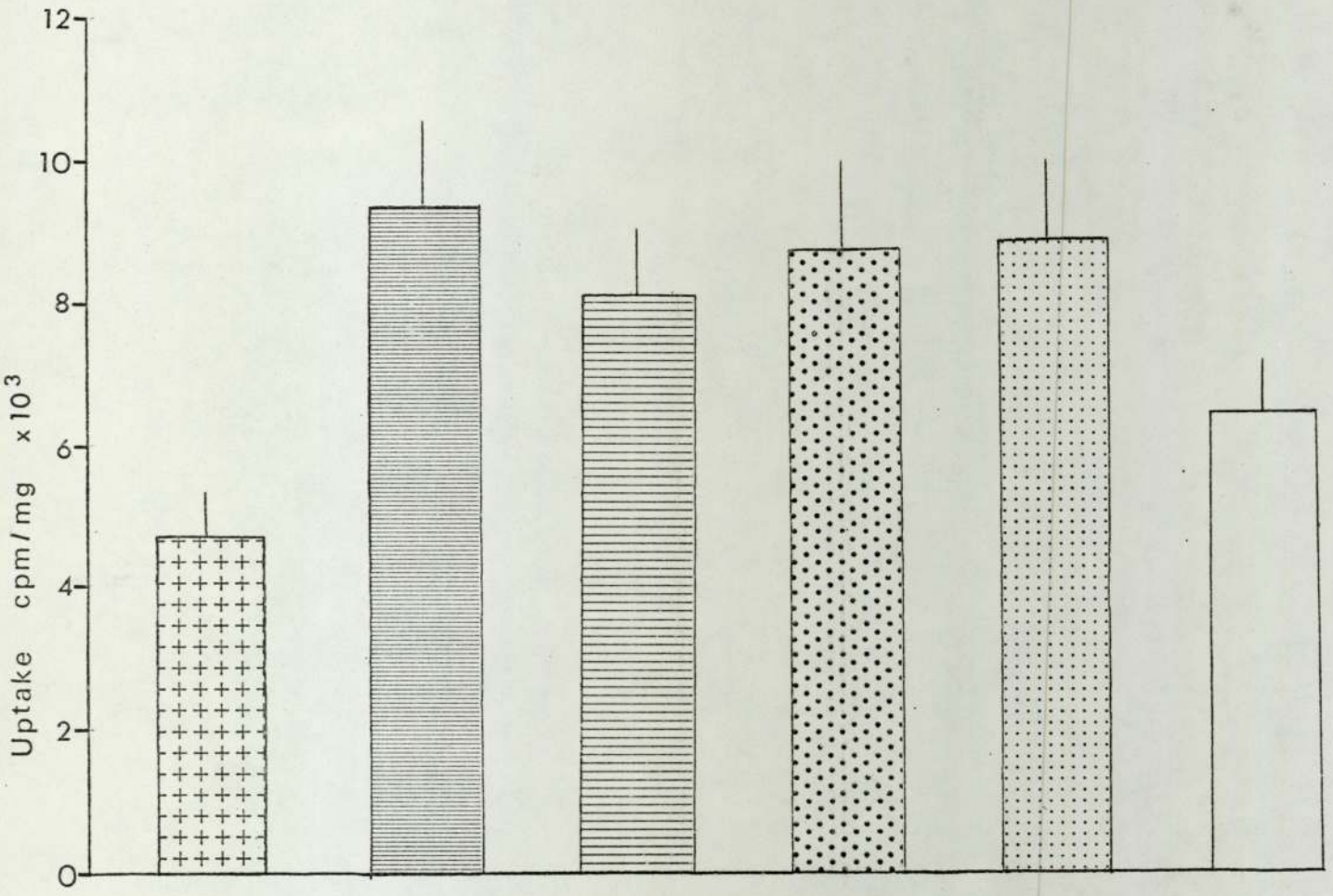
T = synaptosomes incubated at 37°C (Control)(n = 8)

A1 = Amitriptyline 1 uM added to synaptosomes (n = 5)

A2 = Amitriptyline 10 uM added to synaptosomes (n = 6)

Fig. 49

Effect of kynurenine on the in vitro uptake of ^{3}H-5-HT into synaptosomes



= Uptake at 0-4°C (Blank) n = 8

= Uptake at 37°C (control)(n = 8)

= L-Kynurenine 0.1 uM added to synaptosomes (n = 8)

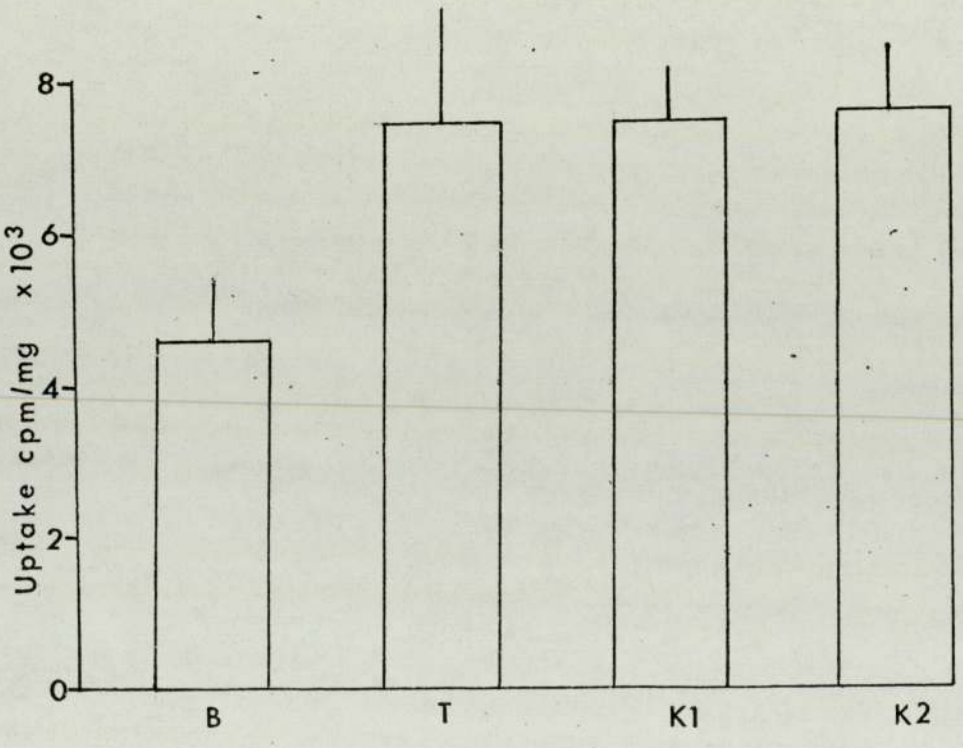
= L-Kynurenine 1 uM added to synaptosomes (n = 8)

= L-Kynurenine 1 mM added to synaptosomes (n = 8)

= dl-kynurenine 1 mM added to synaptosomes (n = 4)

Fig. 50

Effect of kynurenine in vivo on the synaptosomal uptake of ^{3}H-5-HT



B = synaptosomal uptake at 0 - 4°C (Blank)(n = 3)

T = uptake at 37°C in saline-treated controls (n = 3)

K1= uptake after 2 hr. pretreatment with dl-kynurenine 0.5 mg/kg (n = 3)

K2= uptake after 2 hr. pretreatment with dl-kynurenine 20 mg/kg (n = 3)

The results are expressed as the mean ± standard error.

GENERAL DISCUSSION

General Discussion

The purpose of this work was to further investigate the effects of some steroid hormones on tryptophan metabolism and brain 5-HT turnover. Previous workers have found that cortisol and contraceptive steroids affect both animal behaviour and brain 5-HT function.

Cortisol has been reported to reduce brain TP after cortisol (Green et al 1975) accompanied by decreased liver TP, plasma free TP, brain 5-HT and 5-HIAA (ibid.). Many workers have reported a reduction in brain 5-HT levels (Curzon and Green 1968, Green and Curzon 1968, Yuwiler et al 1971, Fuxe et al 1973, Yuwiler and Geller 1974) after 3 - 10 hr. pretreatment with doses of 5 to 20 mg/kg. Brain 5-HIAA levels were also reduced, indicating reduced synthesis (Curzon and Green 1971). These changes were accompanied by an induction of TP pyrrolase in the liver.

A low dose of cortisol (75 ug/kg) was found to affect behaviour in the mouse, causing potentiation of the head-twitch response to 5-HTP and 5-HT after 24 hr. pretreatment, However, this behaviour was inhibited after repeated dosing with cortisol (Handley and Miskin 1972). It was possible that this behavioral change was accompanied by a change in 5-HT turnover, as the head-twitch response to 5-HTP and 5-HT is thought to indicate the "free" 5-HT (Corne et al 1963). However in the present experiments no change in turnover could be detected at this dose of cortisol and in fact a significant reduction in turnover was only seen after 24 hr. pretreatment with cortisol 20 mg/kg. Repeated dosing with a lower dose of cortisol or 24 hr. pretreatment with lower doses was also found to have no effect on 5-HT turnover. It is likely that the

behaviour is more sensitive to subtle changes in serotonergic function or the changes may occur in a particular brain area.

Brain and plasma TP were also measured after 24 hr. pretreatment, but neither of these were significantly affected by cortisol 20 mg/kg. Other workers have also reported brain TP to be unchanged (Azmitia and McEwen 1974, Thierry et al 1968) after corticosteroid treatment. However, after 90 min. pretreatment with cortisol 20 mg/kg brain TP and 5-HT synthesis was found to be increased (Neckers and Sze 1975). This increase in 5-HT turnover was also observed in the present work, when the same pretreatment interval was used.

The effects of cortisol observed seem to be dependent on the dose and pretreatment interval used. Kovacs et al (1975) have shown that hypothalamic 5-HT was increased, unchanged or decreased after 30 min. treatment, depending on the dose used.

Changes in TP and 5-HT levels have also been found in different stages of the oestrous cycle (Greengrass and Tonge 1971). 5-HT levels was maximal in the forebrain and midbrain of the mouse at dioestus and minimal at oestrus (ibid.). Whole mouse brain 5-HT has also been found to be reduced at oestrus compared with dioestrus (Bond 1979). This observation was repeated in the present work but the changes were not statistically significant. Brain 5-HT turnover was also found to be increased at oestrus compared with dioestrus but not significantly. These findings are consistent with a previous report that brain TP was increased at oestrus (Bond 1979). However other authors found decreased tryptophan levels in fore, mid and hindbrain at oestrus (Greengrass and Tonge 1971). Plasma total and % free tryptophan have also been found to be reduced at

oestrus compared with dioestrus values (Bond 1979). It has recently been suggested that increased brain 5-HT synthesis may not always be accompanied by increased brain TP and that variations in 5-HT synthesis correlated well with changes in the active transport of TP (Hamon et al 1974). There may therefore be increased flux of TP into brain at oestrus which may result in increased 5-HT synthesis. There have been suggested to be two pools for TP, only one of which is functionally active and produces 5-HT (Green and Grahame-Smith 1976) so that the relationship between brain TP uptake and 5-HT synthesis is still unclear.

Oestradiol has been found to reduce midbrain 5-HT levels in the rat (Leonard and Hamburger 1974) whereas progesterone is reported to increase 5-HT turnover (Ladisich 1977, Glowinski et al 1973). In these studies very high doses of these steroids were used. In one study where lower doses of a combination of oestrogen and progesterone (40 ug and 4 mg respectively per 100 g rat) were used no effect on 5-HT synthesis was seen in ovariectomised females (Hyyppa 1973). In this work 14 day pretreatment with a combination of norethisterone 200 ug/kg and oestradiol 100 ug/kg in intact female mice was used, and this also failed to affect 5-HT turnover. Intact animals were used because it was intended to resemble the administration of contraceptive steroids to Man. Ovariectomised animals have no basal levels of oestrogen and progesterone and the secretion of pituitary hormones and releasing factors is abnormal, which is not the situation encountered when oral contraceptives are used. It appears that changes in 5-HT synthesis rate only occur at very high doses of these steroids, and these observations are unlikely to be significant in relation to the use of contraceptive combinations in Man. The doses used in these experiments

are still higher than those used in contraception but are much less than those used by other authors.

The mechanisms by which cortisol and norethisterone and oestradiol in combination could affect brain 5-HT turnover were studied. Cortisol had been shown to decrease plasma free TP (Green et al 1975), and this may have occurred by a direct effect of cortisol on TP binding. Plasma oestradiol levels have also been correlated with plasma free TP in peri-menopausal women (Aylward 1976). The effects of cortisol, norethisterone, oestradiol and mestranol on the in vitro binding of TP to albumin were therefore studied. In the methodology used the pH of the solutions could be checked at the start of the experiment after gassing the Kreb's buffer with 95% O_2/5% CO_2. More recent methods are capable of controlling pH during the experiment (Bloxham et al 1977) which leads to more accurate determination of the % free at physiological pH. However the methods used here were capable of determining changes in binding under steady state conditions. Cortisol at a concentration of up to 1 mg/kg had no effect on the binding. Oestradiol or mestranol 50 ug/ml also failed to affect binding, whereas norethisterone 50 ug/ml caused a slight increase in the % free. However it is unlikely that this observation has any physiological significance because there was only a small effect on binding even at this very large dose. It therefore appeared that these hormones did not directly affect TP binding and other possible actions on 5-HT synthesis were investigated.

It had been suggested that cortisol reduced 5-HT synthesis by inducing TP pyrrolase (Green and Curzon 1970) which increases kynurenine production. The evidence for this was the cortisol had maximal effects on pyrrolase activity and reduced brain 5-HT and 5-HIAA levels after a similar

time interval (about 5 hours)(Curzon and Green 1968). Kynurenine also reduced brain 5-HT levels, but after only 2 hr. (Green and Curzon 1970) indicating that kynurenine may have a direct effect on 5-HT turnover, whereas cortisol affected turnover via kynurenine.

The influence of cortisol and a combination of norethisterone and oestradiol on plasma kynurenine were determined. In retrospect, it was unfortunate that the effects of cortisol after 24 hr. treatment were studied. Pyrrolase has been shown to be induced for up to 11 hr. after cortisol 5 mg/kg (Curzon and Green 1968). Pyrrolase activity may therefore be expected to have returned to normal after 24 hr. This time interval was chosen because it was the pretreatment used by Handley and Miskin (1972) in their behavioural experiments. It was intended to determine whether the behavioral changes were accompanied by a change in 5-HT turnover, and whether this could be mediated by increased kynurenine production. An increase in pyrrolase activity would increase kynurenine production and may be expected to increase plasma kynurenine levels. In fact plasma kynurenine levels were markedly reduced compared with saline treated controls 24 hr. after cortisol 5 mg/kg and 20 mg/kg, indicating that pyrrolase was no longer induced. It appears, therefore, that a reduction in turnover was not related to an increase in kynurenine production, since plasma kynurenine levels were reduced. The fact that plasma kynurenine levels were below control levels 24 hr. after cortisol was a surprising observation. It is possible that at this time the enzymes beyond kynurenine in the kynurenine pathway are being induced by cortisol and removing kynurenine from the plasma. The inducibility of these enzymes by cortisol appears not to have been determined previously.

Cortisol reduced brain 5-HT turnover significantly 24 hr. after a dose of 20 mg/kg. This dose is very high and well outside even the therapeutic range, though similar doses have been used by other workers (e.g. Curzon and Green 1968, Green and Curzon 1968, Neckers and Sze 1975).

The effects of 14 day pretreatment with a combination of norethisterone 200 ug/kg and oestradiol 100 ug/kg on 5-HT turnover and plasma kynurenine were determined. No effect on 5-HT turnover was found, but plasma kynurenine levels were markedly increased after this combination. It appeared, therefore, that the effects of these steroids on plasma kynurenine were not parallelled by an effect on turnover.

The effects of kynurenine itself on 5-HT turnover were also investigated. It had been shown that kynurenine reduced the uptake of TP into brain slices (Green and Curzon 1970) and reduced brain 5-HT and 5-HIAA levels indicating reduced 5-HT synthesis (ibid.). It was suggested that increased plasma kynurenine, which would compete for TP uptake into brain, would tend to reduce brain TP and 5-HT synthesis (Green and Curzon 1970). However, other workers failed to confirm the reduction in brain 5-HT or 5-HIAA levels (Joseph and Kadam 1979), and reported essentially normal brain TP levels (ibid.).

Brain TP was found to be increased 30 min. and 2 hr. after dl-kynurenine 0.5 mg/kg and to a lesser extent 30 min., but not 2 hr. after dl-kynurenine 5 mg/kg. These observations were not consistent with an inhibition of TP uptake by kynurenine (Green and Curzon 1970) which occurs *in vitro*, occurring *in vivo*. Kynurenine has been shown to bind to plasma proteins including albumin (Hankes and Schmaeler 1978, Joseph and Kadam 1979). About 20% of plasma kynurenine was found to be bound to albumin (Hankes and Schmaeler 1978) though this % has been suggested to be nearer

50% by other workers (Joseph and Kadam 1979). It is therefore possible that kynurenine could compete with TP for binding to albumin, assuming that both kynurenine and tryptophan bind to the same site on the albumin molecule and that the affinity of kynurenine for the site is similar to or greater than that of TP. If displacement of TP was taking place, the % free TP in the plasma would increase. Since increasing plasma free TP has often been found to increase brain TP and 5-HT synthesis (for review see Curzon and Knott 1977) this may have important consequences. However increases in plasma free TP may also induce the enzyme for the destruction of TP i.e. pyrrolase (Sourkes and Townsend 1955) which tends to remove the "extra" free TP. In this work a marked increase in brain TP was seen after dl-kynurenine 0.5 mg/kg and 5 mg/kg, the increase being greater after 0.5 mg/kg. It is possible that a low dose of kynurenine slightly increases the free TP without affecting pyrrolase, resulting in increased brain TP. The higher dose may affect both the free TP and TP pyrrolase so that the net increase in brain TP is smaller.

Plasma TP was found to be reduced in a dose-dependent manner 2 hr. after kynurenine injection. A significant fall in plasma TP occurred 2 hr. after dl-kynurenine 5 mg/kg. The plasma TP levels did not correlate with the plasma kynurenine levels and this effect was difficult to interpret.

However, it was found that dl-kynurenine had no effect on brain 5-HT levels after 2 hr. pretreatment in the mouse at doses of 0.5 or 5 mg/kg, and a high dose (20 mg/kg) caused an increase in 5-HT levels. The low dose (0.5 mg/kg) significantly reduced 5-HT turnover, whereas the higher doses (5 mg/kg and 20 mg/kg) had no effect on turnover. These

observations were consistent with the effects of dl-kynurenine on animal behaviour. A low dose of kynurenine (0.5 mg/kg) caused marked potentiation of the head-twitch response to 5-HTP or 5-HT (Handley and Miskin 1977). Since potentiation of the head-twitch response is likely to be due to increased release of 5-HT, inhibition of 5-HT reuptake or increased sensitivity of receptors to 5-HT this may be expected to be accompanied by a slowing of 5-HT turnover. However a higher dose of kynurenine (5 mg/kg) inhibited the behaviour (ibid.). These authors suggested that the dual effects of kynurenine may have been due to inhibition of 5-HT reuptake by kynurenine, masked at higher doses by receptor blockade. The effects of kynurenine on the uptake of 5-HT into synaptosomes was therefore studied.

In vivo pretreatment with dl-kynurenine 0.5 mg/kg or 5 mg/kg did not affect the ability of synaptosomes prepared from the animals to take up 5-HT. L-kynurenine had no effect on the in vitro uptake of 5-HT, though dl-kynurenine slightly reduced the uptake of 5-HT at a dose of 1 mM. This observation is unlikely to have physiological significance due to the very high concentration used. Thus the potentiation of the head-twitch response and a reduction in brain 5-HT turnover after kynurenine 0.5 mg/kg could not be explained by a reduction of 5-HT uptake.

A further explanation for the above effects on the head-twitch response and on turnover was the possibility that different concentrations of kynurenine were taken into brain after the different doses. This seemed unlikely because it may be expected that a greater concentration would be taken up as the dose increased, and this would be expected to cause a dose-dependent effect on 5-HT synthesis. However, this possibility was

investigated.

Kynurenine has previously been reported to enter the brain after a peripherally injected dose (Gal and Sherman 1978, Joseph and Kadam 1979, Gould 1979). Brain kynurenine levels were markedly increased 2 hr. after kynurenine 0.5 mg/kg, 5 mg/kg and 20 mg/kg but there was no significant difference between the kynurenine levels after the various doses. Brain 5-HT turnover was either markedly reduced (0.5 mg/kg) or unchanged (5 mg/kg or 20 mg/kg) indicating that it was unrelated to brain kynurenine at 2 hr. after kynurenine injection.

It was possible, however, that brain 5-HT turnover at 2 hr. was related to the fluxes occurring in brain kynurenine levels up to the 2 hr. determination. Therefore, the mechanism of kynurenine uptake up to 2 hr. was studied in more detail. Intraperitoneal injection of dl-kynurenine 20 mg/kg markedly increased plasma levels. Brain kynurenine levels followed plasma levels closely and a constant proportion of the plasma levels was taken into brain. The uptake was occurring along a concentration gradient and was indistinguishable from passive diffusion. Lower doses of kynurenine (0.5 mg/kg and 5 mg/kg) also increased plasma kynurenine levels after 30 min., but levels returned toward normal at 60 min. There was a secondary rise in brain kynurenine levels at 2 hr. At this time plasma levels had declined toward normal and uptake was therefore occurring against a concentration gradient, and was therefore active. Thus, there appear to be at least two mechanisms for taking up kynurenine into brain, one of which is active. It is likely that only the L-isomer of kynurenine is taken up and it may be taken up by the same carrier as TP since it competes with TP for uptake (Green and Curzon 1970).

The biochemical effects of certain steroid hormones on brain 5-HT turnover in animals have been studied in some detail. It is obviously impossible to determine the effects of these hormones on 5-HT turnover in Man. Since changes in plasma free TP have been found to influence 5-HT synthesis in brain (Tagliamonte et al 1971) changes in free and total TP were determined in a group of puerperal women. It was expected that a significant proportion of these women would suffer a dysphoric reaction, known as post-partum "blues", (Yalom 1968) within the first few days after delivery. Pregnant women have a very high plasma cortisol which gradually reduces post-partum. High plasma cortisol levels have been reported in depressive illness (e.g. Board et al 1956, 1957, Gibbons and McHugh 1962) and cortisol has been shown to affect brain 5-HT function (e.g. Curzon and Green 1968). At parturition there is also a sudden reduction in plasma levels of oestrogen and progesterone, and sudden withdrawal of female hormones is associated with a reduction in 5-HT synthesis in animals (Greengrass and Tonge 1975). Thus, post-partum "blues" appeared to be a good model for studying the inter-relationships between these hormones, biochemical variables and mood state. There was found to be a significant correlation between the plasma % free TP and mood state. Plasma cortisol was also positively correlated with the elation score. Thus, biochemical variables were related to mood state.

It appears that these steroid hormones, influence brain 5-HT turnover, though this work indicates that these changes may only occur at very high doses. The mechanism by which the effects on turnover occurred were investigated. It had been suggested that changes in brain 5-HT turnover were related to increased kynurenine production after cortisol,

but evidence has been presented here against this possibility. The changes in turnover were found to occur in the absence of increased kynurenine production and increased plasma kynurenine levels could occur without affecting brain 5-HT turnover.

It has been confirmed that kynurenine can affect brain 5-HT synthesis, but only under certain conditions. Changes in the dose of kynurenine or the pretreatment interval affected the observed changes in brain or plasma TP levels, brain 5-HT levels or turnover. The possible mechanism of these changes was examined, but no conclusions could be reached and further work is necessary.

Suggestions for further work

Further investigations of the effects of low doses of contraceptive steroids on brain 5-HT turnover after extended periods of treatment would be valuable. It would also be of interest to determine whether any effect on turnover could be seen in specific brain areas.

The mechanism by which these steroids, particularly cortisol, affect 5-HT turnover is by no means established, and a more detailed study of the relation between dose and pretreatment interval to change in 5-HT turnover is needed.

Dl-kynurenine was found to cause a dose-dependant decrease in brain 5-HT turnover. Though it appeared that only the L-isomer entered the brain and these effects were likely to be due to L-kynurenine it would be advantageous to confirm these findings using L-kynurenine.

The apparently paradoxical effects of kynurenine on brain 5-HT turnover warrants further investigation. It would be possible to measure % free TP, plasma TP, brain TP and 5-HT turnover after each dose of kynurenine

in the same animals. This would give a valuable insight into the temporal relations between changes in these parameters.

Further work is needed on the uptake of kynurenine into brain, including a description of the kinetics. This could most easily be performed *in vitro* using radiolabelled L-kynurenine.

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