

THE REGULATION BY SEX HORMONES OF TRYPTOPHAN OXYGENASE AND
TWO OTHER AMINO-ACID-METABOLISING ENZYMES IN RAT LIVER.

A THESIS SUBMITTED TO THE UNIVERSITY OF LONDON FOR THE DEGREE
OF DOCTOR OF PHILOSOPHY.

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ABSTRACT

Previous work shows enhanced metabolism of tryptophan to nicotinic acid ribonucleotide in both pregnant women and oestrogen-treated humans while androgenic steroid treatment diminishes excretion of urinary tryptophan metabolites. Sex hormones may produce these effects by changes in the activity of liver tryptophan oxygenase, glucocorticoid-inducible enzyme catalysing the rate-limiting step of tryptophan degradation. This possibility and the effects of sex hormones on tyrosine aminotransferase and alanine aminotransferase, two enzymes similar to tryptophan oxygenase, were therefore investigated in rats. Sex hormones may modify enzyme activity through changes in circulating corticosterone levels, so these were also measured.

Methods for measurement of the three enzymes and a protein-binding technique for the assay of corticosterone are described.

The markedly increased activities of all three enzymes in oestrogen-treated females is not due to raised serum corticosterone levels since these remain unchanged, although the presence of circulating corticosterone appears essential, as full increases in enzyme activity are not found in oestrogen-treated adrenalectomised or hypophysectomised rats.

Circulating androgens may inhibit effects of oestrogen administration to males since oestrogen-treated intact males have lower tryptophan oxygenase activity than do similarly treated females and orchidectomised males.

Castrated and androgen-treated intact females have diminished tryptophan oxygenase activity, perhaps due to observed decreases in serum corticosterone levels. Similar treatment of males did not change enzyme activity or serum corticosterone concentrations.

Neither testosterone propionate nor fluoxymesterone treatment modifies hydrocortisone induction of tryptophan oxygenase although the latter decreases tyrosine aminotransferase induction.

Inhibitory effects of various steroid hormones on tryptophan oxygenase were studied in relation to their structural features.

The relevance of these results to effects of pregnancy on amino acid-metabolising enzymes, and to the significance of increased tryptophan oxygenase activity to changes in levels of nicotinic acid ribonucleotide production, are discussed.

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ABBREVIATIONS AND NOMENCLATURE

Throughout this thesis only those abbreviations included in the 'Policy of the Journal and Instructions to Authors,' published by the Biochemical Journal (Biochem. J. 1971, 121, 1), have been used without prior definition. The nomenclature of chemical compounds and substituents, steroids and other compounds of biological importance are also in accordance with these instructions to authors. Steroids are referred to by their recognised trivial name, while the systematic names, as indicated by the Biochemical Journal, are listed in Appendix II.

L-Tryptophan 2,3, dioxygenase (EC 1.13.1.12) is referred to as tryptophan oxygenase, L-tyrosine:2 oxoglutarate aminotransferase (EC 2.6.1.5) as tyrosine aminotransferase and L-alanine:2 oxoglutarate aminotransferase (EC 2.6.1.2) as alanine aminotransferase.

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CHAPTER 1. A Brief Review of the Metabolism of Tryptophan to Nicotinic
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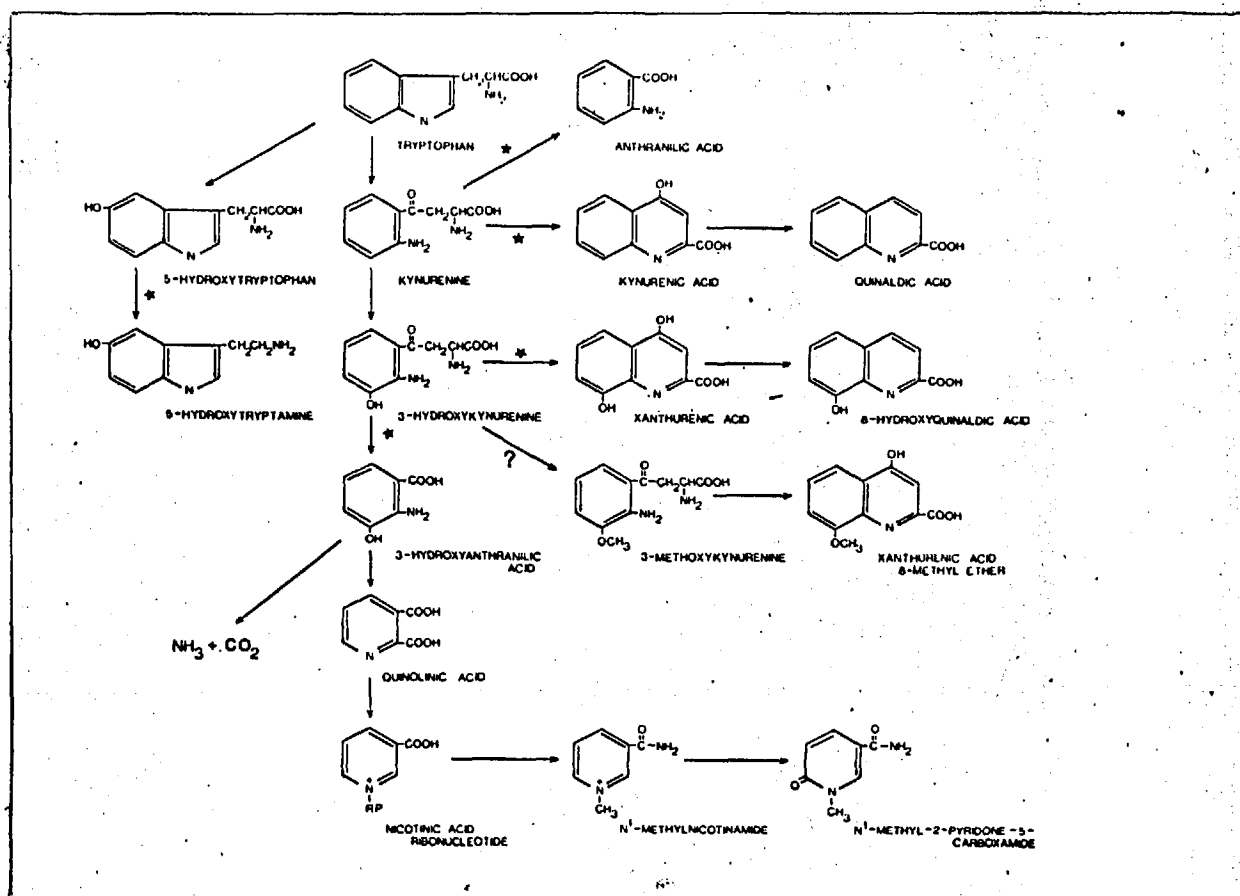
- I. The Tryptophan-Nicotinic Acid Ribonucleotide Pathway.
- II. Factors Affecting the Tryptophan-Nicotinic Acid Ribonucleotide
Pathway.

The Tryptophan-Nicotinic Acid Ribonucleotide Pathway.

Tryptophan is one of the essential amino acids for man and an adequate dietary supply is necessary for normal protein synthesis. There are several pathways available for tryptophan metabolism of which one of the most biologically important is the sequence of reactions culminating in 5-hydroxytryptamine production. Tryptophan is also converted to indoleacetic acid by bacteria in the intestine and this together with other indole derivatives of tryptophan, such as tryptamine, and indolepyruvic acid are excreted in the urine after an oral load of the amino acid.

The pathway by which tryptophan is metabolised to nicotinic acid ribonucleotide is quantitatively the most important, although only a small proportion of the amino acid is utilised along this pathway. Only 1 mg nicotinic acid is obtained from 60 mg L-tryptophan, the remainder is diverted to alternative routes (Horwitt, Harvey, Rothwell, Cutler and Haffron, 1956).

The sequence of reactions which result in nicotinic acid ribonucleotide synthesis are summarised on Fig. 1. The first step consists of rupture of the indole ring of tryptophan and takes place in two stages; tryptophan is initially oxidised to produce formkynurenine and this is followed by rapid hydrolysis to give kynurenine (Knox and Mehler, 1950). The oxidation reaction is catalysed by tryptophan oxygenase, and formamidase is responsible for the second reaction. These stages are discussed in greater detail in Chapter 2.

Fig. 1. The Tryptophan-Nicotinic Acid Ribonucleotide Pathway.

* Reactions requiring Pyridoxal-5 phosphate as cofactor.

Kynurenine is subsequently converted to 3-hydroxykynurenine by an enzyme hydroxylating system present in both liver and kidney, namely L-kynurenine-3-hydroxylase. For this reaction NADPH and molecular oxygen are both required in stoichiometric proportions and oxygen is incorporated into the kynurenine molecule (de Castro, Brown, and Price, 1956; Saito, Hayaishi, Rothberg and Senoh, 1957). An additional requirement for a flavin cofactor was suggested by the observation that hydroxylation was impaired in flavin-deficient rats (Charconnet-Harding, Dagliesh and Neuberger, 1953; Henderson, Koski and D'Angeli, 1955). Okamoto and Hayaishi, (1966) using an outer mitochondrial enzyme fraction treated with ammonium sulphate, have established that FAD acts as a co-enzyme in the reaction.

Alanine is split from the side chain of 3-hydroxykynurenine to yield 3-hydroxyanthranilic acid and it has been generally assumed, on the basis of work by Jakoby and Bonner, (1953a, b) that the enzyme involved in this reaction, kynureninase, is also responsible for formation of anthranilic acid from kynurenine. Pyridoxal 5-phosphate is required as a coenzyme in both reactions (Mason and Berg, 1952).

Evidence that 3-hydroxyanthranilic acid is an intermediate in the conversion of tryptophan to nicotinic acid was provided by the early work of Mitchell, Nyc and Owen, (1948) who found that 3-hydroxyanthranilic acid could replace tryptophan in the diet of rats. At the same time, Albert, Scheer and Daniel (1948) reported increased urinary excretion of N'-methylnicotinamide after administration of 3-hydroxyanthranilic acid to rats. Hanks and Urivetsky (1954) subsequently confirmed these observations by injecting C¹⁴-labelled 3-hydroxyanthranilic acid into rats and isolating labelled N'-methylnicotinamide in urine.

Nicotinic acid is formed from 3-hydroxyanthranilic acid via quinolinic acid (Fig. 1). The benzene ring of 3-hydroxyanthranilic acid is cleaved enzymatically between the carbons 3 and 4 to form the unstable product α -amino- β carboxymuconic- ϵ -semialdehyde (Long, Hill, Weinstock and Henderson, 1954; Mehler, 1956). This unstable intermediate may either recyclise spontaneously to produce quinolinic acid (Miyake, Bokman and Schweigert, 1954) or, by another reaction, catalysed by picolinic carboxylase, enter a completely degradative pathway which terminates in the formation of carbon dioxide via glutaryl CoA (Nishizuka, Ichiyama, Gholson and Hayaishi, 1965; Ichiyama, Nakamura, Kawai, Honjo, Nishizuka, Hayaishi and Senoh, 1965). This alternative route for the metabolism of tryptophan provides a possible control point for the production of nicotinic acid, since high picolinic carboxylase activity may divert the 3-hydroxyanthranilic acid into the degradative pathway producing carbon dioxide, rather than the formation of nicotinic acid ribonucleotide. Ikeda, Tsuji, Nakamura, Ichiyama, Nishizuka and Hayaishi, (1965) showed that the inability of the cat to utilize tryptophan in place of nicotinic acid is explained by its high activity of liver picolinic carboxylase.

Quinolinic acid was found to be an intermediate product in the formation of nicotinic acid ribonucleotide by Hankes and Segal, (1957) who demonstrated that a proportion of quinolinic acid labelled with tritium in the benzene ring was excreted in the urine as N'-methylnicotinamide. The position of quinolinic acid as an intermediate on the pathway of nicotinic acid ribonucleotide synthesis was confirmed by Nishizuka and Hayaishi, (1963) using C^{14} -labelled 3-hydroxyanthranilic acid. In addition, they established that nicotinic acid itself is not involved in this sequence of reactions,

which proceeds via quinolinic acid ribonucleotide. Earlier experiments of Feigelson, Williams and Elvehjem, (1951) had indicated this possibility by showing that rats fed a tryptophan-free diet since weaning had low liver NAD levels, which were restored to normal by supplementation with tryptophan, but not affected by administration of nicotinic acid.

Side-Reactions of the Tryptophan-Nicotinic Acid Ribonucleotide Pathway.

There are several side-reactions which provide alternative metabolic fates for the various intermediates along the tryptophan-nicotinic acid ribonucleotide pathway.

Kynurenine Metabolism

Kynurenine is split by hydrolysis to give anthranilic acid and alanine, a similar reaction to that already described for 3-hydroxykynurenine. Kynurenine also undergoes further transamination to produce kynurenic acid.

3-Hydroxykynurenine Metabolism

Xanthurenic acid is formed as the transamination product of 3-hydroxykynurenine and the reaction depends upon the presence of pyridoxal 5-phosphate as a cofactor (Wiss, 1953). The 3-hydroxykynurenine aminotransferase responsible for this reaction is located both in liver and in kidney cells. The urinary level of xanthurenic acid is high after administration of tryptophan to pyridoxine-deficient animals (Lepkovsky, Robez and Haagen-Smit, 1943) and xanthurenic acid levels are often used as an index of vitamin B₆ deficiency.

3-Hydroxyanthranilic Acid Metabolism

Picolinic acid is formed from 3-hydroxyanthranilic acid, via the same intermediate as in the formation of quinolinic acid, namely α -amino- β -carboxymuconic ϵ -semialdehyde (Mehler, 1956). The production of picolinic acid is catalysed by

the enzyme picolinic carboxylase. The activity of this enzyme is increased in alloxan-diabetic rats, which also excrete less N'-methylnicotinamide in the urine after a tryptophan load than do normal rats (McDaniel, Hundley and Sebrell, 1956).

Administration of insulin reverses this change (Mehler, McDaniel and Hundley, 1958) and these observations together with the investigation into the tryptophan metabolism of the cat, which cannot replace dietary nicotinic acid with tryptophan due to high picolinic carboxylase activity, indicate that the reaction catalysed by this enzyme may prove to be another control point in nicotinic acid ribonucleotide synthesis. Further work by Mehler, Yano and May, (1964) and Ikeda, Tsuji, Nakamura, Ichiyama, Nishizuka and Hayaishi, (1965) have provided additional evidence in support of this conclusion. Both groups of workers found that increased picolinic carboxylase activity in alloxan-diabetic rats is accompanied by diminished NAD synthesis from tryptophan.

The degradation of 3-hydroxyanthranilic acid to carbon dioxide has been studied in detail by Nishizuka, Ichiyama, Gholson and Hayaishi, (1965). They found that the degradation occurs by a diversion from the main tryptophan-nicotinic acid ribonucleotide pathway at the formation of α -amino- β carboxymuconic- ϵ -semialdehyde and that α -oxoadipic acid and glutaryl CoA are both formed prior to carbon dioxide. Picolinic acid is not an intermediate on this pathway although it is formed from α -amino- β -carboxymuconic- ϵ -semialdehyde by spontaneous closure of the benzene ring. The unstable aldehyde is also degraded further via glutaryl CoA to produce carbon dioxide.

Nicotinic Acid

N'-methylnicotinamide and N'-methyl-2-pyridone-5-carboxamide (2-pyridone) are the two principle urinary metabolites of nicotinamide in man (Ellinger and Abdel

Kader, 1949; Knox and Grossman, 1947). Administration of nicotinic acid to human subjects results in the major part of the compound being excreted as 2-pyridone, which is synthesised via amidation, methylation and oxidation (Walters, Brown, Kaihara and Price, 1955). Although most of an oral dose of L-tryptophan is degraded to carbon dioxide via the "glutarate" pathway, as discussed previously in this Chapter, there is also a marked increase in excretion of 2-pyridone after tryptophan administration which is often determined as representative of the end product of the tryptophan-nicotinic acid pathway (Price, 1958).

Factors affecting the Tryptophan-Nicotinic Acid Ribonucleotide Pathway.

1. Vitamin B₆ Deficiency.

Pyridoxal 5-phosphate is required as a coenzyme for several of the reactions on the tryptophan-nicotinic acid ribonucleotide pathway and the production of 3-hydroxyanthranilic acid, kynurenine acid and xanthurenic acid is dependent on pyridoxal 5-phosphate as a coenzyme, as described earlier in this chapter.

The levels of xanthurenic acid excreted in the urine has been regarded as an index of vitamin B₆ deficiency in man, although several workers rely on the ratio of 3-hydroxykynurenine excretion to that of 3-hydroxyanthranilic acid (O'Brien and Jensen, 1963; Heeley, 1965).

The pattern of the urinary excretion of tryptophan metabolites during dietary vitamin B₆ deficiency has been investigated in detail by Yess, Price, Brown, Swan and Linkswiler, (1964). Without administration of a tryptophan load, kynurenic acid levels were lower and kynurenine levels higher than they were before depletion of the vitamin. After tryptophan loading, kynurenine, 3-hydroxykynurenine, kynurenic, xanthurenic and anthranilic acids were all excreted in larger quantities due to vitamin B₆ deficiency. When the effects of the vitamin depletion on tryptophan metabolism were maximal, 3-hydroxykynurenine was excreted in the largest quantity followed by kynurenine and xanthurenic acid, although in all cases, kynurenine, 3-hydroxykynurenine and xanthurenic acid levels were raised. From these results, it may be concluded that a dietary deficiency of vitamin B₆ affected the activity of kynureninase, which converts kynurenine to anthranilic acid and hydroxykynurenine to hydroxyanthranilic acid, more than the kynurenine aminotransferase which catalyses the conversion of 3-hydroxykynurenine to xanthurenic acid. Thus, 3-hydroxykynurenine may accumulate, although it is still metabolised to xanthurenic acid.

Diminished N'-methylnicotinamide excretion, after a tryptophan load in vitamin B₆ deficient rats (Schweigert and Pearson, 1947; Rosen, Huff and Pearlzweig, 1947) and reduced levels of hepatic pyridine nucleotides (Kring, Ebisuzaki, Williams and Elvehjem, 1952) are both consistent with the proposal that depletion of the coenzyme results in a metabolic block on the pathway at the reaction converting hydroxykynurenine to hydroxyanthranilic acid. The work of Yess et al, described previously, indicates that the enzyme catalysing this step is especially sensitive to vitamin B₆ deficiency. In contrast to the results of Schweigert and Pearson, (1947) and Rosen and co-workers (1947), Brown, Yess, Price, Linkswiler, Swan and Hankes, (1965) did not observe any alteration in the levels of nicotinic acid, 2-pyridone or N'-methylnicotinamide, after administration of a tryptophan load to human subjects on a low vitamin B₆ diet. Quinolinic acid levels were raised in these subjects. It is possible that the high protein-low pyridoxine dietary regime used by Brown et al in their study may not have resulted in a sufficiently severe vitamin deficiency to affect nicotinic acid metabolism. Kring and co-workers (1952) employed a combination of vitamin B₆ deficient diet and desoxypyridoxine, a vitamin B₆ antagonist, in order to obtain diminished liver pyridine nucleotide synthesis.

The Administration of Adrenocorticoids

Tryptophan oxygenase, which catalyses the first step on the tryptophan-nicotinamide pathway, is inducible by glucocorticoids. The mechanism and significance of this will be considered in detail in Chapter 2. Studies with

hospital patients have shown that intramuscular injection of hydrocortisone, followed by a 5g tryptophan load results in both increased liver tryptophan oxygenase activity and urinary excretion of kynurenine (Altmann and Greengard, 1966). It is possible that increased tryptophan oxygenase levels may result in the rapid production of large quantities of kynurenine from the tryptophan in the oral load, which may exceed the capacity for metabolism by the other enzymes of the pathway. Raised levels of urinary kynurenine would result from such a situation, and it has been suggested that the amount of this metabolite excreted in the urine is an index of tryptophan oxygenase activity (Curzon and Bridges, 1969). In the rat, treatment with cortisol produced increases in kynurenine, 3-hydroxy-kynurenine and o-aminohippuric acid (derived from anthranilic acid) but xanthurenic acid and N'-methylnicotinamide remained unchanged (Tenconi and Pasquariello, 1967).

Further studies with human subjects treated with hydrocortisone, given as an intramuscular injection, followed by a 5g tryptophan load, resulted in elevated levels of 3-hydroxykynurenine, xanthurenic acid, and 3-hydroxyanthranilic acid, together with kynurenine, although 2-pyridone levels remained unchanged. If pyridoxine was given before administration of the tryptophan load, these increases in the levels of urinary metabolites were prevented (Rose and McGinty, 1968). It may be that the increased kynurenine produced by cortisol induction of tryptophan oxygenase results in a "relative" deficiency of pyridoxal 5-phosphate, so that there is insufficient coenzyme available to metabolise the excess substrate. The pattern of metabolites produced by glucocorticoid administration is similar to that observed by Brown and co-workers in subjects given a diet low in pyridoxine, discussed previously in this Chapter and this seems to support such a conclusion.

The diminished level of 3-hydroxyanthranilic acid observed by Rose and McGinty (1968) after pyridoxine administration to cortisol-treated subjects indicates the possibility that the metabolism of tryptophan was facilitated to a stage beyond the formation of this metabolite by pyridoxine treatment. These authors, however, observed that 2-pyridone was unaffected by this treatment, and Wolf (1969) has shown reduced urinary excretion of both 2-pyridone and N'-methylnicotinamide after cortisol treatment and a 2g dose of L-tryptophan. One possibility is that the increased amount of tryptophan entering the pathway, after administration of the tryptophan load, is diverted away from nicotinic acid ribonucleotide production under the influence of picolinic carboxylase into the degradative pathway of carbon dioxide formation via glutaryl CoA.

Oestrogens

Adult females excrete larger amounts of kynurenine, 3-hydroxykynurenine, xanthurenic, kynurenic and 3-hydroxyanthranilic acids than adult males after a loading dose of 100 mg L-tryptophan/Kg body weight (Michael, Drummond, Doeden, Anderson and Good, 1964). Rose, (1967) has also demonstrated a sex difference in tryptophan metabolism with a fixed 5g dose of L-tryptophan, which was not evident with the smaller dose of 2g L-tryptophan. In the same study Rose investigated the hormonal basis for this sex difference by studying tryptophan metabolites in groups of young women, post menopausal women and men. In the first groups, 3-hydroxykynurenine, xanthurenic acid and 3-hydroxyanthranilic acid levels were all elevated, while with the older women and men the levels were lower and there was no significant difference between the two groups. These observations imply that it is the hormonal activity during the reproductive years that are responsible for the increased levels of metabolites in the younger women.

Women studied throughout the menstrual cycle secreted higher levels of 3-hydroxykynurenine after ovulation than immediately after menstruation (Brown Thornton and Price, 1961). In this study a 2g load was used, but with a 5g load Rose, (1967) demonstrated increased excretion of xanthurenic and 3-hydroxy-authranilic acids, in addition to 3-hydroxykynurenine, after ovulation. It is suggested that the increased oestrogen secretion at ovulation (Billings, Billings, Brown and Burger, 1972) and the higher oestrogen activity during the reproductive years are responsible for these high levels of metabolites.

Sprince, Lowy, Folsome and Behrson, (1957) reported increased xanthurenic acid secretion during pregnancy in the human, and these levels were returned to normal following treatment with pyridoxine. In addition, increased kynurenine, 3-hydroxykynurenine, and kynurenic acid observed in the pregnant women were all reduced by administration of vitamin B₆ (Wachstein and Lobell, 1954). These changes in tryptophan metabolism were considered due to an increased foetal requirement for vitamin B₆ resulting in a vitamin B deficiency in the mother. Support for this proposal was obtained by the demonstration of diminished plasma pyridoxal 5-phosphate levels in maternal blood at term (Wachstein, Moore and Graffeo, 1957). The extensive studies of Brown, Thornton and Price, (1961) of tryptophan metabolism during pregnancy provided an indication that the abnormal metabolite excretion pattern observed during pregnancy was not simply a result of vitamin B₆ deficiency. These workers showed that with no tryptophan load, the levels of kynurenine, 3-hydroxykynurenine and xanthurenic acid in the urine were all elevated in the pregnant women. After administration of a 2g load of L-tryptophan, large quantities of

acetyl kynurenine and 2-pyridone were excreted in addition to the metabolites measured previously. Hernandez, (1964) found that the levels of 3-hydroxy-anthranilic acid and N'-methylnicotinamide were increased in pregnancy after tryptophan loading. Although the increased levels of urinary kynurenine, 3-hydroxy-kynurenine and xanthurenic acid in pregnancy, observed by Brown et al may be due to vitamin B₆ deficiency, the raised excretion of nicotinic acid metabolites reported by these workers and by Hernandez (1964) suggest that the hormones of pregnancy exert some effects on the pathway so that there is an enhanced metabolism of tryptophan to nicotinic acid ribonucleotide.

A similar pattern to that shown for the pregnant women has also been described for human subjects treated with oestrogens. Rose (1966a) and Price, Thornton and Mueller, (1967) found increased excretion of xanthurenic acid after a 5g or a 2g load of L-tryptophan in women taking oral contraceptives containing oestrogen. As part of the same study, Rose showed that male subjects treated with oestrogens alone have an identical pattern of tryptophan metabolites to that described for women taking the contraceptive pill.

Although the excretion of 2-pyridone is increased in pregnancy (Brown et al, 1961) it is normal in women taking oestrogen-containing contraceptive preparations. However, the excretion of N'-methylnicotinamide is raised in these women (Rose, Brown and Price, 1968) as it is in oestrogen-treated male subjects (Wolf, 1969). Administration of pyridoxine did not affect the excretion of nicotinic acid derivatives after oestrogen treatment, although the levels of the other metabolites were returned to normal (Price et al, 1967).

The level of tryptophan metabolites in women taking the progestational contraceptive, megestrol acetate, was not altered from the control group (Rose and Braidman, 1971). This suggests that the progestational components of the contraceptive steroids may not affect tryptophan metabolism, although norethynodrel may be an exception to this, since it is partially metabolised to oestrogenic compounds.

The effects of both pregnancy and oral contraceptive steroids on tryptophan metabolism, outlined in this Chapter appear, therefore, to be a consequence of increased oestrogenic activity. Rose suggested that the abnormalities of tryptophan metabolism were a consequence of the accumulation of oestrogen conjugates. The sulphate esters of these hormones, may inhibit the activity of the pyridoxal 5-phosphate-dependent enzymes on the pathway, by competition with the cofactor for binding sites on the apoenzyme. This explanation accounts for the reversal of the changes in excretion of metabolites by administration of pyridoxine to oestrogen-treated or pregnant women. Mason and Gullekson, (1960) reported that rat kidney kynurenine was competitively inhibited in vitro for cofactor by oestrogen conjugates. Kidney kynurenine aminotransferase activity is higher in males than in females and ovariectomy produced increases in this enzyme (Mason, Ford and Wu, 1969). Although oestrogen administration to rats produced diminished enzyme activity in the kidney, the liver enzyme was not susceptible to this treatment (Mason et al, 1969; Rose and Brown, 1969a).

The activity of kynureninase in the livers of male rats is higher than in females (Mason and Gullekson, 1960). This has been confirmed recently by Rose and Brown (1969a) who showed further that liver kynureninase was decreased in oestrogen-treated male rats, but not in similarly treated female rats.

An alternative explanation was put forward by Rose (1966b) to account for the effects of pregnancy and oestrogen treatment on tryptophan metabolism. He proposed that oestrogens increase the activity of some enzymes on the pathway so that when the amount of tryptophan available for metabolism is increased, after the administration of a tryptophan load, there is insufficient vitamin B₆ available to fulfil the enhanced requirement for cofactors. A state of "relative" vitamin B₆ deficiency would thus arise. The effects of oestrogens on rat liver tryptophan oxygenase, which catalyses the first step on the degradative pathway are reported in Chapters 5 and 7.

The Effect of Androgens

There is little published information on the effects of androgens on tryptophan metabolism. McGinty and Rose (1969) have shown that mesterolone administration to human subjects resulted in diminished levels of 3-hydroxykynurenine, 3-hydroxy-anthranilic acid and kynurenine in urine after a tryptophan load. Methyltestosterone had a stimulatory effect on the excretion of these metabolites and these authors suggest that the presence of a Δ^4 -3 oxo group in this steroid derivative enables metabolism to oestrogens to take place resulting in the increased levels of metabolites. Mesterolone does not possess this group so that aromatization of ring A does not take place. Administration of both steroids impaired the response of the metabolites to cortisol treatment. These authors suggested that full induction of tryptophan oxygenase may have been prevented by treatment with the androgenic steroids. This possibility has been investigated for rat liver tryptophan oxygenase in the work presented in Chapter 6.

CHAPTER 2. The Regulation of the Activity of Amino Acid-Metabolising
Enzymes.

- I. **Control of Tryptophan Oxygenase and Tyrosine Aminotransferase Activities.**
- II. **Control of the Activities of Alanine Aminotransferase and Other Enzymes of Amino Acid Metabolism.**

I. Control of Tryptophan Oxygenase and Tyrosine Aminotransferase Activities

Tryptophan oxygenase (TO) and tyrosine aminotransferase (TAT) both catalyse the first step on their respective pathways of amino acid metabolism.

The first reaction along the tryptophan-nicotinic acid ribonucleotide pathway results in the formation of formylkynurenine, produced by the rupture of the pyrrole ring of tryptophan, and is catalysed by TO (Hayaishi, Rothberg, Mehler and Saito, 1957).

This reaction requires atmospheric oxygen. Formylkynurenine is hydrolysed further by formamidase to produce kynurenine and because this reaction is so rapid and the formamidase is present in excess, the determination of kynurenine is normally used to measure TO activity in crude enzyme preparations.

These two reactions were initially investigated by Knox and Mehler, (1950) and Mehler and Knox (1950). Knox (1952) first showed that TO is an iron porphyrin enzyme. Formation of kynurenine is enhanced by the incorporation of a hydrogen peroxide-generating system, such as glucose oxidase or ascorbic acid, into the assay medium, and it was suggested that this was required to produce the reduced ferroprotein form of the enzyme, which was considered the most active (Tanaka and Knox 1959). Further studies by Feigelson and Greengard (1961) and Greengard and Feigelson (1961) demonstrated the presence of a haem cofactor in the liver cell microsomes. The apotryptophan oxygenase is localised in the cell sap and the haem cofactor, protohaem IX, binds loosely to the apoprotein to form the holoenzyme. Both haem and ascorbic acid are included in assay systems to ensure maximal activation of the TO present in any given liver sample (Chapter 4). Knox and Ogota (1965) and Knox and Piras (1966) have described three forms of the enzyme, namely the apoprotein, the ferri-holoenzyme, both of which are inactive, and the active ferro-holoenzyme. The haem cofactor associates with the apoprotein to form the inactive ferriprotein, which is reduced by ascorbic acid to form the active ferroprotein. The transition

from the ferri-holoenzyme to the ferroprotein is regarded as responsible for the lag in the rate of kynurenine production during the initial stages of incubation, described by Feigelson and Greengard (1961). Adenosine-3'-5'-monophosphate has been shown to promote the formation of the ferroprotein in vitro (Knox, Piras and Tokuyuma, 1966) and Chytil (1968) has found that this takes place via the formation of hypoxanthine, which is catalysed by xanthine oxidase. More recently, Julian and Chytil (1970) have shown that xanthine oxidase may promote the stabilisation of the active ferroprotein in vivo.

TAT catalyses the first step in the degradation of tyrosine to acetoacetate (Knox and LeMay-Knox, 1951). Cammarata and Cohen (1950) identified the product of the reaction as p-hydroxyphenylpyruvic acid, formed after the deamination of tyrosine. The reaction requires pyridoxal 5-phosphate as a cofactor (Cammelakis and Cohen, 1956) and the enzyme is found both in the cell sap and mitochondrial fractions of the liver (Chapter 4).

TO and TAT activities are both responsive to glucocorticoid administration and they have been widely studied as examples of control of mammalian enzyme activity. Increases in activity occur after treatment of intact rats with hydrocortisone, tyrosine and tryptophan. Early studies of Knox (1951) showed that TO activity is elevated by injection of general stressing agents, such as phenylalanine, tyrosine or histidine to intact rats. Tryptophan administration also increases TO activity in intact animals, but whereas the effects of the stressing agents are abolished after adrenalectomy, tryptophan is still effective on removal of the adrenal glands. Treatment of intact and adrenalectomised animals with cortisone results in enhanced TO activity (Knox and Auerbach, 1955) caused

by increased de novo synthesis of the enzyme (Civen and Knox, 1959). Early indication that glucocorticoids and tryptophan increased TO activity through different mechanisms was provided by the observation of Civen and Knox (1957), that the injection of tryptophan augmented the response of TO activity, observed after cortisol injection.

TAT was shown to be increased after treatment of whole animals with glucocorticoid, tryptophan and tyrosine (Lin and Knox, 1957) and it was thought that TAT responded in a similar fashion to TO. Kenney and Flora (1961) found this not to be the case, since adrenalectomy abolished the effects of all amino acids on TAT, and the increases in activity observed after treatment of whole animals were regarded as results of stressing effects. Therefore, only TO is increased by both substrate and glucocorticoids.

Both enzymes respond comparatively rapidly to glucocorticoid treatment and reach a maximal response 4 h to 5 h after administration of a single dose of hydrocortisone (Knox and Auerbach, 1955; Feigelson, Feigelson and Greengard, 1962; Nemeth and De La Haba, 1959). Incorporation of labelled amino acids into immunochemically reactive protein has shown the half-life of both enzymes in the liver cells to be between $1\frac{1}{2}$ h to $2\frac{1}{2}$ h (Nemeth, 1962; Schimke, Sweeney and Berlin, 1965; Boctor and Grossman, 1967).

In the early stages of the investigation into the mechanism for increased TO and TAT activity following administration of glucocorticoids, it was thought that this was similar to the situation in micro-organisms, where induction of various proteins is caused by increases in RNA synthesis, resulting in enhanced protein synthesis (Brenner, Jacob and Meselson, 1961). Both the effects of hydrocortisone on TO and

TAT and of tryptophan on TO were prevented by injection of rats with puromycin, an antibiotic which prevents incorporation of amino acids into protein (Feigelson, Gross and Feigelson, 1962). Similar results were obtained using a liver perfusion system (Goldstein, Stella and Knox, 1962) and it was concluded in both studies that protein synthesis is required for glucocorticoids and tryptophan to be effective in increasing TO activity. A sharp distinction between the action of glucocorticoids and tryptophan was made by Schimke, Sweeney and Berlin (1964, 1965a, 1965b) who showed that after treatment with glucocorticoids, the incorporation of labelled amino acids into highly purified TO is increased, indicating raised de novo enzyme synthesis. In contrast, tryptophan administration results in a diminished rate of enzyme degradation from which, it was concluded that the effect of the substrate is protective against loss of activity. Grøengard, Mendelsohn and Acs (1965) showed further that tryptophan administration increases the degree of association between apoenzyme and the microsomal cofactor, while hydrocortisone injections lead to increased apoenzyme synthesis. Work with another antibiotic, actinomycin D was thought to support the hypothesis that glucocorticoids increased TO and TAT activity by increasing transcription of the gene. Actinomycin D, prevents incorporation of purine bases into DNA, and treatment of rats with actinomycin D given at the same time as the glucocorticoid was found to abolish the increases in enzyme activity, while the results of tryptophan treatment were not affected (Greengard and Acs, 1962; Greengard, Smith and Acs, 1963). Hydrocortisone treatment of rats was shown to increase synthesis of nuclear RNA prior to the observation of increased TO and TAT activity, and this was considered further evidence in support

of a transcription site for the action of glucocorticoids (Feigelson, Gross and Feigelson, 1962; Feigelson, Feigelson and Greengard, 1963; Feigelson and Feigelson, 1963; Kenney and Kull, 1963).

A different approach to the problem of elucidating the mechanism of cortisol induction of the enzymes has been provided by the work of Tomkins and his colleagues. Garren, Howell, Tomkins and Crocco (1964) showed that although actinomycin D abolished increases in TO and TAT activity when given simultaneously with the glucocorticoid, if the antibiotic was given 1 h to 2 h after the cortisol injection the TO and TAT activities were elevated above the normal induction levels and remained so for an extended period of time, after which the enzyme level fell. This response was termed "superinduction" and Garren et al postulated the involvement of two factors in enzyme induction, namely an enhanced rate of enzyme synthesis and a diminished rate of destruction of the enzyme template. They considered that when actinomycin D was given together with the glucocorticoid it prevented increased enzyme synthesis, but when it was given after cortisol administration, it prevented synthesis of the proteins which degrade mRNA which codes for TO and TAT, resulting in the superinduction. These results have also been shown for TAT in rat hepatoma culture cells (Peterkovsky and Tomkins, 1967) and work with synchronised cells in culture indicates that TAT is only available for induction by glucocorticoids at certain times of the cell cycle (Martin and Tomkins, 1970).

The work of Tomkins and his colleagues have been questioned by Rosen and Milholland (1968) and by Mishkin and Shore (1967) who have both been unable to repeat the "superinduction" experiment of Garren and co-workers (1964). Kenney has also investigated the effects of glucocorticoids and dexamethasone on Reuben-strain hepatoma cells in culture cells. Reel and Kenney (1968) showed that the "superinduction" effects produced by cortisol treatment followed by actinomycin D

addition to the cell medium (Peterkovsky and Tomkins, 1967) was an artifactual result of the dose of actinomycin D used, and that high concentrations of the antibiotic inhibit degradation of the enzyme protein. Further experiments confirmed this observation and showed that after cortisol induction, high concentrations of actinomycin D block both synthesis and degradation of TAT and that low concentrations only block TAT synthesis, (Lee, Reel and Kenney, 1970). As part of the same study, the kinetics of the rate of incorporation of ^{14}C -labelled amino acids into TAT after cortisol administration were investigated, and it was shown that hydrocortisone acted solely at the transcriptional stage of TAT synthesis, and that no post-transcriptional factors were involved, as suggested by Martin and Tomkins (1970).

Interpretation of the results from experiments involving the effect of actinomycin D on enzyme induction is difficult since there is evidence for the direct effect of the antibiotic on TAT activity and administration of low doses of the drug to adrenalectomised rats result in elevated TAT activity (Rosen, Raina, Milholland and Nichol, 1964). In addition, the use of actinomycin D in experiments designed to elucidate the nature of enzyme induction has been criticised by Harris (1968), who emphasised that actinomycin D is a highly toxic agent and not all its effects can be ascribed to prevention of m RNA synthesis. Thus, Staehelin, Wettstein and Noll (1963) reported considerable disaggregation of rat liver polysomes, resulting from actinomycin D treatment, and Philips Schwartz, Sternberg and Tan (1960), using doses of the antibiotic similar to those used in the studies mentioned previously, observed a large number of deaths within the first 24 h. Results from experiments involving the use of actinomycin D must therefore be interpreted with care, although use of the antibiotic is sometimes unavoidable since no other agent prevents transcription of DNA to m RNA by interference at the genome.

Use of another inhibitor of RNA synthesis, 8-azaguanine which is less toxic and more specific in action than actinomycin D has supported the work of Kenney et al, but not that of Tomkins and his colleagues. Experiments with adrenalectomised animals and liver perfusion systems showed that the induced TO and TAT levels following treatment with hydrocortisone were increased further when 8-azaguanine was given with the glucocorticoid and were maintained for 18 h afterwards. When 8-azaguanine was administered 4 h after cortisol injection, the induced levels of TO and TAT were maintained for extended periods of time (Levitan and Webb, 1969a, 1969b). The "superinduction" observed by Geller et al (1964) with actinomycin D treatment was therefore separated from the prolonged maintenance of induced TO and TAT levels. Levitan and Webb (1969a) also found that treatment with bases containing primary amine groups such as 8-azaguanine and 5-cytosadenine caused enhancement of cortisol-induced TAT levels, whereas administration of 5-fluorouracil and 8-azaxanthine which do not possess these groups only resulted in an extension of the period of maximal induction. It was concluded that induction of activity may be associated with an increase in the free hepatic amino acid pool, resulting from deamination of those bases with primary amine groups, but that the prolonged period of maximal induction was due to incorporation of purine and pyrimidine bases into RNA. Further experiments by Levitan and Webb (1970a, 1970b) showed that hydrocortisone induced both TO and TAT by utilisation of a pre-existing pool of m RNA. Glucocorticoid administration was therefore shown to increase enzyme levels solely by increased synthesis and not by a diminished rate of template degradation.

At the present time, it is difficult to differentiate between the proposals of Kenney and of Tomkins with regard to the mechanism of cortisol induction and insufficient evidence is available to ascertain whether the transcriptional or the post transcriptional stage is involved in the mechanism. However, from the work of Levitan and Webb, discussed in the preceeding paragraph, it appears that the hypothesis proposed by Tomkins et al, that induction of enzyme synthesis results from both an increased rate of template synthesis and a diminished rate of its degradation, has not been verified by the use of agents inhibitory to m RNA synthesis other than actinomycin D. The effect of glucocorticoid administration therefore seems to be the result increased de novo enzyme template synthesis. However, more evidence must be assembled before this is ascertained.

An association between TO induction by cortisol in the mouse, with an increased binding of polysomes to the endoplasmic reticulum of the liver cells has been established by Cox and Mathias (1969). A similar pattern was found by Fishman, Wurtman and Munro (1969) to occur following increased dietary intake of protein which is associated with raised TAT activity and enhanced aggregation of polysomes. Rosen and Milholland (1968) have previously demonstrated that dietary intake of amino acids in the form of casein hydrolysate elevates TAT but not TO activity in both intact and adrenalectomised rats. A combination of small doses of hydrocortisone with casein hydrolysate is more effective in raising both TO and TAT activities than hydrocortisone given alone. Similar results were obtained by Mavrides and Lane (1970) who found that doses of hydrocortisone too small to increase TAT activity, potentiate the effect of casein hydrolysate administration. It is therefore possible that there is some association between the effects of hydrocortisone on TO and TAT and increased pool size of amino acids in liver cells. However, the observations of

Rosen and Milholland (1968) that casein hydolysate had no effect on TO is surprising in view of the work of Labrie and Korner (1968a, b) who found that treatment of intact and adrenalectomised rats, with a mixture of the essential amino acids or tryptophan alone, given orally or by injection, increased the activities of both TO and TAT and that the increases were sensitive to administration of actinomycin D. These results contrast with those described earlier in this Chapter, when high doses of tryptophan produced increases in TO activity which were resistant to administration of actinomycin D. Labrie and Korner concluded that tryptophan has a dose-dependent affect on TO activity, and that at low doses, administration of the amino acid results in increased enzyme synthesis, while at higher doses, it produces stabilisation of the holoenzyme. They also suggested that cortisol induction of both TO and TAT may occur as a result of increased hepatic amino acid pool size. Yatvin and Pitot (1970) have criticised the work of Labrie and Korner on the grounds that actinomycin D blocked the effects of low doses tryptophan by causing a lack of tryptophan in the hepatic cells. At the dose of tryptophan used by Yatvin and Pitot, namely 20 mg/100g body weight, the increases in TO activity were resistant to actinomycin D. However, Labrie and Korner used 100 µg tryptophan/100g body weight to produce increases in TO sensitive to actinomycin D treatment, so that the effects observed by Yatvin and Pitot may still be due to stabilisation of the holoenzyme.

The possible involvement of intracellular amino acids in the mechanism of cortisol induction of TO and TAT has lead Mavrides and Lane (1969) to postulate a connection between the activity of these enzymes and gluconeogenesis in liver cells.

A similar association was suggested previously by Rosen, Roberts, Budnick and Nichol (1958) as a result of investigations into the effects of alanine aminotransferase and aspartate aminotransferase activities on pyruvate metabolism, and also by Greengard, Weber and Singhal (1963) who observed that liver glycogen deposition increased concomittantly with cortisol induction of TAT. Increased dietary intake of protein (Mavrides and Lane 1969; Szepesi and Freedland, 1967, 1968) and starvation (Mavrides and Lane 1969), both conditions associated with increased gluconeogenesis, result in elevated TAT activity, while increased carbohydrate intake represses activity of the enzyme. Mavrides and Lane also suggested that increased TAT activity under gluconeogenic conditions may result in an increased level of substrate for the Embden-Myerhof pathway. It is possible that this may be due to an enhanced rate of degradation of tyrosine to fumarate and acetoacetate.

The association between TAT activity and carbohydrate metabolism is further supported by the effect of pancreatic hormones on the enzyme. However, TO does not appear to be effected in the same way and may not play a similar role to TAT in cell metabolism. Holton and Kenney (1967) found that insulin administration to both intact and adrenalectomised rats elevates TAT activity but not TO. Similar results for glucagon administration were reported by Greengard and Baker (1966) and confirmed using a liver perfusions system, by Knox and Sharma (1968); Reshef and Greengard (1968) and Staib, Thienhaus, Ammedick and Staib (1969). Reshef and Greengard (1968) also showed that a combination of hydrocortisone and glucagon is more effective in increasing TAT activity than hydrocortisone given alone. Labrie and Korner (1969) observed that insulin or glucagon are effective in raising TAT activity in hypophysectomised rats, but that TO is not effected.

Several groups of workers have used in vitro cell culture systems to investigate the mechanism of the effects of pancreatic hormones. Reel, Lee and Kenney (1970) showed that although hydrocortisone induction of TAT reaches maximal activity 6 h after addition of dexamethasone to the cell medium, the effects of insulin on TAT reach maximal levels 30 mins after addition of the hormone to the cell culture. Simultaneous addition of hydrocortisone and insulin to the cell medium produce synergistic effects on TAT activity. In the same study the effects of both hormones on the incorporation of labelled amino acids into TAT were investigated, and insulin was found to diminish enzyme degradation, in contrast to the increase in TAT synthesis produced by hydrocortisone. Similar observations have been reported by Gelehrter and Tomkins (1970).

Release of the pancreatic hormones, glucagon and insulin, results in increased tissue concentration of the cyclic nucleotide, adenosine-3'-5' monophosphate (Makman and Sutherland, 1964; Robinson, Exton and Park, 1967). Adrenocorticotrophic hormone increases activity of the adrenal enzymes involved in corticosteroid synthesis via adenosine-3'-5' monophosphate (Grahame-Smith, Butcher and Sutherland, (1967) and a similar mechanism has been studied by Wicks (1969) and Yeung and Oliver (1968) to account for the stimulation of the rat liver enzyme phosphoenolpyruvate carboxy kinase by adrenalin or glucagon administration and in alloxan-diabetic or fasted rats. Both the studies of Reel, Lee and Kenney (1970) and Gelehrter and Tomkins (1970) indicate that the effects of insulin, discussed previously, may be independent of adenosine, 3'-5' monophosphate. Further support for the contention that insulin and adenosine 3'-5' monophosphate act by different mechanisms is provided by the work of Barnet and Wicks (1971) which showed that the addition of both insulin and adenosine, 3'-5' monophosphate to the medium of Reuben strain hepatoma culture cells have an additive effect on TAT activity.

Glucagon may be dependent on adenylyl cyclase, since Greengard (1969) reported both glucagon and adenosine 3' 5'-monophosphate administration to intact rats produce similar increases in TAT. Wicks (1969), using foetal liver in organ culture, showed that the administration of glucagon and adenosine 3'5'-monophosphate together did not produce further increases than if either agent was administered separately. Moreover, Reel et al (1970) found glucagon to be ineffective in their cell culture system which was devoid of adenylyl cyclase.

A possible explanation for the difference in response of TO and TAT to pancreatic hormones may be associated with the number and properties of the isoenzymes which have been found for TO and TAT. Holt and Oliver (1969) have separated three isoenzymes for TAT, using acrilamide gel electrophoresis, one of which was separated after administration of pyridoxine and appeared as a slow-moving band, another intermediate band was found after hydrocortisone administration, and a third, fast-moving component was separated after glucagon or adenosine 3'5'-monophosphate treatment. Pitot and Cho Chung (1970) using a similar technique only demonstrated two such isoenzymes for TO, one synthesised constitutively in adrenalectomised animals and the other was separated after treatment with hydrocortisone. It may be that the pancreatic hormones, and hydrocortisone are each able to increase TAT activity by increasing the synthesis of the appropriate isoenzyme while with TO only hydrocortisone is able to increase enzyme activity since the isoenzymes which respond to the pancreatic hormones may not be present. Such a difference between the two enzymes may account for the ability of TAT to respond to a wide range of stimuli while the response of TO is more restricted.

The effect of hydrocortisone administration on both TO and TAT may be dependent on growth hormone. Thompson and Mikuta (1954) found that hypophysectomy results in enhanced response of TO to cortisol and similar observations were made by Grossman and Mavrides (1967) for TAT. These findings were confirmed by Labrie and Korner (1968b) and Csanyi and Greengard (1968). Both studies showed that growth hormone administration diminishes the response of TO and TAT to cortisol. Labrie and Korner also found that hypophysectomy enhances the effect of amino acids on both enzymes and which is diminished on treatment with growth hormone. A possible explanation, put forward by both groups of workers was that growth hormone is a repressive factor acting at the genome and is able to prevent full induction of TO and TAT by cortisol. Another possibility, which was not considered is that growth hormone, by increasing protein synthesis (Geschwind and Evans, 1949) is able to synthesise such a factor. Labrie and Korner also suggested that since growth hormone raises the level of protein synthesis in the liver, this may decrease the amino acid pool size and, developing their argument that cortisol may induce TO and TAT via action of amino acids, they proposed that this reduction of available amino acids may also prevent full induction by cortisol.

Hypophysectomy and adrenalectomy were found to be ineffective in changing the levels of TO and TAT in immature rats (Labrie and Korner, 1968; Csanyi and Greengard, 1968). However, in mature females adrenalectomy caused substantial decreases in TO activity (Knox, Piras and Tokuyuma, 1966). These results are discussed further in Chapter 5.

Both enzymes are known to vary diurnally and in rats kept under conditions of a 12h light/dark cycle, maximal levels of TO and TAT activity occur between 1500 h and 2400 h (Hardeland and Rensing, 1968; Mavrides and Lane, 1969). TO was also shown to have a circadian rhythm in the mouse, but in this case maximal enzyme

activity is between 1100 h and 1800 h (Rapoport, Feigin, Bruton and Beisel, 1966).

Civen, Brown and Granner (1970) have recently shown that the diurnal variation

of TAT is a result of changes in de novo protein synthesis. Although there is a circadian rhythm in circulating corticosterone levels (Cheifetz, Gaffaud and Dingman, 1968) it is not clear if it is involved in the mechanism of enzyme variation.

Adrenalectomy in the mouse results in a partial damping, but not elimination of the rhythm in TO activity (Rapoport et al, 1966) and this was also found for TAT in the rat (Mavrides and Lane, 1969b). Both groups of workers concluded from these results that corticosterone was not solely responsible for the circadian variation of either enzyme.

Comparison between the pattern of variation in rat and mouse suggests that environmental factors are associated with the mechanism for this variation, since the rat is a nocturnal animal while the mouse is active during periods of light.

Consequently variation in food intake has also been suggested as a possible cause and continuous feeding throughout the period of light results in a complete abolition of TAT variation (Cohn, Joseph, Larin, Shoemaker and Wurtman, 1970).

Further support for this suggestion is provided by the observation of Fishman, Munro and Wurtman, (1970) that both the rhythm of TAT activity and diurnal variation in the degree of polysome aggregation depend on the time of dietary protein intake.

Wurtman, Shoemaker and Larin, (1967) also found that tryptophan is the most potent amino acid in producing the effects on TAT. Mavrides and Lane (1969b), in addition to their observation that adrenalectomy produces only a partial damping of diurnal variation of TAT in the rat, showed that doses of hydrocortisone, too small to induce TAT, potentiate the increases in enzyme activity following administration of casein hydrolysate. They suggested that cortisol plays a permissive role in the mechanism for the circadian variation of TAT and enhances the effect of changes in amino acid levels on TAT activity. The relevance of this concept to the results obtained in the

present study is discussed further in Chapter 8. The suggested role for amino acids, in particular tryptophan, has been criticised by Fuller, (1970) who measured plasma tryptophan and corticosterone levels and found no correlation between these and the diurnal variation of either TO or TAT activity in the rat. However, the concentration of tryptophan in the hepatic cell was not estimated, and it may be that it is the changes in intracellular concentrations of amino acid, rather than increased blood levels, which raises activity of liver enzymes. Treatment of rats with a monoamine oxidase inhibitor, which increases noradrenaline or with L-dihydroxyphenylalanine, a precursor of noradrenaline, abolished diurnal rhythm of TAT, (Axelrod, 1968). It is possible therefore, that apart from corticosterone, other hormones secreted by the adrenal gland control the circadian variation of TAT activity.

Further control of TO and TAT activity in vivo may be exerted by the end product or other metabolites formed on the respective pathways of tryptophan or tyrosine metabolism. Cho Chung and Pitot (1967) investigated the effects of NADH on TO and found that in vitro, NADH inhibited purified TO by interaction at an allosteric site, rather than by competition with the apoprotein for either a substrate or cofactor. Subsequently, Pitot and Cho Chung (1970) showed that administration of NADH to rats decreased TO activity so that it is possible that increased NADH levels may diminish TO activity together with the turnover rate along the tryptophan-nicotinic acid ribonucleotide pathway. A comparable situation exists for TAT, since although fumarate does not inhibit TAT activity, another metabolite formed on this pathway of tryosine metabolism, homogentisate, does suppress activity of this enzyme both in vivo and in vitro (Civen, Wilson and Brown, 1970). Thus, both TO and TAT may be subject to some measure of feedback control.

II. Control of the Activities of Alanine Aminotransferase and Other Enzymes of Amino Acid Metabolism

Alanine aminotransferase (AAT) catalyses the formation of pyruvate from alanine by transfer of an amino group to α -oxoglutaric acid. The enzyme requires pyridoxal 5-phosphate as a cofactor and, in many respects, is characteristic of several enzymes such as serine dehydrase, and aspartate aminotransferase, which are concerned both with amino acid degradation and carbohydrate metabolism.

Although many of these enzymes are inducible by glucocorticoids, typically they have a prolonged delay before reaching maximal levels of activity. Liver AAT activity reaches its peak 48 h after hydrocortisone administration and the increased activity persists for 5 days (Rosen, Harding, Milholland and Nichol, 1963). The maximal level of serine dehydrase occurs 24 h after glucocorticoid treatment, (Pitot and Peraino, 1964) and aspartate aminotransferase activity is only increased in male rats after daily injections of 2.5 mg hydrocortisone/100g body weight given for 5 days, (Herzfeld and Greengard, 1971). Arginase activity is also increased on administration of glucocorticoids, although peak levels of activity were only observed 2 days after injection of hydrocortisone (Schimke, 1963). This type of response contrasts to the rapid increases in TO and TAT activity after glucocorticoid treatment discussed previously in this Chapter.

Since the response of these enzymes occurs either after repeated injections of hydrocortisone or some time after the last corticosteroid injection, it is questionable whether the observed increases in enzyme activity result from the direct action of glucocorticoids. The relationship between the effects of glucocorticoids on these enzymes and other factors such as pancreatic hormones and dietary composition has been widely investigated. Rosen, Roberts and Nichol (1959) showed increased AAT activity in the livers of alloxan-diabetic and fasted rats, and similar results have

been obtained for serine dehydrase activity (Ishikawa and Nakajima, 1965). Rats fed diets high in protein content but low in carbohydrate have raised activities of AAT (Szepesi and Freedland, 1967; Rosen, Milholland and Nichol, 1959), serine dehydrase (Freedland and Avery, 1964; Freedland and Szepesi, 1967; Peraino, 1967; Cheung, Cotropia and Sallach, 1969; Ku, Rogers and Harper, 1970) ornithine transcarbamylase (Pitot and Peraino, 1964), and arginase (Schimke, 1962).

The role of dietary protein intake has been studied by Pitot and Peraino (1964; 1967) with respect to the control of serine dehydrase synthesis. Glucose administration to rats represses increased serine dehydrase activity after high protein diets or glucocorticoid administration. Similar observations with AAT and TAT activities were made by Freedland and Szepesi (1968). Hanoune, Chambaut and Josipowicz (1971) showed that a single glucose load represses TAT activity in both control and glucocorticoid treated rats and these authors found this effect resulted from diminished enzyme synthesis. Amino acids and glucagon treatments are also effective in increasing serine dehydrase activity, several hours after administration (Jost, Khariallah and Pitot, 1968). These results contrast with the effects of glucocorticoid on this enzyme but bear more similarity to the results obtained with the administration of these agents on TAT activity, described earlier in this chapter. Adenosine, 3'-5' monophosphate administration produced similar effects on serine dehydrase activity to glucagon, and repeated injections of the cyclic nucleotide prevents repression of enzyme activity by glucose (Jost, Hsie and Rickenberg, 1969).

Peraino (1967) considered the possibility that glucocorticoids increased serine dehydrase activity by increasing the uptake of amino acids into the liver. This suggestion is similar to that put forward by Rosen and Milholland (1968) to explain cortisol-induction of TAT and by Mavrides and Lane (1970) to explain diurnal variation of TAT, both of which have been discussed earlier in this Chapter. It would seem, therefore, that the general mechanism for the induction of liver amino acid-metabolising enzymes by glucocorticoid is dependent on a potentiation by corticosteroids of increased enzyme activity due to the action of amino acids. This mechanism may be of especial importance in the regulation of both amino acid and carbohydrate metabolism, since when dietary carbohydrate intake is low and protein intake is relatively high, amino acids are able to promote amino acid degradation during gluconeogenesis. Conversely, a high carbohydrate intake may depress both the results of glucocorticoid- and amino acid- induction of amino acid-metabolising enzymes, and may therefore prevent unnecessary gluconeogenesis from taking place.

CHAPTER 3. The Effects of Sex Hormones on Circulating Corticosterone Levels.

The existence of a binding protein in human plasma with a high affinity for cortisol and corticosterone has been demonstrated by Daughaday (1958) and Sandberg and Slaunwhite (1959a). Daughaday characterised the protein as an α_1 globulin and it has been referred to by Daughaday as corticosteroid-binding globulin, and by Sandberg and Slaunwhite as transcortin. Most of the cortisol in plasma is bound to this protein (Sandberg and Slaunwhite, 1959a; 1959b) and the results of several studies support the conclusion that the transcortin-bound cortisol is physiologically inactive, while the unbound steroid is active. Slaunwhite, Lockie, Back and Sandberg (1962) found that the liver glycogen level is increased after treatment of adrenalectomised mice with unbound cortisol, but that these increases are not obtained when transcortin is administered together with the cortisol. Comparable work by Matsui and Plager (1966) showed that the rate of oxidation of glucose in vitro to carbon dioxide by isolated mouse ear strips was proportional to the amount of free cortisol present in plasma samples added to the in vitro system. Kawai and Yates (1966) showed that corticosteroid-binding globulin impeded the feedback inhibition of adrenocorticotrophin hormone release exerted by free corticosterone during periods of stress. Thus, transcortin may have two effects; firstly it may control the degree of cortisol-action on carbohydrate metabolism and secondly it may exert a control on the synthesis of cortisol by preventing feedback inhibition of adrenocorticotrophic hormone release.

Slaunwhite and Sandberg (1959b) observed increased levels of total plasma 17-hydroxycorticosteroids and transcortin in oestrogen-treated human subjects and similar results were reported by Daughaday, Kozak and Biederman (1959) and Wallace and Carter (1960). Further, oestrogen treatment was reported to decrease both the rates of cortisol synthesis and cortisol degradation (Wallace and Carter, 1960;

Doe, Zinneman, Flink and Ulstrom, 1960; Layne, Meyer, Vaishwana and Pincus, 1962; Peterson, Nokes, Chen and Black, 1960). Pregnancy results in similar alterations in total plasma cortisol levels and transcortin (Daughaday et al, 1960). An increased level of unbound cortisol in oestrogen-treated subjects was not reported in these early studies, nor was it found in pregnant women (Booth, Dixon, Gray, Greenaway and Holness, 1961) and it was suggested by both Wallace and Carter (1960) and Peterson et al (1960) that the increased level of 17-hydroxycorticosteroids seen during pregnancy and in oestrogen-treated subjects was due to the decreased cortisol degradation resulting from the increased level of transcortin, rather than increased cortisol synthesis. Raised production of corticosteroids usually results in high levels of unbound cortisol, as is the case for patients with Cushing's Syndrome (Doe et al, 1960; Burke, 1970) and after adrenocorticotrophic hormone administration (Doe et al, 1960). Thus Doe and co-workers (1960) and Daughaday et al (1960) proposed that the absence of the features of Cushing's Syndrome in pregnant women and oestrogen-treated subjects was due to the fact that much of the raised plasma 17-hydroxycorticosteroids was protein-bound.

Later work, however, has shown that both pregnancy and oestrogen-treatment results in small increases in the level of unbound plasma cortisol (Plager, Schmidt and Staubitz, 1964; Murray, 1967; Doe, Dickinson, Zinneman and Seal 1969; O'Connel and Welsh, 1969; Burke, 1970; Rosenthal, Sandberg and Slaunwhite, 1969). It is possible that improved techniques for ultrafiltration have enabled more precise measurements of free and protein-bound cortisol to be made and this has been responsible for the results of the more recent studies. Burke (1970) has shown further that there is a relation between the dose of oestrogen used and the response observed in the corticosterone levels, such that treatment with high levels of oestrogen

result in increased amounts of free cortisol while lower doses of oestrogen have no effect. Oestrogens, therefore, may have two effects on cortisol levels in man; firstly, the level of transcortin may be increased, which prevents degradation of cortisol leading to raised plasma concentration of the glucocorticoid and secondly, oestrogens may increase cortisol synthesis, possibly due to increased adrenocorticotrophic hormone secretion, which results in the elevation in unbound cortisol in oestrogen-treated subjects and pregnant women.

In the rat, oestrogen treatment of male rats and ovariectomised females, but not intact females, produces increased levels of plasma corticosterone (Kitay, 1963a, 1963b; Gala and Westphal, 1965; D'Angelo, 1968). Kitay (1963a) and Kitay, Coyne and Newsom (1965) suggested that the effects observed for oestrogen-treated male rats and castrated females are due to the results of both adrenocorticotrophic hormone secretion and raised corticosterone secretion due to enhanced activity of adrenal hydroxylating enzymes by oestrogens. Coyne and Kitay (1969) have more recently demonstrated the ovariectomy diminished both synthesis and release of adrenocorticotrophic hormone, which were increased by oestrogen treatment of spayed females. It would seem, therefore, that oestrogens may stimulate corticosteroidogenesis by increasing adrenocorticotrophic hormone levels, in addition to their effects on the adrenal gland enzyme systems.

Oestrogen treatment of female rats has no effect on either the level of transcortin (Gala and Westphal, 1965) or total plasma corticosterone, as mentioned previously. These results are in contrast to the observations made with oestrogen-treated women, where both cortisol-binding globulin and cortisol levels in plasma

were increased. Kitay (1963a; 1963b) proposed that for the rat, there is a relation between the dose of oestrogens administered and the response of corticosterone, such that low doses are stimulatory to corticosteroidogenesis while larger doses are inhibitory. In male rats, Kitay suggested, the doses are sufficiently low to stimulate corticosteroid synthesis and thus raise the level of plasma corticosterone, while in female rats the combination of endogenous oestrogen and the administered dose are inhibitory to corticosteroidogenesis and so the concentration of corticosterone remains unchanged.

It may be that a possible cause for the difference in response of circulating corticosteroid levels in female rats and in women following oestrogen administration is a species difference in the response of transcortin to this treatment. In oestrogen-treated human subjects, cortisol-binding globulin levels are raised, which may contribute to the increased plasma cortisol levels by impeding degradation of the steroid, as discussed previously. In the oestrogen-treated rat, transcortin levels are raised in the males but unchanged in females (Gala and Westphal, 1965), which may allow degradation of corticosterone to proceed uninhibited in the female rat injected with oestrogens and thus offset any other stimulatory effects oestrogens may have.

In pregnant rats, plasma corticosterone levels are raised (Hoet, Pagni, Ekka, and Sabba, 1965). This is a surprising observation, in view of the results of oestrogen treatment of female rats, discussed in the preceding paragraphs. It is possible that the increases in circulating corticosterone are not due to the action of oestrogen but are due to the conversion of the increased plasma progesterone levels of pregnancy to corticosterone in the adrenal gland.

In contrast to the effect of oestrogens, androgen administration to female rats diminishes the level of plasma corticosterone and the production of corticosterone by the adrenal (Kitay, 1963b, 1963c; Kitay, Coyne, Nelson and Newsom, 1966). Addition of oestrogens to adrenal homogenates in vitro, stimulates the activity of the adrenal hydroxylating enzyme (Ruhmann-Wenhold, Lauro and Nelson, 1970). This effect is impaired if androgens are also included in the incubation medium (Ruhmann-Wenhold and Nelson, 1970). It may be that this type of inhibition of adrenal enzyme activity is responsible for the results observed by Kitay (1963c) with androgen-treated intact female rats. More recently, Coyne and Kitay (1971) have shown that castration of male rats increases synthesis and release of adrenocorticotrophic hormone, both of which are diminished in androgen-treated orchidectomised males. It is possible that these effects of testosterone on adrenocorticotrophic hormone levels may also contribute to the decreases in plasma corticosterone levels in androgen-treated females.

Little work has been published on plasma cortisol levels in androgen-treated human subjects except for one study by Sandberg, Slaunwhite and Carter (1960). These authors found that administration of testosterone and androgenic steroids to patients with carcinoma of the breast had no effect on the level of plasma cortisol. However, since breast cancer patients are known to have both elevated plasma levels of 17-hydroxycorticosteroids, and unbound cortisol (Bell, Bulbrook, and Deshpande, 1967) it is uncertain whether this affected the results obtained, and prevented androgen treatment from resulting in decreased circulating cortisol levels.

Oestrogen treatment of human subjects results in marked elevations in levels of urinary tryptophan metabolites, discussed previously in Chapter I, and also in decreased levels of plasma tyrosine (Rose and Cramp, 1970). Although much of the increased concentration of plasma corticosterone observed after oestrogen administration to human subjects is protein-bound and therefore regarded as physiologically inert (Slaunwhite, Lockie, Back and Sandberg, 1962), Rose, (1970) and Rose and Cramp (1970) proposed that it was the raised plasma cortisol levels, rather than the oestrogens, which were responsible for the changes in enzyme activity that resulted in altered metabolism of tryptophan and tyrosine.

Oestrogen administration to male rats increases the activity of alanine aminotransferase (Rosen, Roberts, Budnick and Nichol, 1959; Keller, Richardson and Yates, 1969) which raised the possibility that oestrogens either directly or indirectly via another hormone may have a generalised effect on the activity of a large number of glucocorticoid-inducible enzymes of amino acid metabolism. Keller et al (1969) proposed that the increased liver alanine aminotransferase in oestrogen-treated male rats was due to the elevated level of plasma corticosterone. They further suggested that the vascular membrane of the liver tissue was sufficiently permeable to allow bound hormone into the liver cells where the free hormone is released to induce alanine aminotransferase. The relevance of this hypothesis to the results presented in this thesis is discussed in Chapters 5 and 8.

CHAPTER 4. Methods Used During This Investigation

- I. Animals
- II. Statistical Methods
- III. Tryptophan Oxygenase Assay
- IV. Tyrosine Aminotransferase Assay
- V. Alanine Aminotransferase Assay
- VI. Serum Corticosterone Assay

I. ANIMALS

The male and female Sprague-Dawley rats used throughout this study were mostly from a colony maintained in the animal house at St. Mary's Hospital Medical School, although occasionally these were supplemented by animals obtained from a commercial supplier, A. Tuck and Son Ltd., L.A.B.S., Rayleigh, Essex. Unless otherwise stated, they had a freely available diet of Oxoid 41 pellets and tap water.

CONDITIONS OF ANIMAL MAINTENANCE

Introduction

Several of the hormone treatments and gland ablations included in the present study decreased the level of serum corticosterone and attempts were also made to compare changes in serum corticosterone concentrations with those observed in enzyme activity. It was therefore necessary to measure serum levels of corticosterone in rats at rest rather than under conditions of stress. Care was therefore taken to standardise the conditions of animal maintenance during experiments so that true resting values for serum corticosterone and reproducible levels of enzyme activity could both be obtained.

(1). Light and Temperature

Serum corticosterone levels and the activities of TO and TAT vary diurnally (Chapter 2) and circulating corticosterone levels were also reported to vary with environmental conditions (Glenister and Yates, 1961; Ader and Friedman, 1968). Accordingly, rats were kept under conditions of standardised temperature (21°C) and a 12h. light/dark cycle (0700-1900h.).

(2). Caging

Gala and Westphal (1965) caged rats separately in order to obtain true resting levels of plasma corticosterone. Dunn and Scheving (1971) showed that where groups of rats were kept in the same cage, opening the cage and removing the first animal was sufficient to give increased pituitary-adrenal activity in the remaining animals. This resulted in a wide variation of plasma corticosterone levels in the morning, when resting levels were low, but not later in the afternoon when they were higher due to diurnal variation. These authors used a fluorimetric method which was not sufficiently sensitive to record small differences in corticosterone levels and thus the full effect of handling in the afternoon may not have been observed. During the present investigation, the precaution was taken to cage rats singly for the duration of their injection schedule, whenever corticosterone was to be measured. In experiments involving cortisol injections, rats were caged in groups.

(3). Killing

Glenister and Yates (1961) reported that a 10 sec. interval between initial handling of the rat and its death does not allow the stress of the procedure to affect plasma corticosterone levels. Plasma corticosterone levels in rats at the resting state were obtained from blood samples taken after decapitation, following a 20 sec. (Kitay, 1961) and 30 sec. (Gala and Westphal, 1965) time interval between initial handling of the rat and its death.

In all experiments in the present study where corticosterone was assayed, rats were killed by decapitation within 30 secs. of approaching the cage. In cases where a longer time interval elapsed, the corticosterone result was discarded.

INJECTIONS

(1). Sex Hormones

As a consequence of the diurnal variations discussed previously (Chapter 2) all injections of sex hormones were administered between 0900 and 10.00h. and the rats were killed between 14.00 and 15.00h. Oestradiol benzoate (Nutritional Biochemicals Corp.), testosterone propionate (Sigma Chemical Co.) and fluoxymesterone (Sigma Chemical Co.) were dissolved in corn oil for subcutaneous injection. Injections were administered for 14 days, except where otherwise stated, and the rats were killed on the day after the last injection.

AAT and TAT activities, expressed in terms of the tissue concentration, vary according to body weight, such that AAT activity increases (Harding, Rosen and Nichol, 1961) and TAT activity decreases (Lin, Rivlin and Knox, 1959) with increasing body weight. In the present study, the composition of the experimental groups was arranged so that the body weights of rats in the test groups were similar to those in the control groups at the end of the injection period. Oestradiol benzoate administration decreases rat body weight (Keller, Richardson and Yates, 1969) so that in experiments involving oestrogen treatment the rats in the test groups were heavier than those in control groups at the beginning of the injection schedule. In contrast, androgen treatment increases body weight (Harding, Rosen and Nichol, 1961) so that in these studies, the rats in the control groups were heavier than those in test groups at the beginning of the injection period. Hormone-treated animals were always matched with a control group which were injected on the same days with a corresponding volume of oil.

(2). Hydrocortisone

Hydrocortisone hemisuccinate (Sigma Chemicals Co.) was administered by intraperitoneal injection at a dose of 2.5 mg/100g body weight. Control rats were injected with a corresponding volume of saline. Injections were given at 0900h. and the rats were killed at 14.00h. as the maximal increases in TO and TAT are known to occur 5h. after glucocorticoid treatment (Chapter 2).

SURGICAL PROCEDURES

All surgical procedures required for the experimental work reported in this thesis were performed by the author with the exception of hypophysectomy.

Hypophysectomised rats were obtained from a commercial supplier, Scientific Products Farm Ltd., Ash, Canterbury, Kent.

(1). Anaesthetic

Ether was used as the anaesthetic throughout the operations performed by the author.

(2). Bilateral Adrenalectomy

The rat was anaesthetised, placed prone and a small vertical mid-line incision 1 cm. long was made through the skin over the neural spine of the last thoracic vertebra. The skin was retracted on both sides of the spine and the angle between the last rib and the vertebra on one side was carefully explored. With the tip of scissors, the fascia was incised and the adrenal gland exposed. The adrenal vein and blood supply were exposed, ligated and severed before the removal of the adrenal gland. The same procedure was used to remove the other gland.

Rats were kept on 0.9% saline in place of drinking water following the operation and injections were begun 4 to 5 days after surgery. Successful removal of the adrenal glands was confirmed by a post mortem examination of the peritoneal cavity for adrenal tissue and measurement of serum corticosterone levels at the end of the injection regime.

(3). Bilateral Ovariectomy

The rat was anaesthetised, placed prone and a small vertical mid-line incision made through the skin over the first lumbar vertebra. The skin was retracted on either side of the spine so that the peri-ovarian fat deposits could be identified. After incising the fascia over the fat deposits with the tip of the scissors, the ovary was exposed and the fallopian tubes and ovarian blood supply ligated and severed, before removal of the ovary. The same procedure was used to remove the remaining ovary.

The operations were carried out on weanling females which were then kept for 2 to 3 months before being used for experiments. During this time the completeness of removal of the ovaries was ascertained by examination of vaginal smears for red blood cells.

(4). Orchidectomy

After anaesthesia, the rat was placed supine and a small vertical mid-line incision 0.25 cm. long was made through the skin at the tip of the scrotal sacs. Through this incision, the fascia and muscular sheath enclosing one of the testes was incised. The cauda epididymis was identified and the vas deferens and testicular blood supply were exposed, ligated and severed so that the testis could be removed. The same procedure was used to remove the remaining testis.

The operation was performed on mature males. After orchidectomy, they were kept 7 to 10 days before injections were begun.

(5). Hypophysectomy

This operation was performed on mature female rats by the supplier, Scientific Products Farm Ltd. Hypophysectomised rats were given 5% glucose in place of tap water while they were in the animal house at St. Mary's Hospital Medical School and were kept for 2 weeks before injection schedules were started.

(6). Procedure for obtaining adrenal weights

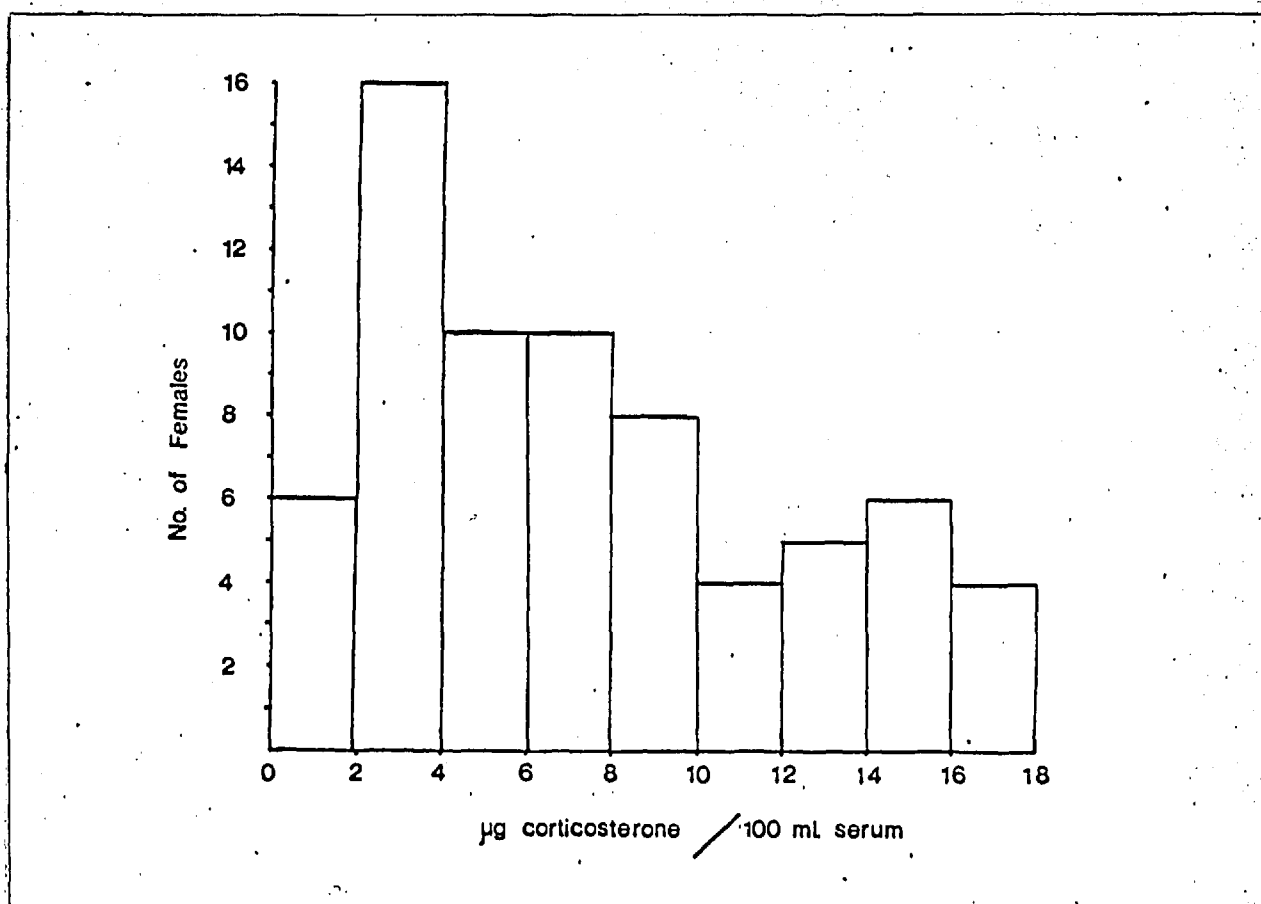
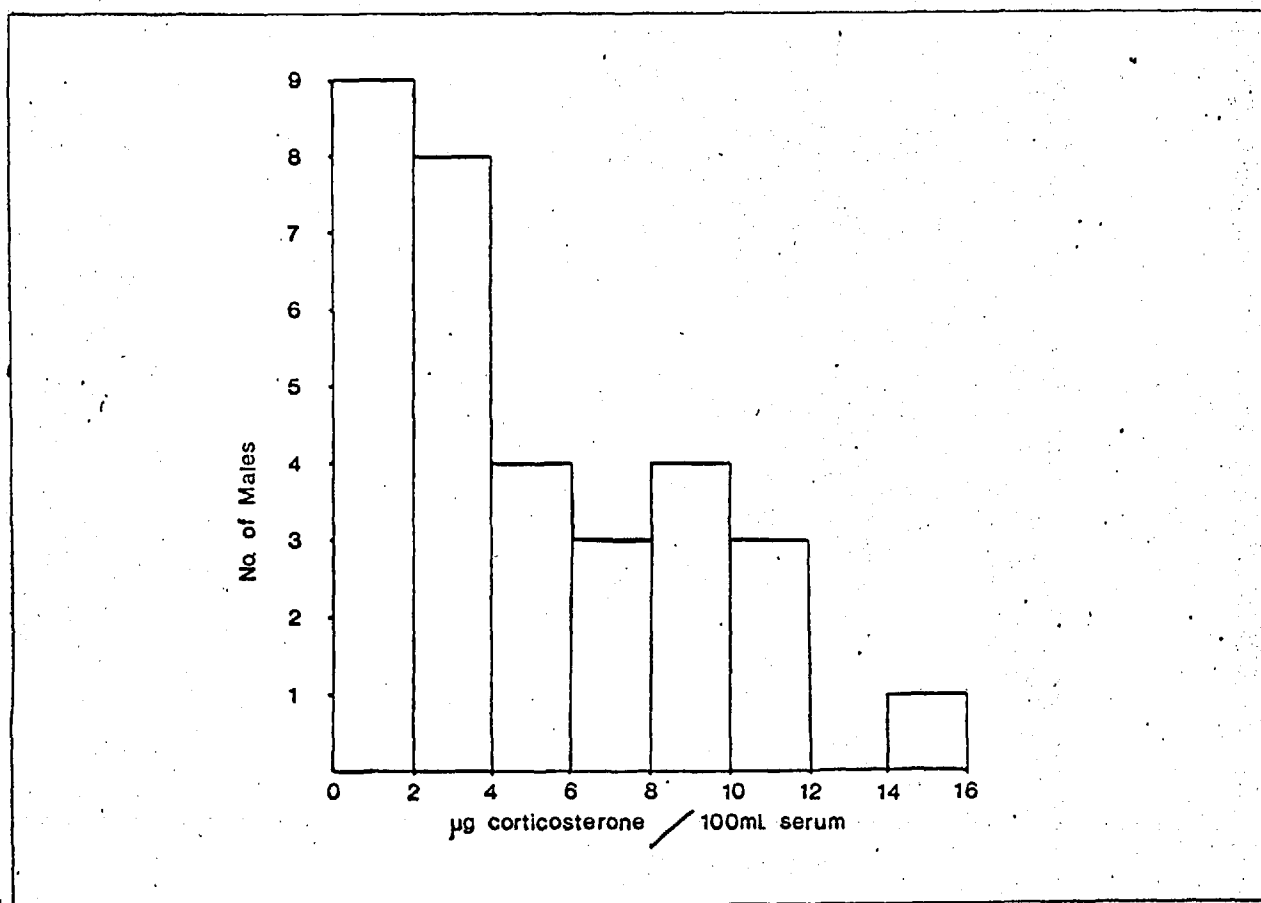
Whenever possible, adrenals were weighed in experiments where serum corticosterone was assayed. Adrenals were removed from the peritoneal cavity at death, separated from the surrounding fat tissue, blotted and weighed on a Gallenkamp torsion balance.

II. STATISTICAL METHODS

Levels of statistical significance were determined by the Students' t test and differences between test and control groups with $p > 0.05$ were not regarded as significant.

As shown on fig. 2 the normal range of serum corticosterone values gave a skew distribution so that mean values and standard deviations were calculated after determination of the $\log_{10}$ for each result as described by Wootton (1964). Results are presented both with and without this skew correction.

Fig. 2. Distribution of Serum Corticosterone Levels in Male and Female Control Rats.



III. TRYPTOPHAN OXYGENASE ASSAY

Introduction

The method used in the present study was similar to that described by Leklem, Woodford and Brown (1969) and depends upon the measurement of kynurenine produced after incubation of the substrate with liver enzyme extract, haem and ascorbic acid.

Assay Procedure

(1). Preparation of liver extract

Homogenising medium 0.02M - Sodium dihydrogen orthophosphate
 0.25M - Sucrose
 0.14M - Potassium chloride

The pH of the solution was adjusted to pH 7.0 with 4N sodium hydroxide.

The animals were killed by decapitation, the liver was quickly removed and chilled in an ice-cold vessel containing the homogenising medium. Throughout this procedure and the following preparation, livers and liver extracts were kept between 0 to 4°C. 10% W/V homogenates were prepared by homogenisation of the liver in a Waring Blender operated at 3/4 of its maximum speed for 45 secs. Microsome-free supernatant was obtained by centrifugation of the homogenate in a Beckman model L-2 preparative ultracentrifuge at 105,000 x g for 60 mins.

(2). Incubation Method

(a). Incubation Medium

0.3 ml 0.2M-sodium dihydrogen orthophosphate adjusted to pH 7.0 with 4N sodium hydroxide.

0.1 ml 50 mM-L-tryptophan (Koch-Light Laboratories Ltd.); the amino acid was dissolved in glass distilled water and the pH adjusted to pH 7.0 with saturated sodium bicarbonate.

0.2 ml 12 mM-ascorbic acid; this was dissolved in glass-distilled water and was freshly prepared before each assay.

0.2 ml 75 μ M-haematin (Sigma Chemicals, London); 4.8 mg haem was converted to haematin by the addition of 2 drops of 1.5N sodium hydroxide before it was dissolved in water to a final volume of 100 ml. This was also freshly prepared before each assay.

0.2 ml glass distilled water.

All reagents, with the exception of ascorbic acid and haematin were stored between 0 to 4°C, and remained stable for 2 weeks.

(b). Procedure

2 ml liver supernatant was added to 1 ml of the above reaction mixture so that the final volume of 3 ml contained 0.8 mM-ascorbic acid, 5 μ M-haematin and 1.66 mM-L-tryptophan. The reaction was carried out in 25 ml stoppered Ehrlenmyer flasks which contained the reaction mixture and the liver supernatant and were kept at 0°C in an ice bath, before the assay was begun. The reaction was started by transferring the flasks to a Dubnoff incubator at 37°C. Duplicate flasks were incubated for 60 and 90 mins with air as the gas phase and a zero time flask was included for each assay. The reaction was stopped by adding 10 ml of 5% (W/V) trichloroacetic acid to each flask and the precipitate was removed by centrifugation.

Conditions of Assay

(a). pH

The maximal conversion rates of tryptophan to kynurenine occur between pH 6.5 and 8.0 with an optimal pH of 7.0 (Knox and Mehler, 1950; Seglen and Jervell, 1969). Both homogenisation and incubation media were adjusted to this pH in the present study.

Fig. 3. Effect of Variation of Haem Concentration on Rate of Kynurenine Production in the Presence of 0.8 mM-Ascorbic Acid.

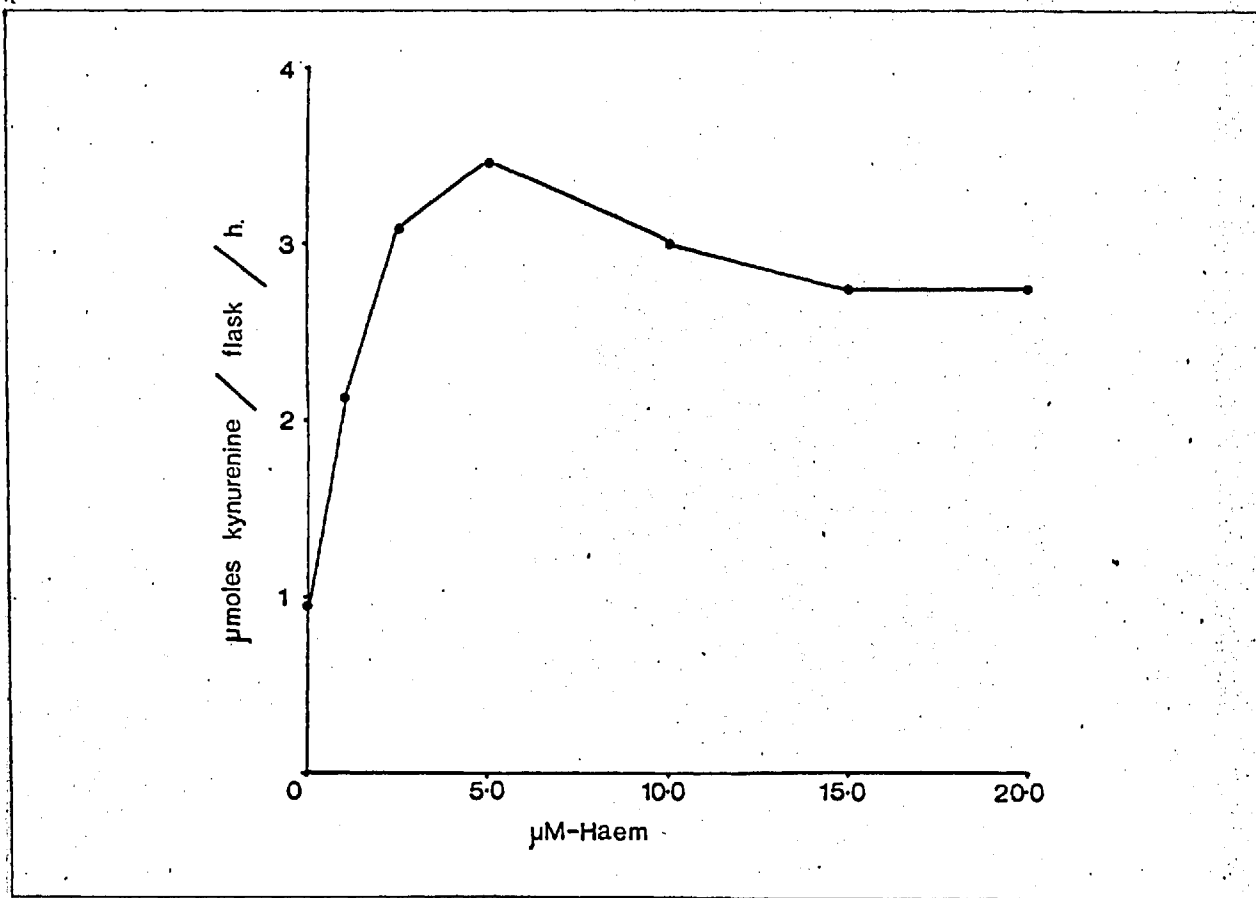
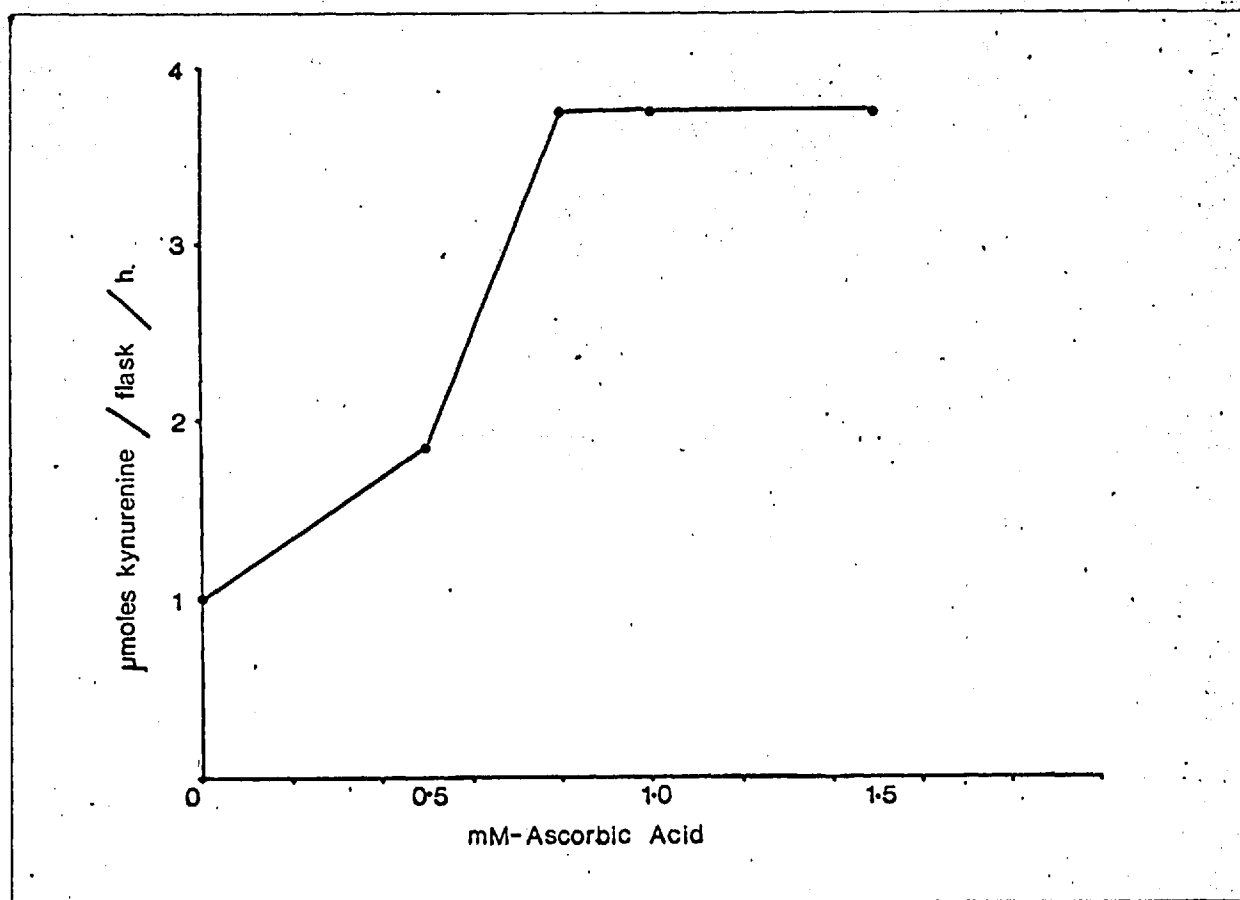


Fig. 4. Effect of Variation of Ascorbic Acid Concentration on Rate of Kynurenine Production in the Presence of 5 μM Haem.



(b). Concentration of haem and ascorbic acid

Knox and Piras (1967) and Seglen and Jervell (1968) used haem concentrations within the range 2 to 8 μM with respect to the final volume of liver extract and incubation medium. In the present study, the combination of 5 μM -haem and 0.8 mM-ascorbic acid was optimal for the enzyme reaction (Figs. 3 and 4).

(c). Time of Incubation

The rate of kynurenine formation is not linear during the first 20 mins of the reaction with cell sap (Feigelson and Greengard, 1961) or homogenate (Seglen and Jervell, 1968). Piras and Knox (1967) overcame this problem by allowing the incubation to proceed for 30 mins. before measurement of kynurenine over a 25 mins. period, during which time the reaction rate was linear. This procedure results in high blank values and a low sensitivity (Morrill, Feiner, and Kostellow, 1968) and was therefore unsuited to the present study, since in vitro inhibitions, gland ablations and hormonal treatments, which caused low levels of enzyme activity, were to be investigated. The rate of kynurenine formation was found to be linear over the first 90 mins. of incubation (Fig. 5) and in a series of 8 assays, duplicate flasks were incubated for 60 mins. and 90 mins. There was no difference between the enzyme activities, expressed in μmoles kynurenine/g wet liver wt/h., in these two flasks (Table 1) indicating that the initial 20 mins. period, during which the reaction was non-linear, did not affect the overall reaction rate in these longer incubation times. The procedure of including 60 mins. and 90 mins. incubation flasks was therefore adopted for each assay, and enzyme activity was expressed in μmoles kynurenine/g wet liver wt/h.

Fig. 5. Relation Between Kynurenine Production and Duration of Incubation.

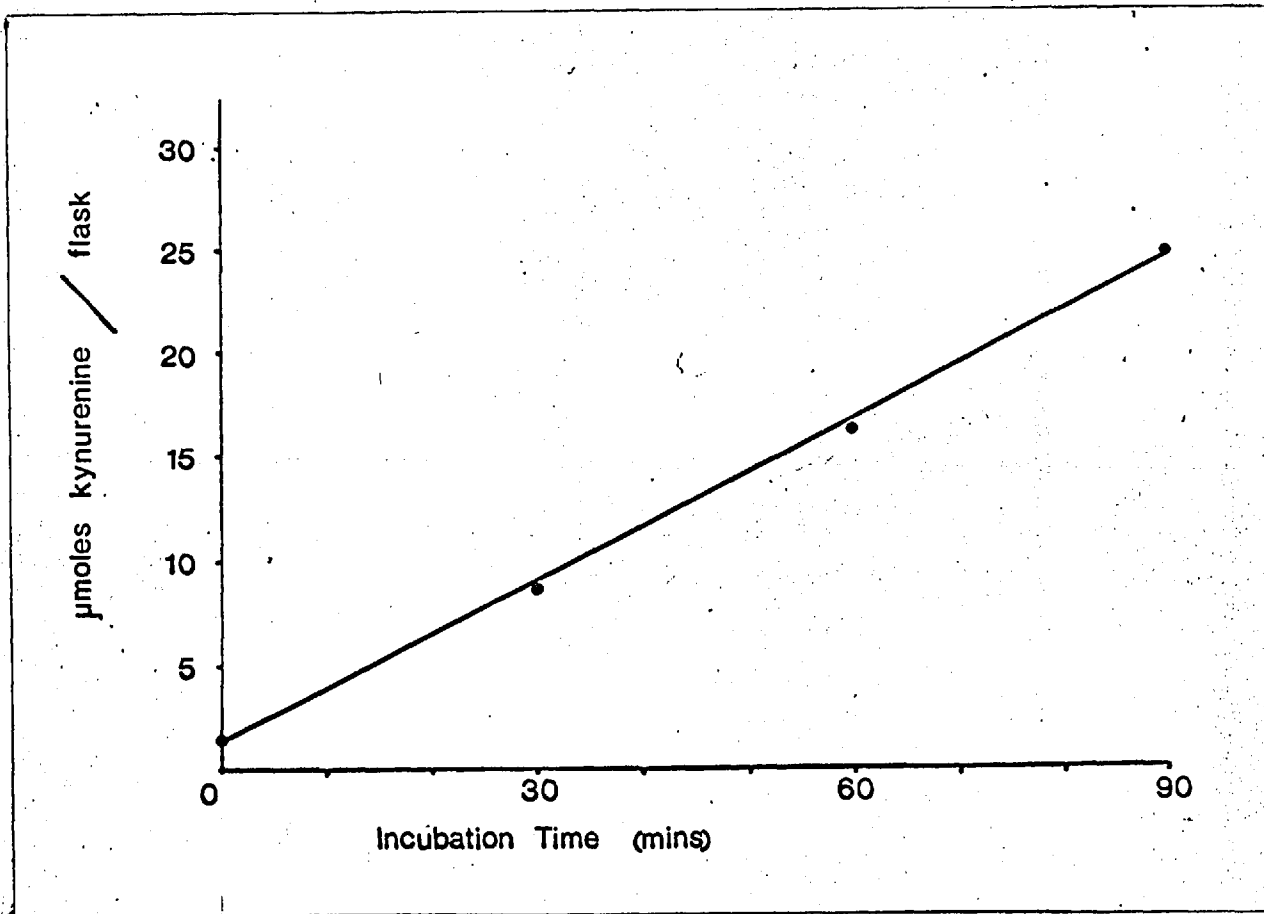


Table 1 The effect of different incubation times on the rate of tryptophan oxygenase activity

<u>Time of Incubation</u>	<u>No. of Assays</u>	<u>Tryptophan Oxygenase Activity</u> <u>μmoles kynurenine/g/h.</u>
60 mins.	8	3.4 ± 0.5
90 mins.	8	3.4 ± 0.3

Values are stated as the mean ± standard deviation

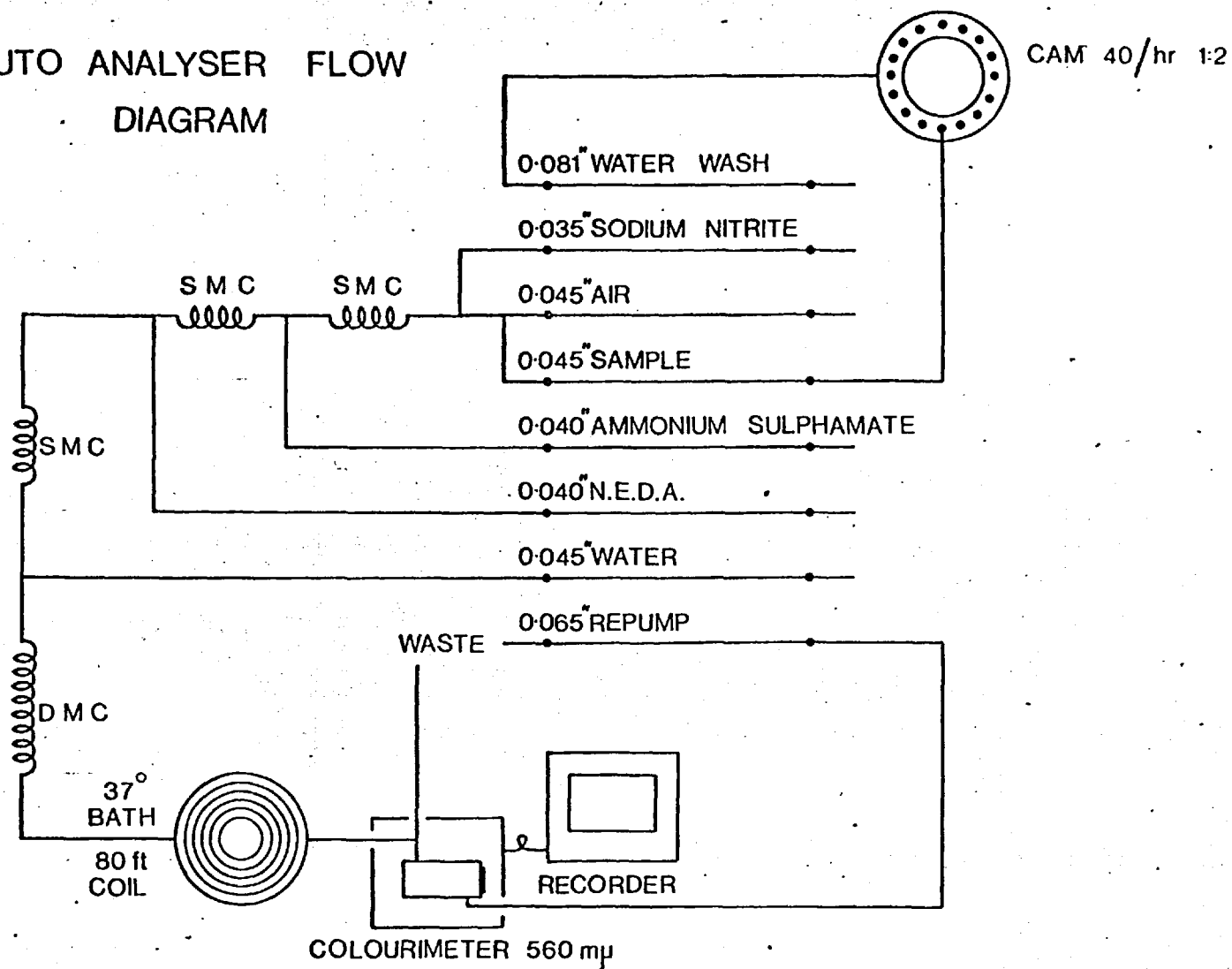
(4). Measurement of Kynurenine

For the purpose of TO assay, kynurenine is normally measured by its spectrophotometric absorption at 360 mμ (Piras and Knox, 1967; Greengard and Baker, 1966; Labrie and Korner, 1968; Rosen and Milholland, 1968). However, a compound formed by a complex of tryptophan and ascorbic acid interferes with the absorption of kynurenine at this wavelength (Seglen and Jervell, 1968; Morrill, Feiner and Kostellow, 1968). The addition of ascorbic acid to the incubation medium increases enzyme activity (Tanaka and Knox, 1959). Morrill et al (1968) calculated that the absorption of the tryptophan-ascorbic acid complex at 320 mμ accounted for 25% of the total increased optical density produced by addition of ascorbic acid, previously thought due to kynurenine production. In the present study, therefore kynurenine was measured by a colorimetric method based on the diazotisation of aromatic amines (Bratton and Marshall, 1939). In this method, kynurenine was diazotised with sodium nitrite and ammonium sulphamate before the formation of a purple compound, with maximal absorption at 550 mμ, on the addition of N'(1-naphthyl) ethylene diamine hydrochloride. Optimal colour development occurred

Fig. 6.

AUTO ANALYSER FLOW DIAGRAM

Internal tube diameters
are shown in inches
SMC = Single Mixing Coil
DMC = Double Mixing Coil
NEDA = N' (1-naphthyl) ethylene
diamine hydrochloride



after a 3h. period. This method, modified by Arend, Leklem and Brown (1970) for the use of a Technicon autoanalyser, was used in the present study.

(a). Autoanalyser Assay Method

(i). Solutions

0.05% Sodium nitrite in glass distilled water.

0.75% Ammonium sulphamate in glass distilled water.

0.10% N'(1-naphthyl) ethylene diamine hydrochloride.

All solutions were stable for 2 weeks on storage between 0 to 4°C.

(ii). Standards

Kynurenine was dissolved in 3.85% (W/V) trichloroacetic acid, the final concentration in the assay medium after deproteinisation with 5% (W/V) trichloroacetic acid, so that a stock solution of 100 µg/ml was prepared, which was further diluted for a standard concentration range of 1 µg to 10 µg kynurenine/ml.

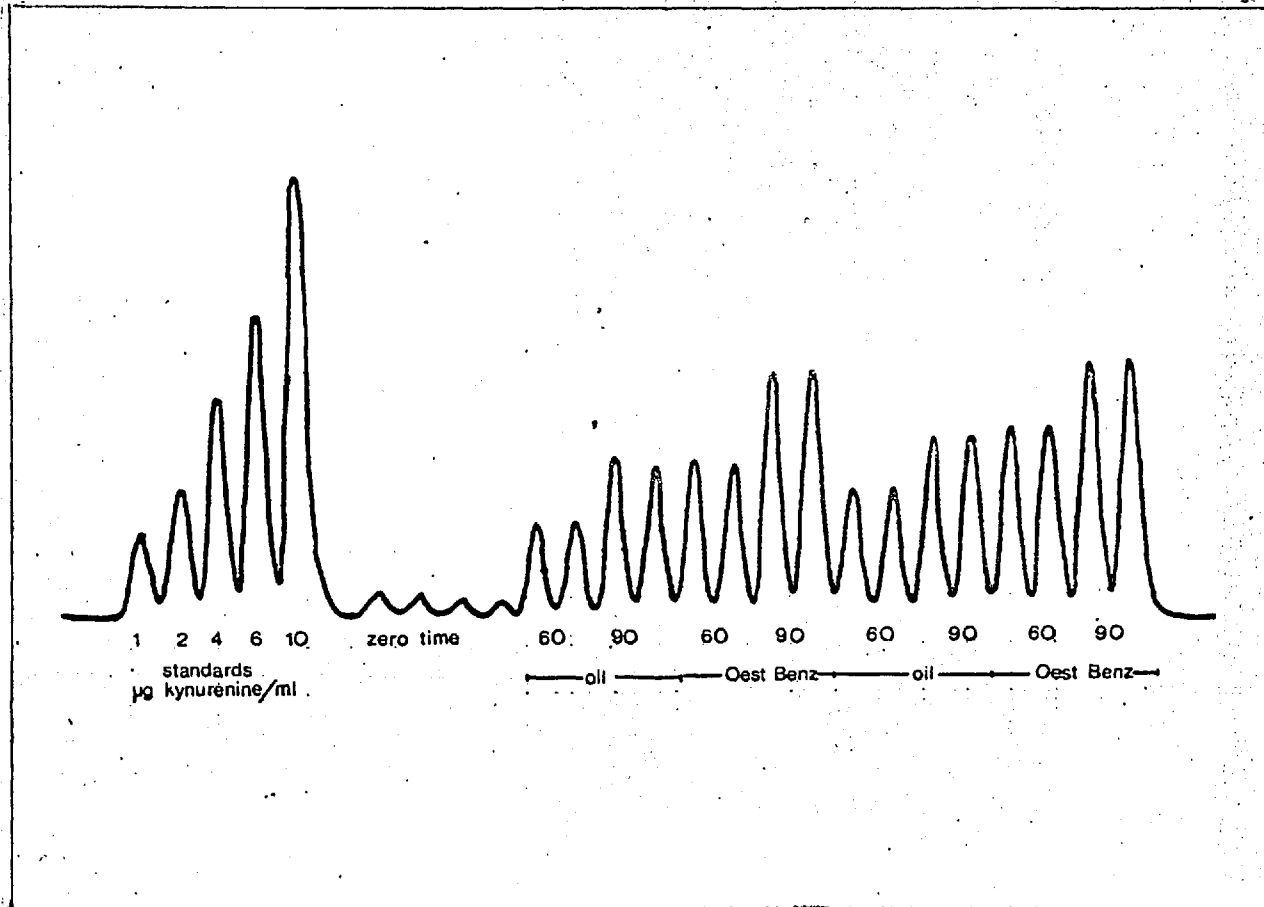
(iii). Instrumentation

The Technicon components used included a Sampler No. III, proportionating pump, heating bath, colorimeter with a 15 mm tubular flow-cell and recorder.

(iv). Procedure

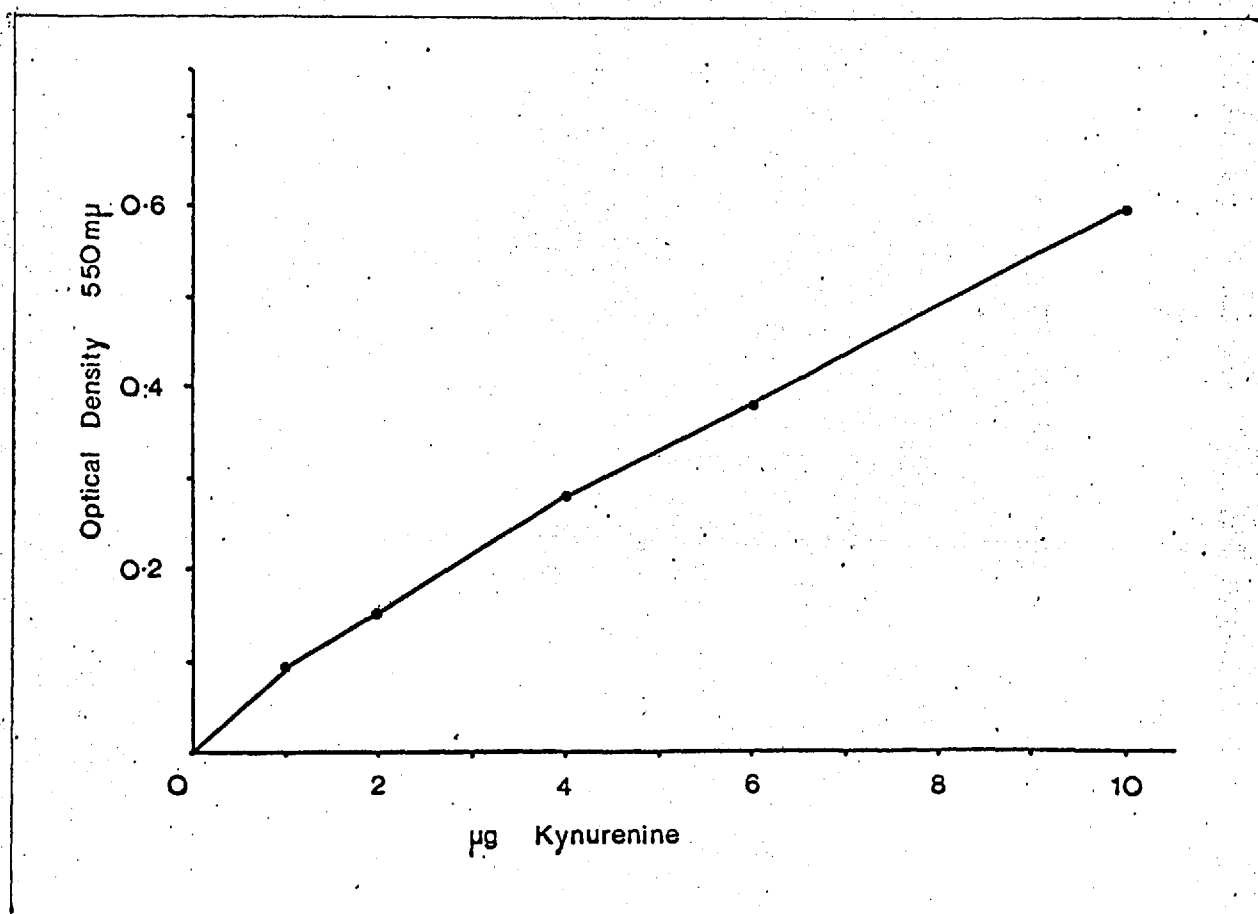
The autoanalyser was assembled as shown on the flow-diagram in Fig. 6. After the mixing of the sodium nitrite, ammonium sulphamate, N'(1-naphthyl) ethylene diamine hydrochloride, satisfactory colour development was obtained by the use of a double time delay coil kept at 37°C in the water bath. Optical density was read in a standard colorimeter using a 15 mm tubular flow-cell with 550 mµ interference filters. A sampling rate of 40/h. was used to produce clearly defined peaks, and the sample to wash ratio was 2:1. A recorder chart for this assay is shown in Fig. 7.

Fig. 7. Chart Record from Autoanalyser



Test samples were incubated for zero time, 60 mins, (60) and 90 mins (90). Samples from corn oil-treated control female rats (oil) were diluted 1:4, and those from female rats treated with a daily dose of 10 µg oestradiol benzoate (Oest. Benz.) were diluted 1:8 with 3.85% trichloroacetic acid.

Fig. 8. Standard Curve for Kynurenine



(v). Standard Curve

The standard curve for this procedure was not linear (Fig. 8). Further dilution of each sample, after deproteinisation with 5% trichloroacetic acid, with 3.85% trichloroacetic acid was necessary so that it was within the 1 μ g to 10 μ g kynurenine/ml range of the standard curve. Samples from control rats were routinely diluted a further 1:4, from oestradiol benzoate treated rats a further 1:8 and cortisol treated rats a further 1:10.

Evaluation of Method

Table 2. Comparison of mean values for tryptophan oxygenase in this and other studies.

<u>Study</u>	<u>Sex</u>	<u>No. of rats</u>	<u>Tryptophan Oxygenase</u> <u>μmoles Kynurenine/g/h.</u>
Present	Male	39	2.3 ± 0.6
Study	Female	78	3.7 ± 1.1
Greengard, Baker and Friedell, (1967)	Male	9	3.7 ± 0.6
	Female	5	4.6 ± 0.9
Knox, Piras and Tokuyuma, (1966)	Female	5	4.5 ± 1.2
Reshef and Greengard, 1968	Male	6	3.9 ± 0.3

A comparison between the mean values for TO obtained by the method used in the present study and those reported by other workers is difficult since many authors have expressed activities as μ moles kynurenine/mg protein/h. In addition, the numbers included in the experimental groups in these other studies were small, compared with the total group size of the present investigation.

However, from the limited data available, it appears that the levels of TO activity obtained in the present study are lower than those of other authors, possibly due to the elimination of extraneous interference due to ascorbic acid by using a colorimetric method for kynurenine measurement. Both Reshef and Greengard (1968) and Greengard, Baker and Friedell (1967) measured kynurenine by its spectrophotometric absorption at 360 m μ which is subject to such interference. The care taken during the present study to keep animals in controlled conditions, devoid of stressful stimuli, as described earlier in this Chapter, also may have resulted in low TO activity in control rats.

IV. TYROSINE AMINOTRANSFERASE ASSAY

Introduction

Tyrosine aminotransferase activity is normally measured by estimating the rate of formation of p-hydroxyphenolpyruvic acid, the initial product of tyrosine transamination identified first by Cammarata and Cohen (1950). These authors used a modification of the method described by Briggs (1923) similar to Fiske-Subbarow's assay for phosphate (Fiske and Subbarow, 1924). The assay of Cannellakis and Cohen (1956b) was modified by Rosen, Harding, Milholland and Nichol (1963) and it is this method with some minor modifications that was used in this present study.

P-hydroxyphenylpyruvic acid has also been estimated by measuring the optical density of the tautomer produced on addition of a tautomerase to the assay system, the "enol-borate method", (Lin, Pitt, Civen and Knox, 1958). Since this is essentially for use with a continuous direct reading spectrophotometer, which requires a clear solution, it was unsuitable for the rapid measurement of tyrosine aminotransferase in whole homogenate, necessary in the present study.

A third method described by Diamondstone is based on the spectrophotometric measurement of p-hydroxybenzaldehyde produced from p-hydroxyphenylpyruvic acid in the presence of alkali (Diamondstone, 1966). This method was devised to measure TAT activity in low concentrations of homogenate, and since the reaction rate was not linear with high concentrations of homogenate it was therefore not acceptable for the present study.

Method

(1). Incubation medium

36.4 ml 4mM-L-tyrosine (Sigma Chemicals, London) in 0.2M potassium dihydrogen orthophosphate. The buffer was adjusted to pH 7.3 with 4N sodium hydroxide. Due to difficulty experienced with the solubility of tyrosine, the amino acid was dissolved in buffer before commencing each assay and then added to the remainder of the incubation medium.

4.2 ml 0.03M- α oxoglutaric acid (Sigma Chemicals, London) in glass distilled water.

2.8 ml 2.8 mM pyridoxal phosphate (Sigma Chemicals, London) in glass distilled water.

This solution was prepared afresh each week and stored in a dark bottle to prevent decomposition by ultra violet light.

1.4 ml 0.15M diethyldithiocarbamic acid in glass distilled water.

5.6 ml 0.25M sucrose in 0.2M potassium dihydrogen orthophosphate at pH 7.3.

All solutions, with the exception of tyrosine were stored between 0 to 4°C.

Procedure

10% (W/V) liver homogenates were obtained as described for the TO assay.

An assay medium was prepared as indicated above. The volumes listed were sufficient to assay homogenate samples from 4 rat livers. 3.6 ml of the medium were pipetted into conical flasks, at 0°C in an ice bath, and to each of these 0.4 ml liver homogenate was added. The reaction was begun by placing the ice-cold flasks in a Dubnoff shaking water bath at 37°C and was stopped by the addition of 0.4 ml 100% (W/V) trichloroacetic acid. The flasks were incubated for 5 and 15 mins.

(3). Standards

A solution of p-hydroxyphenylpyruvic acid, concentration 100 $\mu\text{g}/0.1$ ml glass distilled water, was freshly prepared each week. Two standard flasks were included for each assay and consisted of 0.1 ml and 0.2 ml, respectively, of the standard solution, assay medium with 0.25M-sucrose in place of pyridoxal phosphate and 0.4 ml homogenate. The mixture was immediately deproteinised with 0.4 ml 100% (W/V) trichloroacetic acid.

(4). Conditions of Assay

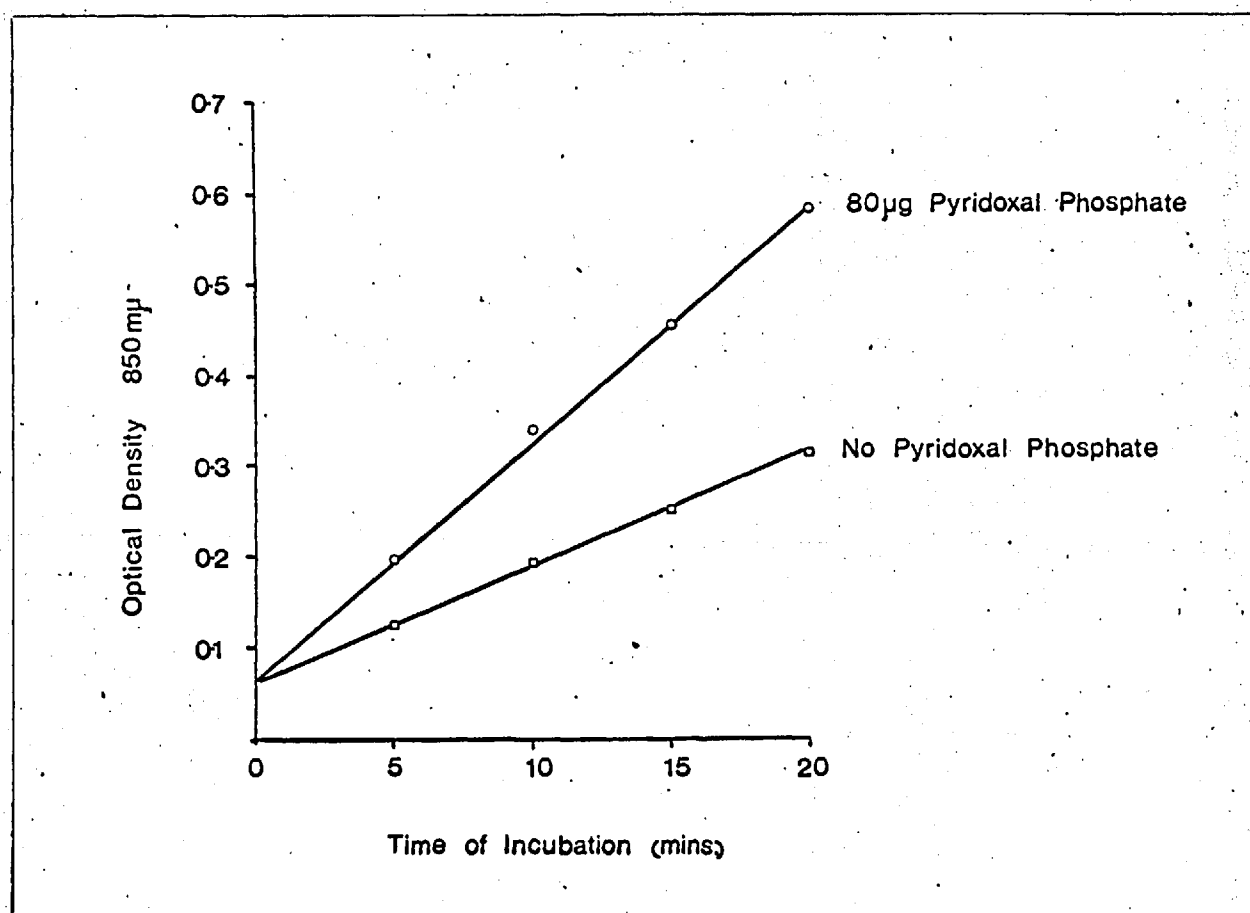
(a). Concentration of tyrosine

In the original method of Rosen and co-workers (1963) the enzyme reaction was initiated, after a three mins. preincubation period, by the addition of 0.6 ml of an aqueous solution of 10 mM-L-tyrosine. The flasks already contained buffer, homogenate and α -oxoglutarate. Much difficulty was experienced with this procedure due to the poor solubility of the amino acid. This was overcome by dissolving a more dilute solution of tyrosine in buffer and including it in the assay medium before the incubation was begun. The concentration of tyrosine solution was decreased from 10 mM, used by Rosen et al (1963), to 4 mM and the proportion of tyrosine added to the incubation medium was increased so that the final concentration of tyrosine in each flask was the same as that used by Rosen and co-workers.

(b). Time Course of Incubation

Initiation of the reaction by the addition of substrate, according to the procedure of Rosen and co-workers (1963) results in high blank values (Greengard and

Fig. 9. Relation Between p-Hydroxyphenylpyruvic Acid Production and Time of Incubation.



In Figs. 9, 10 and 12 p-hydroxyphenylpyruvic acid was measured as the molybdenum blue complex by its absorption at a wavelength of 850 mμ.

Gordon, 1963). Greengard and Gordon (1963) discarded this procedure and began the reaction by transferring the flasks from an ice bath to a Dubnoff incubator at 37°C , and this was also adopted in the present study. Production of p-hydroxyphenylpyruvic acid was linear with time over a 20 min. period, (Fig. 9), and the concentration of product obtained between 5 and 15 mins. incubations was routinely used to calculate the rate of enzyme activity expressed as $\mu\text{moles p-hydroxyphenylpyruvate/g wet liver wt/h}$.

(c). Enzyme and cofactor concentrations

TAT was assayed in the presence of pyridoxal phosphate throughout the present study to obtain maximal expression of enzyme activity (Chapter 2). The combination of 0.4 ml homogenate with 80 μg pyridoxal phosphate provided optimal conditions for the enzyme reaction (Fig. 10).

(d). pH

The production rate of p-hydroxyphenylpyruvic acid is maximal with a pH between 7.0 and 8.5, and optimal at 7.6 (Lin, Pitt, Civen and Knox, 1958). However, in the present study the pH of the homogenising medium was 7.0, since this was critical for the TO assay. In order to avoid subjecting the enzyme protein preparation to an extreme shift in pH, the TAT assay was carried out at pH 7.3.

(5). Measurement of p-hydroxyphenylpyruvic acid

Colour Reagent

12 ml 3% (W/V) ammonium molybdate in 5N HCl.

20 ml 1% (W/V) potassium monohydrogen phosphate in glass distilled water.

32 ml glass distilled water.

Fig. 10.

Effect of Homogenate and
Pyridoxal 5-Phosphate
Concentration on
p-Hydroxyphenylpyruvic
Acid Production.

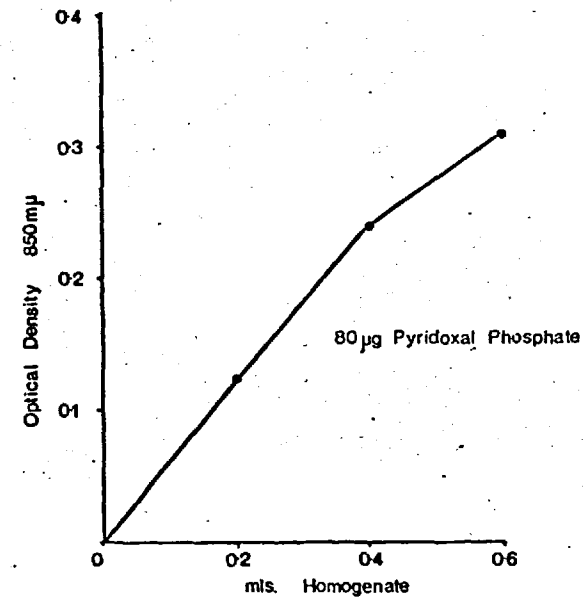
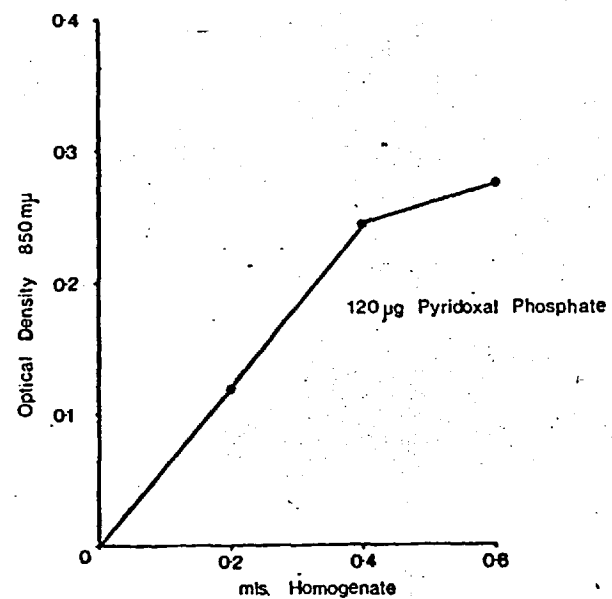
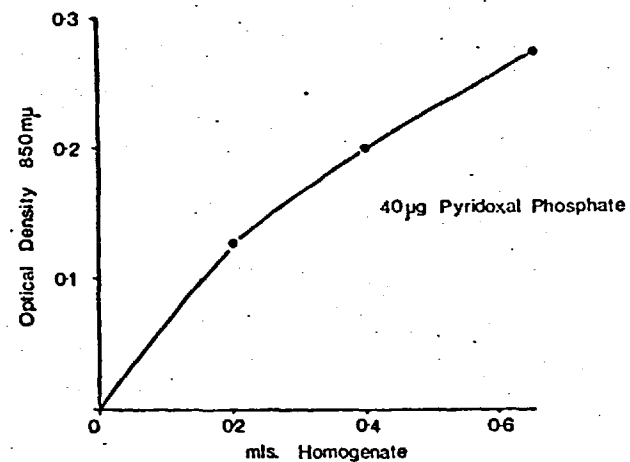
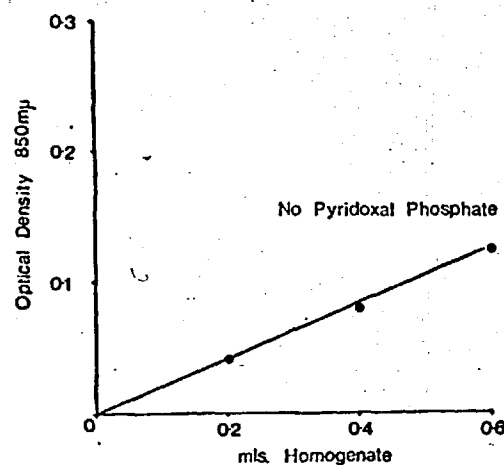
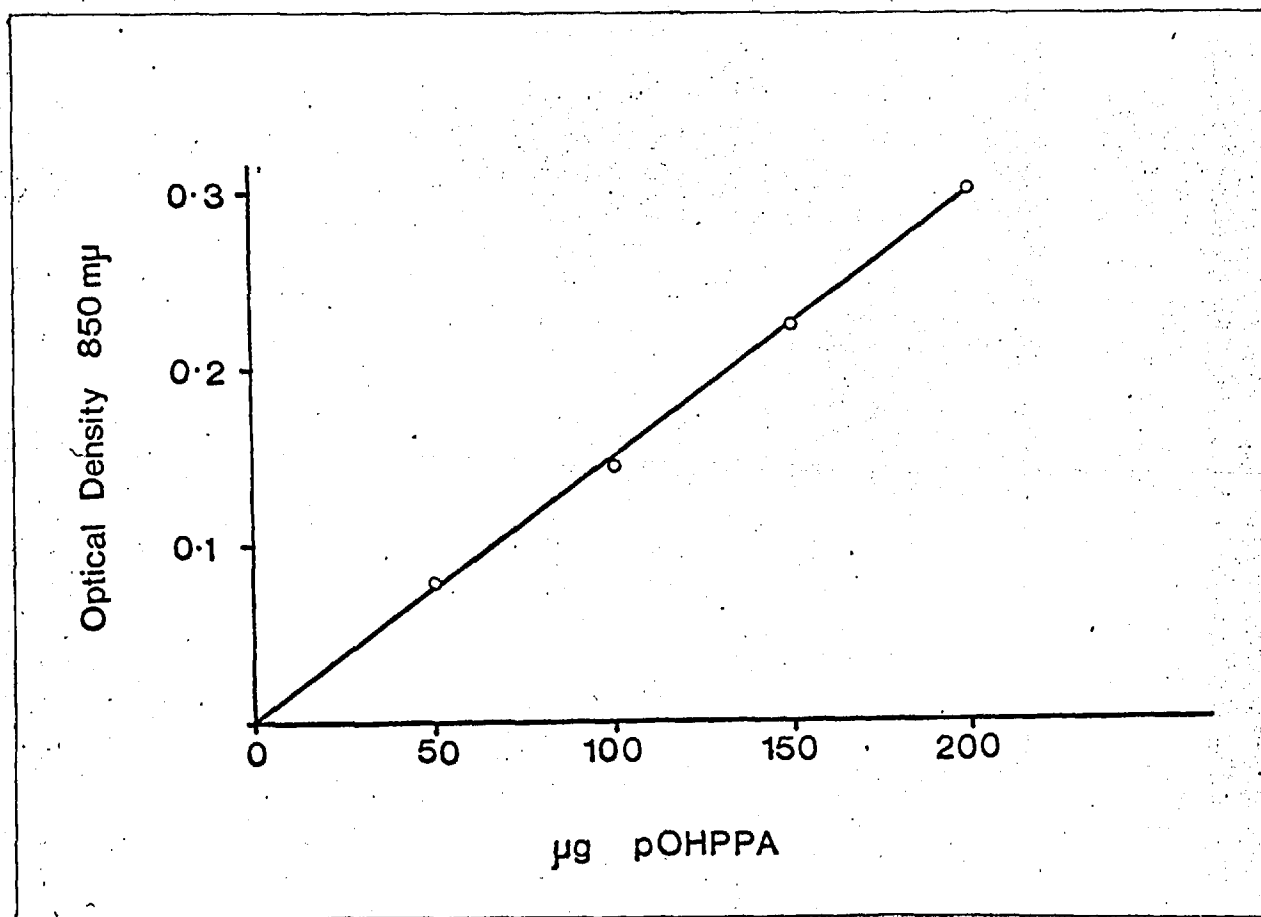


Fig. 11. Standard Curve for p-Hydroxyphenylpyruvic Acid (pOHPPA)



Colour reagent was freshly prepared before each assay as indicated above. 1 ml of the clear supernatant from the deproteinised assay medium was added to 2 ml of the colour reagent. The optical density of the molybdenum blue, formed after the colour reaction had proceeded for 1h was measured in a Unicam S.P. 600 spectrophotometer at a wavelength of 850 m μ . A standard curve is shown on Fig. 11.

Cellular distribution of Enzyme

Preparation of particle-free supernatants from liver homogenates, by the same method as that used in the TO assay, resulted in a substantial loss of TAT activity, compared with whole homogenates, when pyridoxal phosphate was omitted from the assay medium. Addition of pyridoxal phosphate increased TAT activity in supernatant, although it was still below the activity in whole homogenate assayed under the same conditions (Fig. 12). Since mitochondria are removed on preparation of a particulate-free supernatant, the possibility that this was responsible for the low activity in supernatant, was investigated.

Mitochondria were prepared from whole homogenate by the method of Hogeboom (1955) modified by Ueda (1967). Mitochondria were separated as a pellet after centrifugation of the whole homogenate at 10,000 x g for 15 mins. in a Beckman model L2 preparative ultracentrifuge. Mitochondrial TAT activity was greater than in 10,000 x g supernatant when pyridoxal phosphate was omitted from the assay system. However, on addition of the cofactor, activity in the supernatant increased whereas mitochondrial activity remained unaltered, (Table 3, Fig. 13).

Fig. 12. Comparison Between p-Hydroxyphenylpyruvic Acid Production in Liver Homogenate and 100,000 x g Cell Sap (Supernatant).

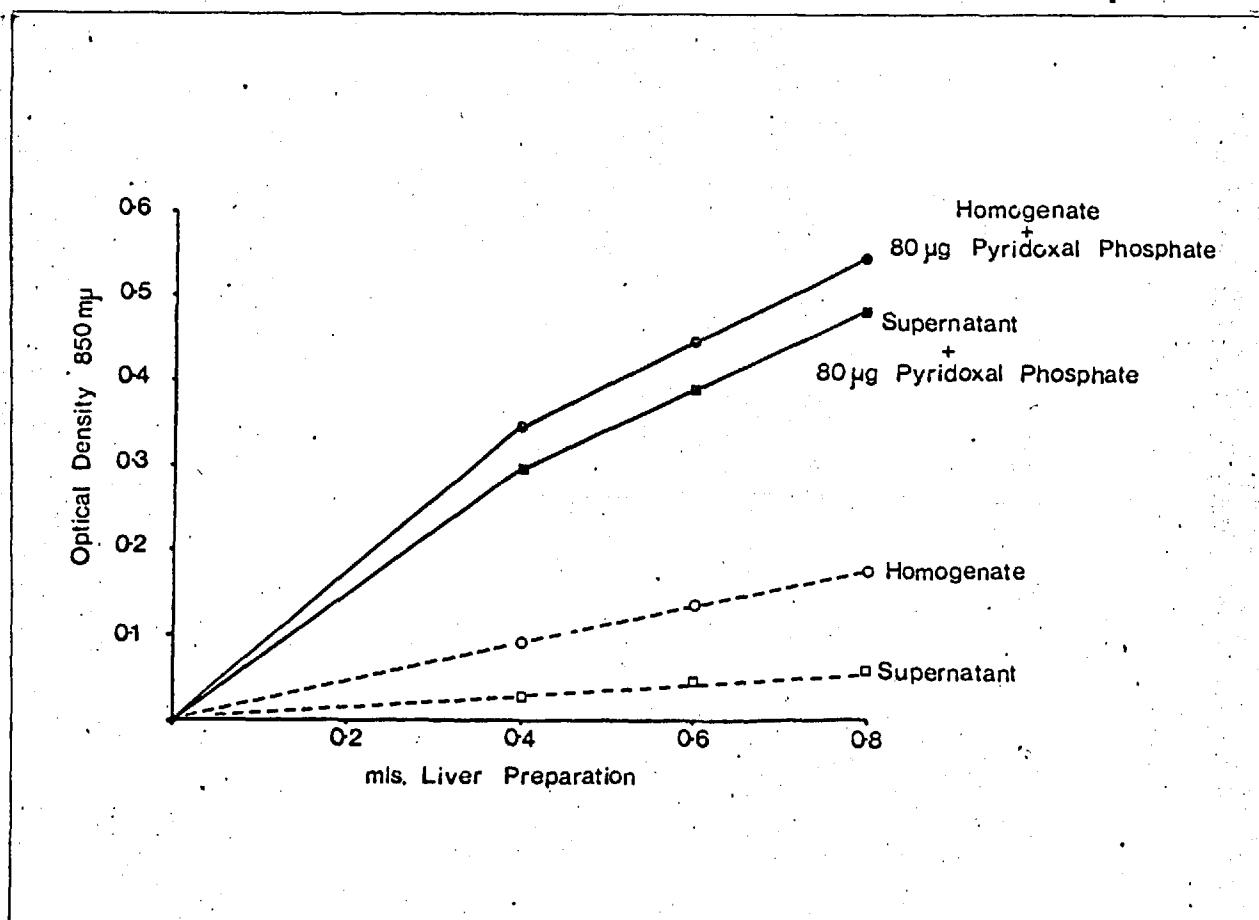


Table 3. Tyrosine Aminotransferase Activity in Cell Fractions

Cell Fraction	Tyrosine aminotransferase activity $\mu\text{g p-hydroxyphenylpyruvate/flask/10 mins}$	
	No Pyridoxal Phosphate	With Pyridoxal Phosphate
Mitochondria	19.9	19.8
10,000 x g Supernatant	5.6	88.4

Mitochondrial TAT activity was therefore responsible for the higher activity in homogenates than in supernatant. The inability of pyridoxal phosphate to stimulate mitochondrial activity may be due either to the saturation of mitochondrial TAT with pyridoxal 5-phosphate prior to the addition of exogenous cofactor or to the presence of an isoenzyme of TAT in the mitochondria, which is unresponsive to the addition of the coenzyme. A further possibility is that the mitochondrial membrane is impermeable to pyridoxal 5-phosphate.

Evaluation of Method

Table 4. Comparison of mean values for Tyrosine aminotransferase between this and other studies

<u>Study</u>	<u>Sex</u>	<u>No. of animals</u>	<u>Tyrosine aminotransferase activity $\mu\text{moles OHPPA/g/h}$</u>
Present study	Female	72	90 ± 23
Present study	Male	25	95 ± 21
Greengard and Dewey (1967)	Female	6	66 ± 4
Greengard and Gordon (1963)	Male	8	54 ± 4

The mean values for TAT activity in both male and female rats calculated from results collected during the study are shown on Table 4. In comparison to the work of Greengard and Dewey (1967) these results are high. TAT activity was shown to vary seasonally, (Chapter 5) and this may account for these differences since the observations of Greengard and Dewey may have been made during October to December when activities are low.

Greengard and Dewey (1967) also used particulate-free supernatant in their assays. The present study shows that some activity is lost on removal of the mitochondrial fraction, and since whole homogenate was used throughout the current investigation this may also explain the difference in activities. Several workers have measured liver TAT activities in the course of their investigations (Rosen et al, 1963; Kenney and Flora, 1961; Kupfer and Partridge, 1970; Singer and Litwack, 1971; Mavrides and Lane, 1968; Labrie and Korner, 1968a.b), although these authors have expressed activities as μ moles product/g protein/h so that comparison between their results and those obtained in the present study was impossible.

V. ALANINE AMINOTRANSFERASE ASSAY

Introduction

The method used in this study was that of Rosen, Roberts and Nichol (1959) which was a modification of the colorimetric procedure described by Tonhazy, White and Umbreit (1950) for the measurement of aspartate and alanine aminotransferases.

Assay Method

(1). Solutions

Reagent A 12 ml 0.17M-2 amino-2-methyl-1-3 propanediol in glass distilled water, adjusted to pH 8.9 with 5N HCl.

2.5 ml 0.5M-L-alanine (BDH) in glass distilled water.

1.5 ml 0.1M- α -oxoglutaric acid (Sigma Chemicals, London) in glass distilled water.

75 μ l 10% (W/V) bovine plasma albumin (BDH) in glass distilled water.

Reagent B This was of the same composition as Reagent A except that L-alanine was replaced by water.

All solutions were kept between 0 to 4°C.

(2). Procedure

10% (W/V) liver homogenates were prepared as described for the TO assay and were diluted further with ice-cold distilled water so that concentrations of 1% and 0.5% were assayed for each liver homogenate.

Two 50 μ l portions of both 1% and 0.5% concentrations were each added to 0.5 ml Reagent A and one 50 μ l portion of each concentration was added to 0.5 ml Reagent B for the blanks. Before the incubation was begun, tubes containing the assay mixtures were kept between 0 to 4°C in an ice bath, and the reaction was started by

Fig. 14. Production of Pyruvate (Optical Density of Hydrazone at 530 m μ) Over a 30 mins Incubation Time.

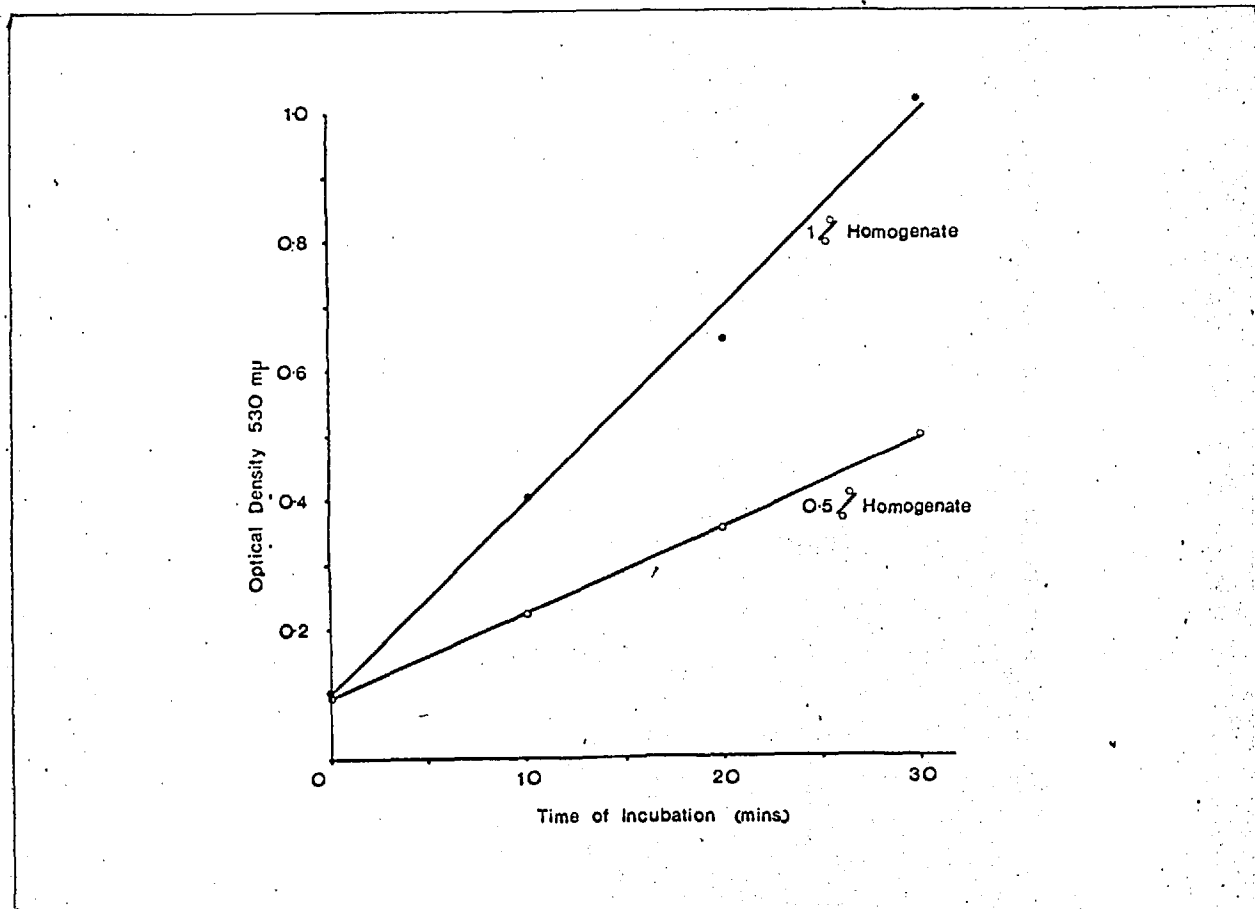
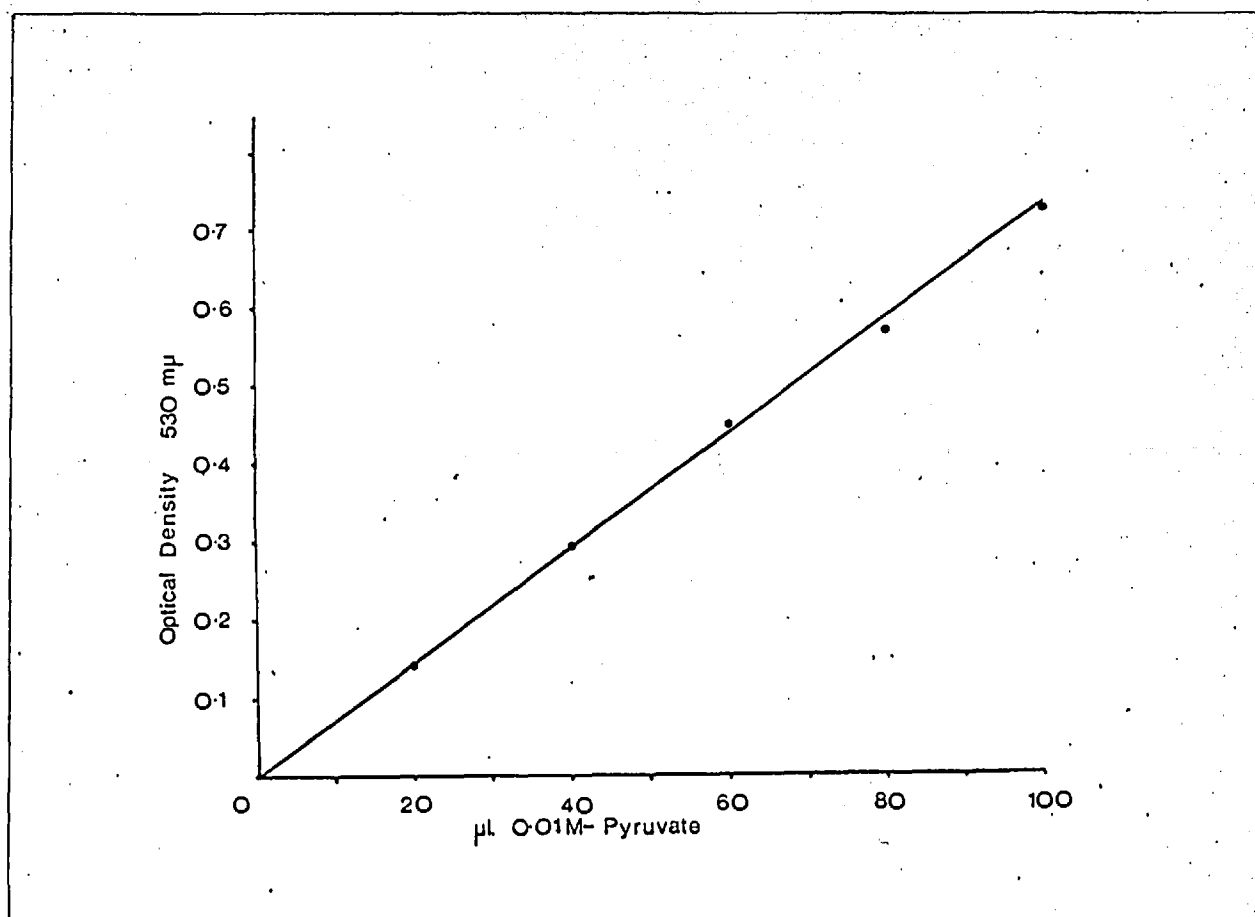


Fig. 15. Standard Curve for Pyruvate.



placing the tubes in a water bath at 37°C . After 30 mins. the reaction was stopped by the addition of 0.05 ml 100% (W/V) trichloroacetic acid.

The reaction rate was linear over the 30 min. incubation period for both concentrations of homogenate (Fig. 14).

(3). Treatment of standards

25 μl and 50 μl of 0.01M-pyruvate were routinely added to 0.5 ml Reagent B and treated similarly to test samples. A standard curve was linear over the range 0-100 μl 0.01M pyruvate/tube (Fig. 15).

(4). Colour Reaction

(a). Solutions

1% (W/V) 2,4 dinitrophenyl hydrazine in 2N HCl

Water-saturated toluene

2.5% (W/V) potassium hydroxide in 95% ethanol

(b). Procedure

0.5 ml dinitrophenylhydrazine was added to the deproteinised assay mixture and incubated at 37°C for 5 mins. The pyruvic acid hydrazone thus formed was extracted into 1 ml water-saturated toluene. 0.6 ml of this upper layer was pipetted out into 3 ml alcoholic potassium hydroxide, to give a reddish colour read at 530 $\text{m}\mu$ on a Unicam SP 600 spectrophotometer.

Evaluation of method

AAT requires pyridoxal phosphate to act as a cofactor for the transamination of alanine. Keller, Richardson and Yates (1969) showed that, under conditions similar to those in the present study, there was a linear relation between the amount of

pyruvate formed and concentration of homogenate without addition of pyridoxal phosphate cofactor. After inclusion of cofactor in the assay system this reaction rate was no longer linear with respect to the concentration of homogenate, as the enzyme was already fully saturated with cofactor in the liver. These observations confirm earlier work of Rosen et al (1959). In the present study, pyridoxal phosphate was routinely omitted from the assay medium.

The mean values for AAT activity calculated from results obtained throughout the present study are summarised on Table 5.

Table 5. Comparison of mean alanine aminotransferase values between this and other studies

<u>Study</u>	<u>Sex</u>	<u>No. of animals</u>	<u>Alanine aminotransferase</u> <u>mmole pyruvate/g wet liver</u> <u>Wt/h.</u>
Present Study	Male	10	4.1 ± 0.8
Present Study	Female	43	2.1 ± 0.6
Keller, Richardson and Yates (1969)	Male	30	2.3 ± 0.8
Keller, Richardson and Yates (1969)	Female	14	2.3 ± 0.6

VI. CORTICOSTERONE ASSAY METHOD

Introduction

Corticosterone was measured throughout this study by a radioactive protein-binding method based on a combination of the techniques developed by Murphy (1964, 1967) and Mayes and Nugent (1966) for the assay of cortisol.

Corticosteroids have also been estimated by fluorimetric methods (Guillemin, Clayton, Lipscomb and Smith, 1959; Mattingly, 1962) but there are still sufficient non-specific fluorogens extracted from plasma to interfere considerably with the measurement of cortisol using the method of Mattingly (Stenlake, Davidson, Williams and Durnie, 1970). In an investigation of the method of Guillemin et al, Dunn and Scheving (1971) found a residual fluorescence in the plasma of adrenalectomised rats equivalent to 6 μ g corticosterone/100 ml plasma. Keller, Sendelbeck, Moore and Yates (1966) reported a similar residual fluorescence in plasma from hypophysectomised and adrenalectomised rats equivalent to 2 μ g corticosterone/100 ml plasma.

Since experiments with hypophysectomised and adrenalectomised rats and the estimation of low levels of plasma corticosterone resulting from gland ablations and hormonal treatments, formed a major part of the study, methods involving fluorimetry were not considered suitable. The double isotope method of Fraser and James (1968), although specific for corticosteroids, was thought too complex for the present investigation.

Principle of Protein Binding Method

This method is based on competition for binding sites on a specific steroid-binding globulin. A plasma protein with binding properties specific for corticosterone is first allowed to reach equilibrium with a known quantity of the labelled hormone. When the unknown cold sample is added, a new equilibrium is reached in which some of the labelled corticosterone is displaced from the binding globulin. The bound and free hormones are now separated, using an inert material with a high specificity for the free hormone such as dextran-coated charcoal, or florisol, a siliceous earth. Almost all the free hormone now becomes bound to this solid phase and may be centrifuged down to form a precipitate, while the protein-bound hormone remains in the supernatant. The loss in radioactivity from the supernatant is used to indicate the level of corticosterone present in the unknown sample.

Assay Method

(1). Materials and Solutions

(a). Redistilled dichlormethane

(b). Binding solution

1-2H³ corticosterone, specific activity 30-40 Ci/mM from the Radiochemical Centre, Amersham. This was of 98% purity as analysed by a Bush B chromatography system and diluted 1:10 with ethanol to form the stock solution which was stored at -20^o C.

Corticosterone Binding Globulin (C.B.G.) was obtained by using human plasma as described later in this Chapter. Sufficient plasma for optimal binding, normally between 4 to 5 ml, was pipetted into a 100 ml volumetric flask. Distilled water was added to within 1 ml of the mark followed by 0.4 ml of the stock 1-2H³ corticosterone

solution, and the volume was then made up to 100 ml with distilled water. Such a procedure ensured that addition of the ethanolic corticosterone did not precipitate any of the binding plasma. The binding solution was freshly prepared before each assay.

(c). Dextran-coated charcoal

0.5% charcoal (activated Norit OL from Hopkins and Williams) in glass distilled water.

0.05% Dextran of Molecular Weight 80,000 (from Pharmacia, Upsala, Sweden) in glass distilled water. Dextran-coated charcoal solution was prepared by mixing equal volumes of the charcoal and dextran solutions, and remained stable for 4 days if stored between 0 to 4°C.

(d). Scintillant

The scintillant consisted of 25g naphthalene, 7g 2,5 diphenyloxazole, 0.25g 1,4 di-(2-(4 methyl 5 phenyloxazolyl)-benzene and 50 ml toluene per litre of 1-4 dioxan and had a counting efficiency of 40% for tritium in a Packard Tricarb Liquid Scintillation Counter.

(2). Procedure

0.5 ml serum samples were extracted with 10 ml redistilled dichlormethane in stoppered glass vials by shaking them horizontally for 20 mins. in a mechanical shaker. The vials were left to stand for a further 30 mins. to ensure separation of the upper aqueous and the lower steroid-containing phase. The aqueous phase was removed under vacuum suction and duplicate 2 ml aliquots of the residue were pipetted into assay tubes. After standing the tubes in a water bath at 45°C and

and evaporating the solvent under nitrogen, 0.5 ml of the binding solution was added to each tube using a Hamilton repeating syringe calibrated at 100 μ l intervals. The tubes containing the sample and binding solution were incubated in a water bath at 40°C for 5 mins., transferred to an ice bath for a further 10 mins. and 0.4 ml of the dextran-charcoal mixture was then added to each tube using a spring-loaded syringe. The dextran-coated charcoal was kept in suspension by constant stirring for at least 20 mins. before use and throughout its addition to the assay tubes. The tubes were left to stand for 30 mins. and centrifuged for 5 mins. in a Mistral Model 6L at 2,000 r.p.m. Using an Eppendorf automatic pipette, 0.2 ml of the supernatant were added to 5 ml of scintillant in glass screwcap vials and these were counted at 40% efficiency for 5 mins. each in a Packard Tricarb Spectrometer, Model 574.

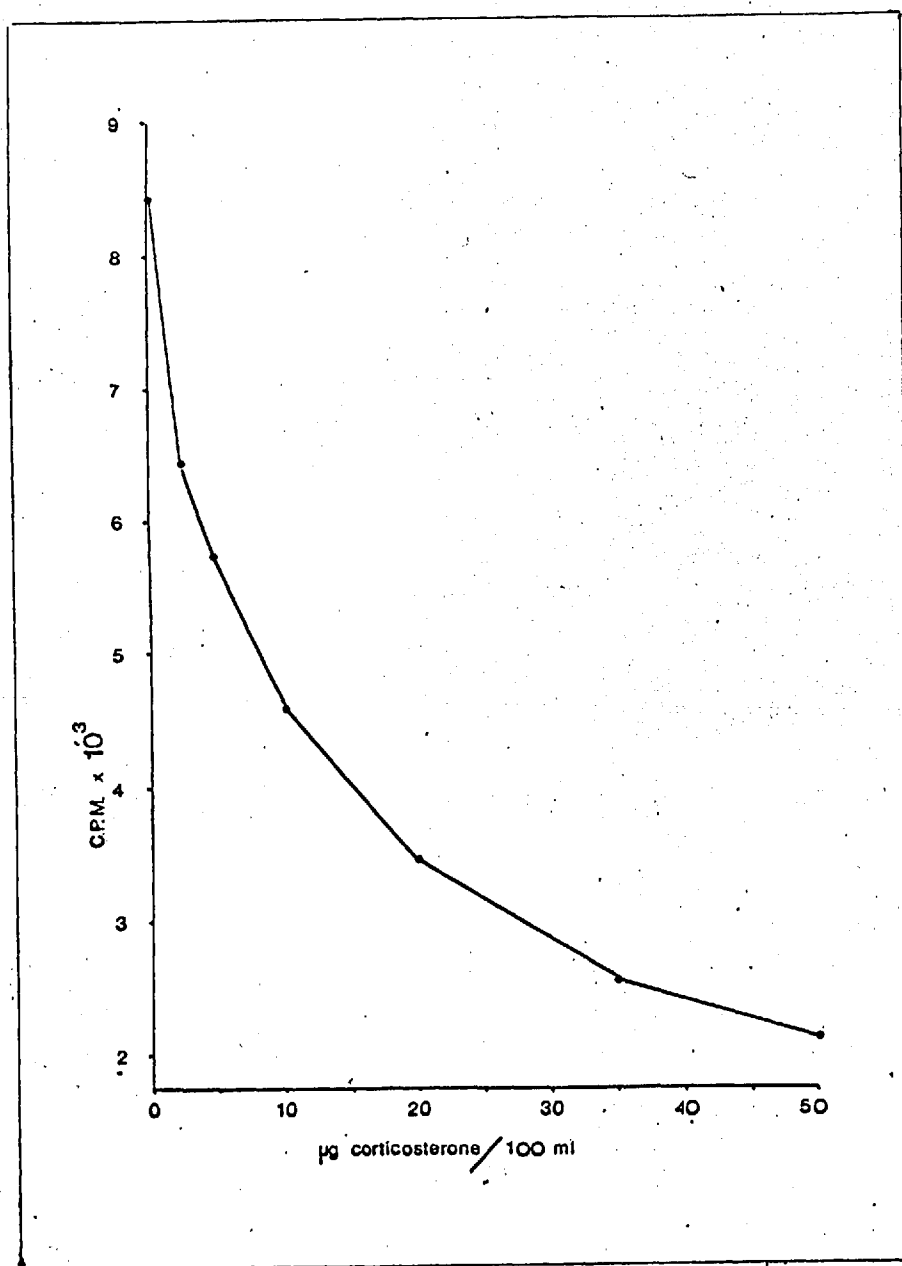
(3). Preparation of samples

During the present investigation, much difficulty was experienced in obtaining rat plasma samples which were free from fibrin clots and it was found more convenient to measure serum levels of corticosterone.

(4). Treatment of standards

The range of concentration of standards, found to be most appropriate to the levels of corticosterone routinely measured, was 0 to 50 μ g corticosterone/100 ml. A solution of corticosterone at a concentration of 5 mg/100 ml ethanol was further diluted to give concentrations of 2.5 ng, 5.0 ng, 10.0 ng, 20.0 ng, 35.0 ng, 50.0 ng in 0.5 ml. Each standard 0.5 ml sample was assayed in duplicate with the same extraction, binding and counting procedures as the samples. A typical standard curve, obtained by plotting radioactivity, expressed in c.p.m. against concentration of corticosterone, is shown in Fig. 16.

Fig. 16. Standard Curve for Corticosterone.



(5). Internal standards and quality controls

In order to check the variability of assays and their precision, serum samples containing known levels of glucocorticoids were included in each assay. Ideally, these should have been obtained from the rat, but since insufficient material was available, horse serum (Borouhgs Wellcome No. 3 Serum) was used. Each batch was divided into two portions on arrival from the suppliers and sufficient corticosterone was added to one portion to increase the concentration of glucocorticoids by 5 ng/0.5 ml so that two sets of standard control samples, each containing a different concentration of corticosteroids, were obtained. Each portion was pipetted into 0.5 ml aliquots and these were stored at -20°C until required for assay. These quality controls were treated in the same way as the standards and samples. The concentration of corticosterone obtained in these samples throughout the study are shown in Table 6.

The percent recovery was routinely calculated from the difference between the two internal standards.

Table 6. Mean values for quality control

<u>Sample</u>	<u>Corticosterone</u> <u>$\mu\text{g corticosterone}/100\text{ ml serum}$</u>
Sample 1	(15) 6.7 ± 2.5
Sample 2	(15) 11.3 ± 2.7

No. of assays are shown in paranthesis. Values are given as mean $\pm$ standard deviation.

(6). Calculation of Results

The concentration of corticosterone equivalent to the radioactivity present in each sample is obtained directly from the standard curve and divided by the percent recovery. Corticosterone levels are expressed in terms of μg corticosterone/100 ml serum.

(7). Conditions of Assay

(a). Extraction Procedure

Mayes and Nugent (1966) used 13 ml dichlormethane to extract 1 ml of plasma. In the present study, the volume of serum obtained from each rat varied between 0.5 ml and 2.0 ml and assays were normally performed on 0.5 ml serum. When these samples were extracted with 10 mls redistilled dichlormethane, the recovery was 100%, which was confirmed by the experiment summarised in Table 7, when a volume equivalent to 2.5 ng $1\text{-}2\text{H}^3$ corticosterone was extracted with dichlormethane after addition to 0.5 ml serum from adrenalectomised rats.

(b). The Binding Procedure

The time sequence and temperature used in the present method were essentially those originally described by Murphy (1964, 1967) as modified by Tait, Schulster, Okamoto and Tait (1970) for corticosterone and by Reeves, de Souza, Thompson and Diczfalusy (1970) for progesterone. Strict adherence to these time intervals and temperatures was observed.

(c). Corticosterone-Binding Globulin

A highly specific binding protein was regarded by Murphy (1967) as essential for protein binding assays, in order to obviate interference from extraneous factors.

Table 7. Efficiency of extraction of serum samples with dichlormethane

<u>Sample</u>	<u>c.p.m.</u>	<u>% recovery</u>
A	3200	
	3150	
B	3300	104
	3200	101
	3150	99
	3200	101

- A. Control samples in duplicate. 0.02 ml $1-2H^3$ corticosterone (equivalent to 2.5 ng) were added to 0.5 ml ethanol which was further diluted by the addition of 10 ml ethanol. 2 ml of this solution were added to 5 ml scintillant and counted for 5 mins as described previously.
- B. 0.02 ml $1-2H^3$ corticosterone were added to 0.5 ml serum from adrenalectomised female rats in quadruplicate and extracted with 10 ml dichlormethane as described in the text. 2 ml samples of the lower steroid-containing solvent layer were added to 5 ml scintillant and counted for 5 mins.

In the present study, corticosteroid-binding globulin (C.B.G.) from a human source was used. Other workers have employed human plasma in the assay of cortisol and dog plasma for corticosterone assays (Murphy, 1967). The affinity of human C.B.G. for corticosterone is similar to that for cortisol (Reeves, de Souza, Thompson and Diczfalusy, 1970) but since Braunsberg and James, (1960) have shown that the levels of circulating cortisol in rat plasma, determined by chromatography, are barely detectable there was no possibility of interference from cortisol with the binding of corticosterone to human C.B.G.

Preliminary investigations showed that of the other steroids testosterone and oestrogen had little binding affinity for human C.B.G. (Table 8). Although there was significant binding of progesterone, Hashimoto and Wiest (1969) have reported that the concentration of progesterone in the ovarian vein, measured by a chromatographic method, was less than 1.5 $\mu\text{g/ml}$ blood throughout the oestrus cycle. Interference by progesterone with the binding assay was therefore not regarded as a significant factor.

(d). Preparation of Corticosterone-Binding Globulin

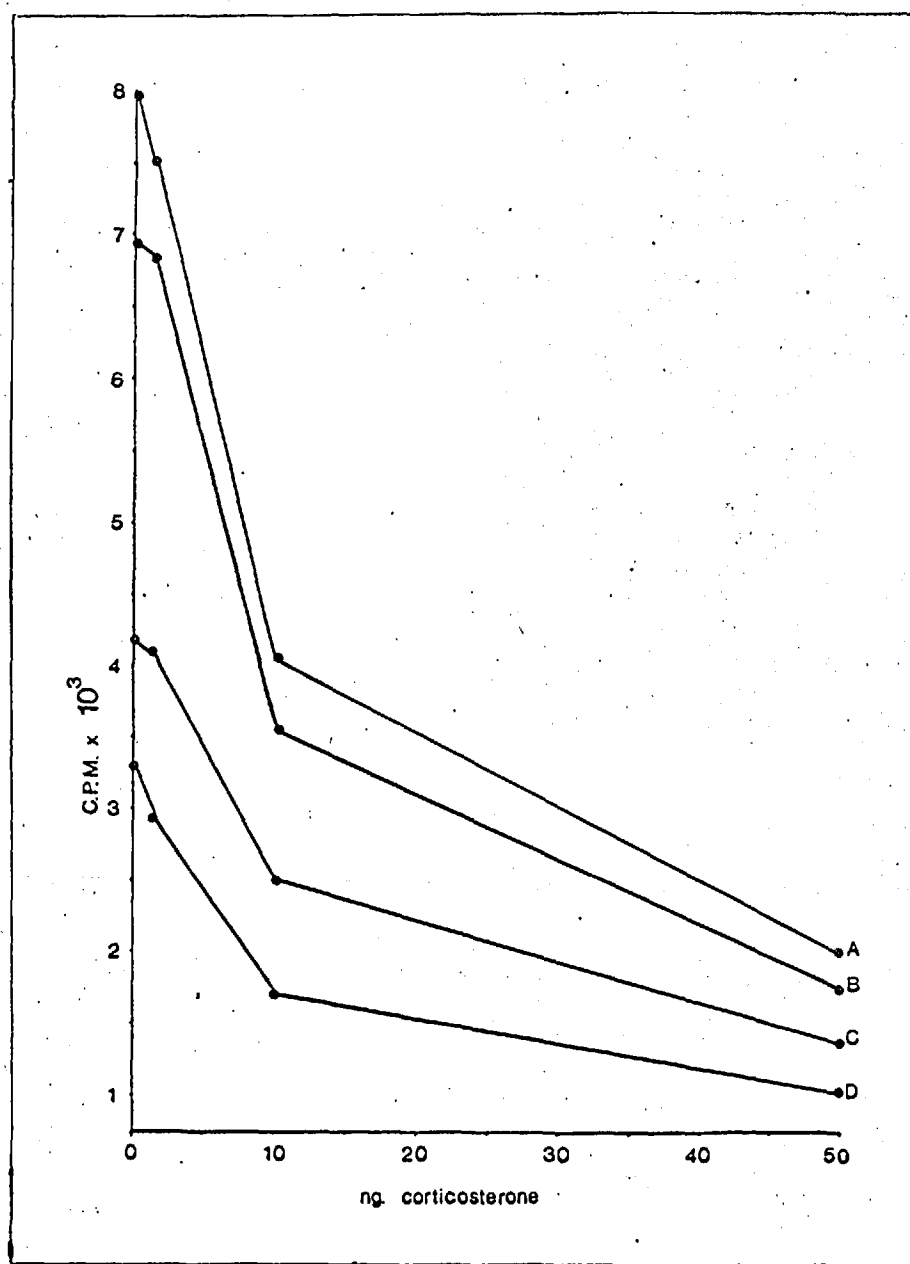
Previous workers achieved increased binding specificity by treating donors with dexamethasone before blood collection (Murphy, 1967). Accordingly, plasma was obtained from a healthy human female subject after treatment with 3 mg dexamethasone at 6.0 p.m. and 3 mg at 12 midnight, before blood was taken the next morning at 9 a.m.

Table 8. Interference of steroid hormones with corticosterone binding

<u>Steroid Hormone/0.5 ml</u>	<u>Equivalent in μg corticosterone/100 ml</u>
Testosterone	3 μg
Oestradiol-17 β	2.5 μg
Progesterone	10 μg
Cortisol	48 μg

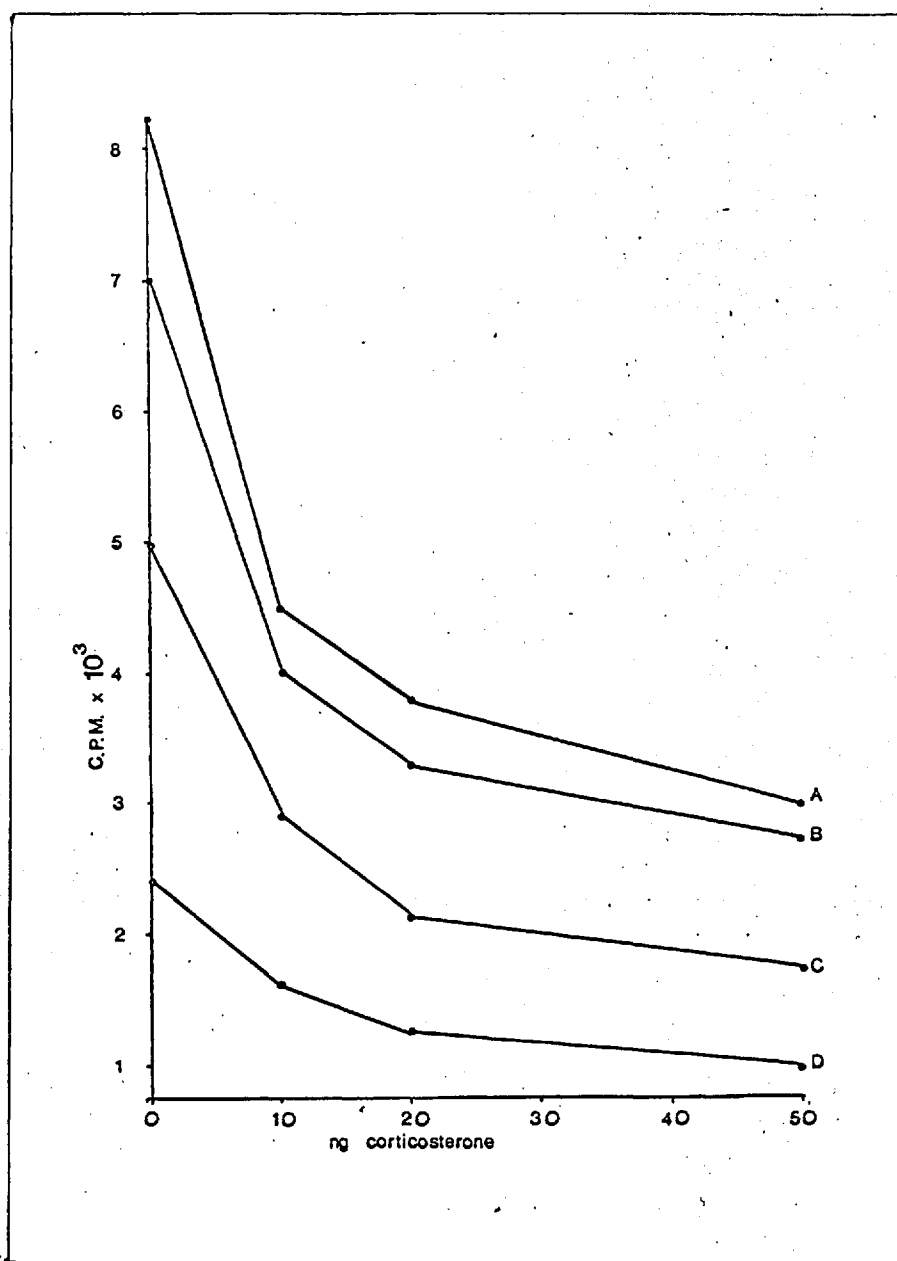
0.5 ml of Binding Solution was added to duplicate tubes containing 50 ng of the above steroid hormones. After the binding procedure described in the text, the degree of displacement of labelled corticosterone from the binding globulin was read off from a standard curve for corticosterone and expressed in terms of the equivalent in μg corticosterone/100 ml.

Fig. 17. Effect of Different Concentrations of Corticosteroid-Binding Globulin (CBG) in the Binding Solution on the Standard Curve for Corticosterone.



- A: Binding solution contained 5% CBG and gave 50% binding capacity.
- B: Binding solution contained 4% CBG and gave 43% binding capacity.
- C: Binding solution contained 3% CBG and gave 26% binding capacity.
- D: Binding solution contained 2% CBG and gave 19% binding capacity.

Fig. 18. Effect of Different Concentrations of 1-2H³ Corticosterone in the Binding Solution on the Standard Curve for Corticosterone.



- A: Binding solution contained 1-2H³ corticosterone at a concentration equivalent to 2 ng/assay and gave 53% binding capacity.
- B: Binding solution contained 1-2H³ corticosterone at a concentration equivalent to 1.5 ng/assay and gave 44% binding capacity.
- C: Binding solution contained 1-2H³ corticosterone at a concentration equivalent to 1.0 ng/assay tube and gave 31% binding capacity.
- D: Binding solution contained 1-2H³ corticosterone at a concentration equivalent to 0.5 ng/assay tube and gave 21% binding capacity.

For each batch of plasma thus obtained, it was necessary to establish the optimal concentration of C.B.G. for the binding solution. A range of binding solutions was prepared as described previously, each with a different proportion of plasma. 0.5 ml of each solution was pipetted into duplicate assay tubes containing 1 ng, 10 ng and 50 ng corticosterone and to 4 blank tubes. The binding procedure was carried out as described previously in this Chapter, although 0.4 ml water was added to 2 blank tubes, in place of dextran-coated charcoal to indicate the total radioactivity present in the assay. The aim was to find which concentration of plasma in the binding solution allowed sufficient displacement of $1\text{-}2\text{H}^3$ corticosterone by the cold sample to give a sensitive curve but also allowed adequate binding to the C.B.G. Binding capacity was routinely calculated for each assay from the difference between radioactivity in the blank tube with water added (c.p.m. _{total}) and the blank tube with charcoal added (c.p.m. _{Ostd.}) and was expressed in the form

$$\% \text{ Binding} = \frac{\text{c.p.m.}_{\text{total}} - \text{c.p.m.}_{\text{Ostd.}}}{\text{c.p.m.}_{\text{total}}} \times 100$$

Under optimal conditions this was usually 50% (Murphy, 1964) and was usually obtained by a 4 to 5% concentration of plasma in the binding solution (Fig. 17)

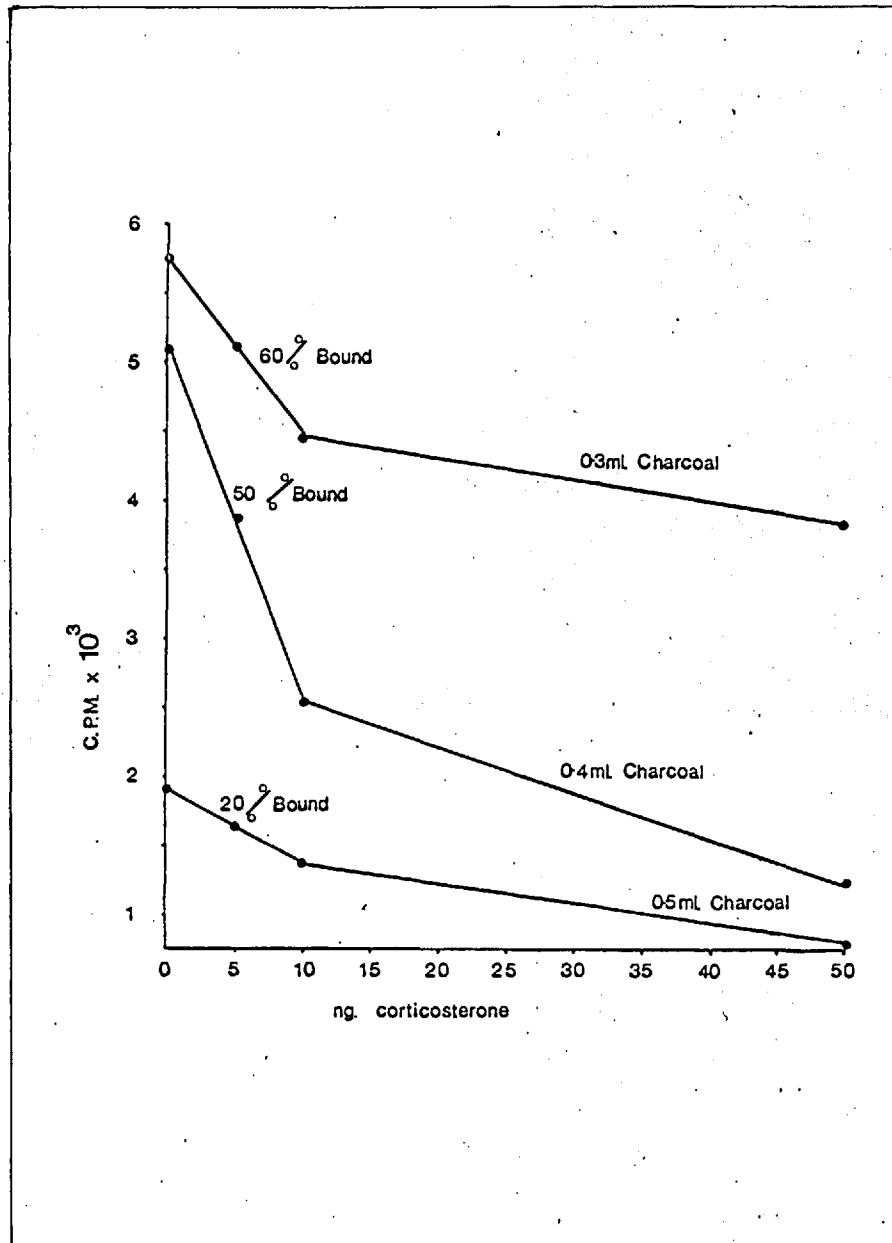
(e). $1\text{-}2\text{H}^3$ Corticosterone

The concentration of $1\text{-}2\text{H}^3$ corticosterone in the binding solution sufficient for a sensitive standard curve was determined by the same procedure used for C.B.G. and was found to be 2 ng/assay tube (Fig. 18).

(f). Dextran-coated charcoal

Charcoal coated with dextran was used as a solid phase in the separation of biological material by Herbert, Gottlieb, Larr and Wasserman (1964). Nugent and Mayes (1966) adapted the dextran-coated charcoal technique for use in the cortisol assay, adding 0.5 ml of the 0.5% charcoal - 0.05% dextran mixture to each assay tube.

Fig. 19. Effect of Different Amounts of the 0.5% Charcoal-0.05% Dextran Mixture
On the Standard Curve and Binding Capacity (% Bound).



However, as shown on Fig. 19 , under the assay conditions employed in the present study 0.4 ml was found to give optimal results.

Evaluation of Method

Table 9. Comparison of mean levels of circulating corticosterone in this and other studies

<u>Study</u>	<u>Sex</u>	<u>No</u>	<u>Corticosterone</u> <u>µg/100 ml serum</u>	
			<u>Not corrected for</u> <u>skew distribution</u>	<u>Corrected for</u> <u>skew distribution</u>
Present study	Male	24	5.1 $\pm$ 4.2	3.3 $\pm$ 2.3
Present study	Female	67	7.4 $\pm$ 5.3	6.3 $\pm$ 2.5
Kitay (1963b)	Male	8	8.9 $\pm$ 2.1	
Kitay (1963c)	Female	8	22.7 $\pm$ 6.9	
Keller et al (1966)	Male	5	9.5 $\pm$ 1.8	

The mean values for both male and female rats calculated from all the control results obtained during the study were lower than reported by other workers using fluometric methods even when uncorrected for skew distribution, as shown in Table 9. . In the present investigation both adrenalectomised males and females had levels of corticosterone too low for detection by the protein binding method, whereas Keller et al (1966) observed a residual fluorescence in these rats equivalent to 2 µg corticosterone/100 ml serum. It was therefore concluded that the method used in the present investigation was both more sensitive and more specific for corticosterone than the fluorimetric method .

CHAPTER 5. A Study of the Effects of Oestrogen Administration on Liver
Tryptophan Oxygenase, Tyrosine Aminotransferase and Alanine
Aminotransferase Activities.

- I. Sex Differences and Seasonal Variation in Tryptophan Oxygenase, Tyrosine Aminotransferase and Alanine Aminotransferase Activities and Serum Corticosterone Levels.
- II. The Effect of Oestrogen Administration on Tryptophan Oxygenase, Tyrosine Aminotransferase and Alanine Aminotransferase Activities and Serum Corticosterone Levels.
- III. Mechanism of Increases in Tryptophan Oxygenase, Tyrosine Aminotransferase and Alanine Aminotransferase Activities in Male and Female Rats.

I. Sex Differences and Season Variations in Tryptophan Oxygenase, Tyrosine Aminotransferase, Alanine Aminotransferase and Serum Corticosterone.

INTRODUCTION

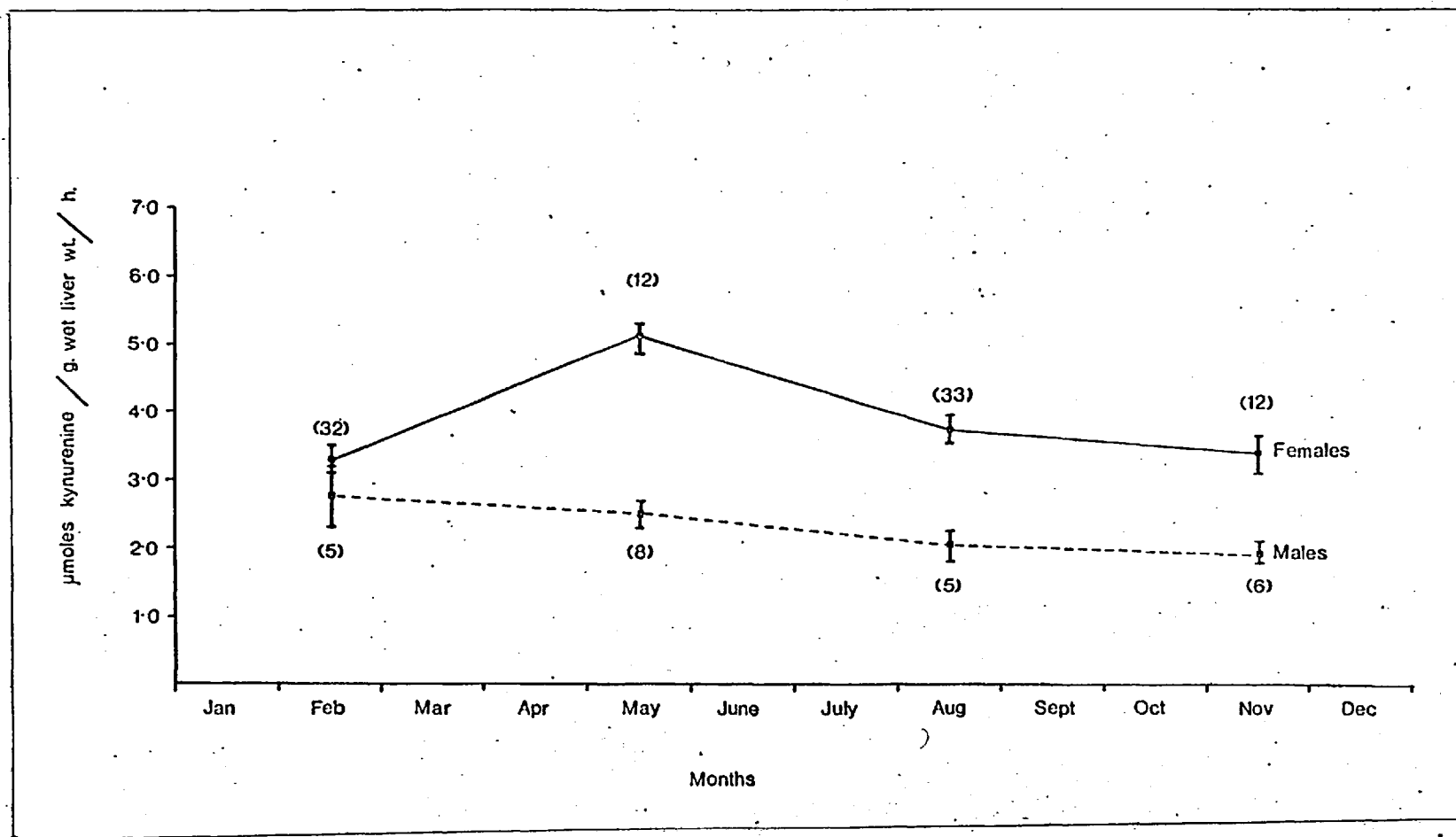
TO and TAT activities were assayed in control male and female rats kept under standardised conditions, throughout the two years of the present study. It was therefore possible to investigate sex differences and seasonal variations in the activities of these two enzymes by examination of these results. Since AAT activity and serum corticosterone levels were not assayed continuously, these data were unsuited to investigation for seasonal variations, although sufficient results were available to establish the presence of sex differences.

RESULTS

The results for TO and TAT are shown on Table 10, and Figs. 20 and 21. There was a marked seasonal variation for TO activity in female rats, with maximum enzyme activity between April and June, while from January to March activity was at its lowest level. In contrast, TO activity in male rats did not vary throughout the year. From January to March enzyme activity in males and females was similar, but thereafter higher levels were maintained in females. TAT activity also varied seasonally; activity increased in females between January and June, but for males it was maximal between July and September. It was only during this latter period that activity in male rats was significantly greater than in females.

AAT activity was higher in male rats than in females although serum corticosterone levels in females were elevated above the males (Table 11).

Fig. 20. Seasonal Variation of Tryptophan Oxygenase Activity in Male and Female Rats.



In Figs. 20 and 21 the numbers in parentheses are the numbers of rats killed during each 3 monthly period. Each point represents the mean value for each group and vertical lines represent SEM, levels of significance are shown on Table 10.

Fig. 21. Seasonal Variation of Tyrosine Aminotransferase Activity in Male and Female Rats.

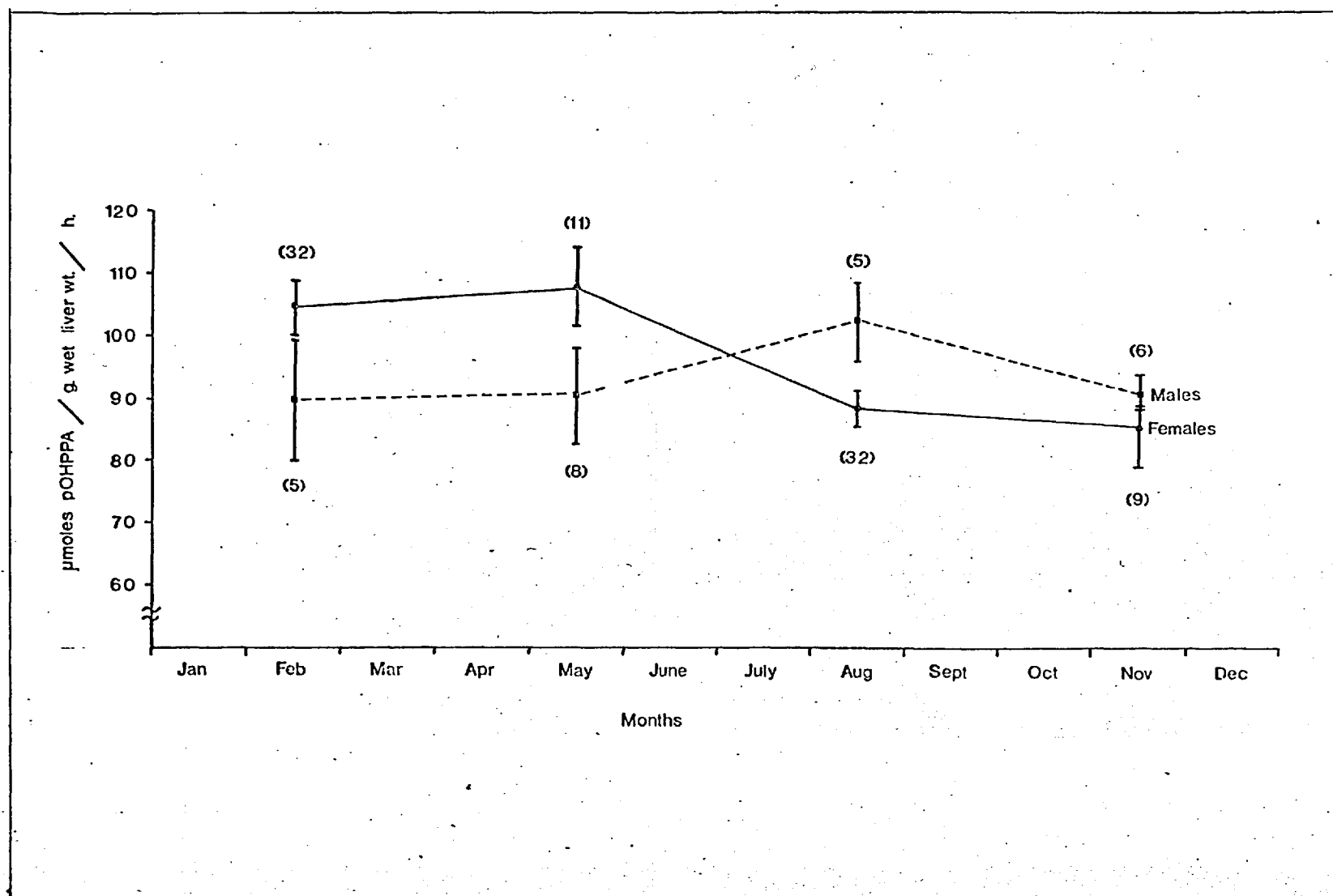


Table 10. Sex Differences and Seasonal Variation in Tryptophan Oxygenase and Tyrosine Aminotransferase Activities Over 1 Year.

<u>Time of Year</u>	<u>Sex</u>	<u>Tryptophan Oxygenase</u> <u>μmoles kynurenine/g/h.</u>		<u>Tyrosine Aminotransferase</u> <u>μmoles p OHPPA/g/h.</u>	
Jan-March	Male	(5)	2.8 ± 0.5	(5)	90 ± 10
	Female	(32)	3.3 ± 0.2	(32)	105 ± 4
April-June	Male	(8)	2.4 ± 0.2	(8)	91 ± 4
	Female	(12)	5.1 ± 0.2* †	(11)	108 ± 6
Jul-Sept	Male	(5)	2.0 ± 0.1	(5)	116 ± 7**
	Female	(33)	3.8 ± 0.2 †	(32)	89 ± 3 †
Oct-Dec	Male	(6)	1.9 ± 0.2	(6)	92 ± 2
	Female	(12)	3.4 ± 0.3*	(9)	86 ± 7

In the tables in Chapters 5 and 6, in which levels of tryptophan oxygenase activity are included, they are expressed in μmoles kynurenine/g wet tissue wt/h. (μmoles kynurenine/g/h) and in those which include levels of tryosine aminotransferase activity they are expressed as μmoles p hydroxyphenylpyruvic acid/g wet tissue wt/h. (μmoles p OHPPA/g/h). Mean values ± SD are given for each group and the numbers included in each group are shown in parentheses.

Key to Table 10.

- * $p < 0.001$ compared with corresponding male controls.
- † $-0.01 > p > 0.001$ compared with corresponding male controls.
- ‡ $0.02 > p > 0.01$ compared with corresponding male controls and with female controls killed between April-June.
- † + $p < 0.001$ compared with female rats killed between Jan-March and between July-Sept.
- ** $p = 0.02$ compared with male controls killed between April-June and Oct-Dec.

Table 11. Sex Differences in Alanine Aminotransferase Activity and in Serum Corticosterone Levels.

<u>Sex</u>	<u>Alanine Aminotransferase</u> <u>mmoles pyruvate/g/h</u>		<u>Serum Corticosterone</u> <u>µg corticosterone/100 ml</u>	
			<u>Uncorrected for skew-</u> <u>distribution</u>	<u>Corrected for skew</u> <u>distribution</u>
Male	(10)	4.1 ± 0.8*	(24) 5.1 ± 4.2 [†]	(24) 3.3 ± 2.3*
Female	(43)	2.1 ± 0.6	(62) 7.4 ± 5.3	(62) 6.3 ± 2.5

In the tables in Chapter 5 in which levels of alanine aminotransferase activity are included, they are expressed as mmoles pyruvate/g wet liver wt/h (mmoles pyruvate/g/h) and in those where serum corticosterone levels are included they are expressed as µg corticosterone/100 ml serum.

* p < 0.001 compared with female controls.

† 0.05 > p > 0.02 compared with female controls.

DISCUSSION

The breeding season in the wild rat, *Rattus norvegicus*, from which the Sprague-Dawley strain is derived, extends from March to October, when the level of circulating sex hormones is highest (Asdell, 1965). An explanation for the results obtained with TO activity in females is that despite controlled environmental conditions of 12 h light/dark cycle and constant temperature, oestrogen levels still vary seasonally and are elevated between March and October as in the wild strain. Results reported later in this Chapter show that oestrogen administration to female rats increased TO activity so that the raised levels of oestrogen may be responsible for the elevated TO activity during these months. In male rats, variation of androgen levels did not affect TO activity (Chapter 6) which therefore remained at the same level throughout the year.

A sex difference in TO activity was not apparent between January and March, but at all other times, females had significantly higher activity than in males. Pinto and Rosenthal (1965) also reported a sex difference in TO activity, but in a later study Greengard, Baker and Friedell (1967) showed that the level of activity was higher in females than in males although this difference was not indicated as significant. It is possible that this study was performed in the January to March period when the sex difference was not apparent.

TAT activities in males and females each varies according to a distinct pattern, although the period of maximum activity for both sexes falls between March and October. It may be that the variation in sex hormone levels discussed in relation to TO activity is also responsible for the seasonal changes in TAT levels. The sex difference in TAT activity was only observed between July to September when the activity in females was lower than in males.

AAT activity is increased in direct proportion to changes in liver weight and precautions described in Chapter 4 were taken throughout this study to ensure that variations in liver weight were small. Nevertheless, male rats had elevated AAT activity above the levels in females, contrary to the observations of Keller, Richardson and Yates (1969), who reported no sex difference, although these authors took similar precautions with regard to liver and body weight to those described in the present study. It may be that the sex difference in AAT activity is only apparent at certain times of the year, as in the case of TO and TAT. Keller et al may therefore have made their observations at a time when the sex difference was not significant.

Female rats had higher serum corticosterone levels than males, which has been widely reported in previous studies, (Kitay, 1961; Glenister and Yates, 1961). This may offset the more rapid metabolism of corticosterone by the liver Δ^5 -5 α dehydrogenase-enzymes (Yates, Herbst and Urquart, 1958) in the female, to produce a net increase in circulating corticosterone relative to the male. The higher adrenal corticosteroid production in the female is due to the circulating level of oestrogens (Kitay, 1963a, 1963b, 1965) although the studies of Yates et al (1958) show that there is an unidentified non-gonadal factor associated with the development of the sex difference in the rate of hepatic corticosteroid degradation. Therefore, the sex difference in circulating corticosterone levels may not be entirely the result of sex hormone action.

The sex difference in serum corticosterone levels is first apparent at 30 days in weanling rats and continues to increase until puberty. The same pattern of development is reported for corticosterone-binding globulin (Gala and Westphal, 1965).

Considerable variation was observed throughout the present study in the levels of serum corticosterone in females. Similar findings were made by Gala and Westphal (1965) who suggested that this could be attributed to the influence of the oestrous cycle which effects serum corticosterone concentrations (Critchlow, Liebelt, Bar-Sela, Mountcastle and Lipscomb, 1963).

II. The Effect of Oestrogen Administration on Tryptophan Oxygenase, Tyrosine Aminotransferase and Alanine Aminotransferase in the Livers of Female Rats and on Serum Corticosterone Levels.

INTRODUCTION

Pregnant women have an increased rate of degradation of tryptophan along the tryptophan-niacin pathway which results in elevated levels of tryptophan metabolites being excreted in the urine (Brown, Thornton and Price, 1961). Treatment of human subjects with oestrogens raises the excretion of urinary metabolites (Rose, 1966a, 1966b) and diminishes the plasma tyrosine levels (Rose and Cramp, 1970). These changes in amino acid metabolism were attributed by Rose to increased activity of the appropriate rate-limiting enzymes on the respective degradative pathways, namely TO and TAT. Oestrogen administration in man also raises the plasma level of protein-bound cortisol (Sandberg and Slaunwhite, 1959) and it was suggested that this is capable of inducing TO and TAT synthesis and is thus responsible for their elevated activity (Rose, 1970; Rose and Cramp, 1970).

This hypothesis is supported by observations that the pregnant rat has both increased TO activity (Auricchio, Rigillo and Di Toro, 1960) and plasma corticosteron levels (Hoet, Pagni, Ekka and Saba, 1964). A similar mechanism was proposed by Keller, Richardson and Yates (1969) to explain the elevated activity of another glucorticoid-inducible enzyme of amino acid metabolism, AAT, observed after oestrogen treatment of male rats. They postulated that the increased levels of plasma-bound corticosterone, resulting from oestrogen administration, induced liver AAT synthesis.

The present study was therefore undertaken to investigate the effects of oestrogen treatment on liver TO, TAT and AAT in relation to serum corticosterone levels in female rats.

Table 12. Effects of Ovariectomy on Liver Enzyme Activities and Serum Corticosterone Levels.

<u>Treatment</u>	<u>Tryptophan Oxygenase</u> <u>μmoles kynurenine/g/h.</u>		<u>Tyrosine Aminotransferase</u> <u>μmoles p OHPPA/g/h.</u>		<u>Alanine Aminotransferase</u> <u>mmoles pyruvate/g/h.</u>		<u>Serum Corticosterone</u> <u>μg/100 ml</u>	
							<u>Uncorrected</u> <u>for skew</u>	<u>Corrected</u> <u>for skew</u>
Intact females 0.1 ml corn oil	(12)	5.1 ± 0.7	(12)	107 ± 21	(12)	1.6 ± 0.4	(7) 9.2 ± 5.7	7.3 ± 2.2
Ovariectomised females 0.1 ml corn oil	(5)	2.0 ± 0.4*	(5)	86 ± 6†	(5)	1.4 ± 0.2	(5) 1.9 ± 0.7*	(5) 1.8 ± 0.7*

* p < 0.001 compared with intact female controls.

† p = 0.05 compared with intact female controls.

Table 13. The Effect of Various Doses of Oestradiol Benzoate (Oest Benz) on Tryptophan Oxygenase , Tyrosine Aminotransferase and Alanine Aminotransferase Activities in Ovariectomised Females.

<u>Treatment</u>		<u>Tryptophan Oxygenase</u> <u>μmoles kynurenine/g/h.</u>		<u>Tyrosine Aminotransferase</u> <u>μmoles p OHPPA/g/h.</u>		<u>Alanine Aminotransferase</u> <u>mmoles pyruvate/g/h.</u>	
0.1 ml corn oil	(12)	1.2 $\pm$ 0.4		(6)	73 $\pm$ 6	(6)	1.1 $\pm$ 0.2
0.1 μg Oest Benz	(12)	1.6 $\pm$ 0.3		(6)	88 $\pm$ 12	(6)	1.4 $\pm$ 0.5
0.1 ml corn oil	(6)	2.1 $\pm$ 0.8		(6)	80 $\pm$ 21	(6)	1.1 $\pm$ 0.1
1 μg Oest Benz	(6)	6.6 $\pm$ 0.8*		(6)	89 $\pm$ 7	(6)	1.9 $\pm$ 0.4*
0.1 ml corn oil	(6)	1.8 $\pm$ 0.6		(6)	90 $\pm$ 24	(6)	1.9 $\pm$ 0.8
5 μg Oest Benz	(6)	8.8 $\pm$ 2.6*		(6)	130 $\pm$ 34*	(6)	2.8 $\pm$ 0.4†
0.1 ml corn oil	(6)	2.0 $\pm$ 0.8		(6)	86 $\pm$ 6	(6)	1.4 $\pm$ 0.4
10 μg Oest Benz	(6)	13.1 $\pm$ 3.4*		(6)	145 $\pm$ 21*	(6)	1.8 $\pm$ 0.3‡
0.1 ml corn oil	(6)	1.1 $\pm$ 0.4		(6)	72 $\pm$ 14	(6)	1.0 $\pm$ 0.2
20 μg Oest Benz	(6)	10.0 $\pm$ 1.5*		(6)	136 $\pm$ 17*	(6)	1.0 $\pm$ 0.3
0.1 ml corn oil	(6)	1.4 $\pm$ 0.4		(6)	77 $\pm$ 18	(6)	1.5 $\pm$ 0.3
50 μg Oest Benz	(6)	11.0 $\pm$ 2.6*		(6)	163 $\pm$ 45*	(6)	2.5 $\pm$ 0.7†

* p < 0.001 compared with corresponding control group.

† 0.05 > p > 0.02 compared with corresponding control group.

‡ 0.05 > p > 0.02 compared with the 10 μg Oest Benz-treated group.

Table 14. Effect of Various Doses of Oestradiol Benzoate (Oest Benz) on Serum Corticosterone Levels and Adrenal Weights in Ovariectomised Rats.

<u>Treatment</u>	<u>Adrenal Weights</u> (mg)	<u>Serum Corticosterone Levels</u> <u>µg corticosterone/100 ml</u>	
		<u>Uncorrected for skew</u>	<u>Corrected for skew</u>
0.1 ml corn oil	(7) 84 ± 10	(9) 2.3 ± 1.1	2.1 ± 1.6
0.1 µg Oest Benz	(7) 70 ± 15	(10) 2.7 ± 1.1	2.5 ± 1.6
0.1 ml corn oil	(6) 88 ± 14	(5) 1.7 ± 0.3	1.7 ± 0.1
1 µg Oest Benz	(6) 93 ± 20	(6) $4.8 \pm 2.2 \dagger$	$4.4 \pm 1.6 \dagger$
0.1 ml corn oil	(6) 98 ± 22	(6) 1.8 ± 0.7	1.7 ± 0.1
5 µg Oest Benz	(6) 80 ± 14	(6) $6.2 \pm 2.4 \dagger$	$5.7 \pm 1.6^*$
0.1 ml corn oil	(6) 76 ± 12	(6) 1.9 ± 0.7	1.8 ± 0.7
10 µg Oest Benz	(6) 84 ± 15	(6) $5.4 \pm 1.3^*$	$5.2 \pm 1.3^*$
0.1 ml corn oil	(6) 82 ± 9	(6) 2.5 ± 0.7	2.5 ± 1.2
20 µg Oest Benz	(6) 71 ± 12	(6) $5.1 \pm 1.2 \dagger$	$4.9 \pm 1.2^*$
0.1 ml corn oil	(6) 90 ± 12	(6) 1.8 ± 0.8	$1.7 \pm 0.1 \dagger$
50 µg Oest Benz	(6) 84 ± 14	(6) $3.8 \pm 0.9 \dagger$	$3.7 \pm 0.1^{**}$

* $p < 0.001$ compared with corresponding controls.

† $0.01 > p > 0.001$ compared with corresponding controls.

‡ $p = 0.01$ compared with corresponding controls.

** $p = 0.05$ compared with 5 µg Oest Benz-treated group.

Fig. 22. Effect of Various Doses of Oestradiol Benzoate on Liver Tryptophan Oxygenase in Ovariectomised Rats.

In Figs. 22, 23, 24 and 25, cross-hatched columns represent mean values for oestrogen-treated rats and black columns denote those for corresponding controls. Vertical lines represent SEM. Oestrogen was administered in the daily doses shown for 14 days. Unless otherwise stated, there were 6 animals in each group.

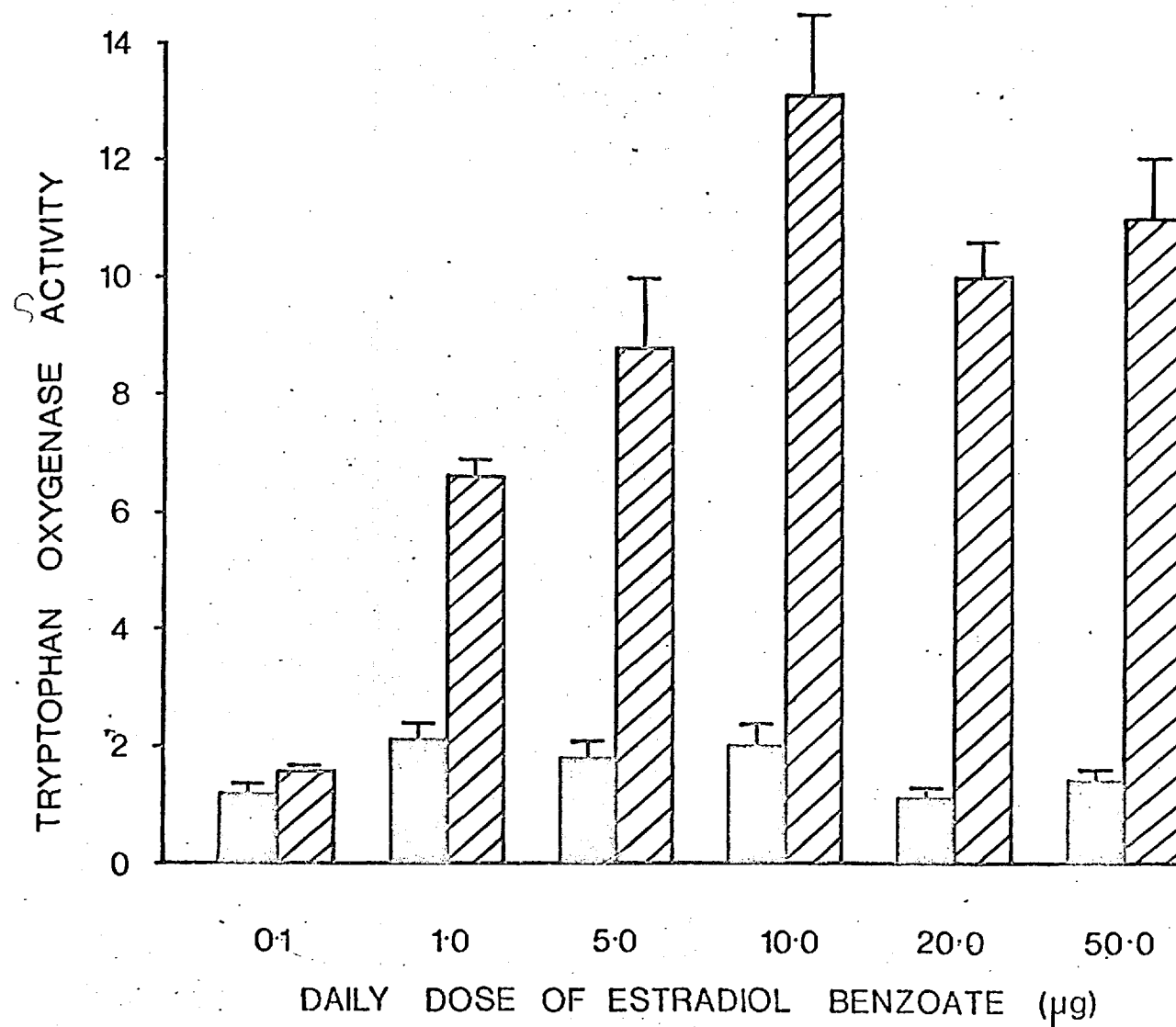


Fig. 23. Effect of Various
Doses of Oestradiol
Benzoate on Liver
Tyrosine Amino-
transferase in
Ovariectomised Rats.

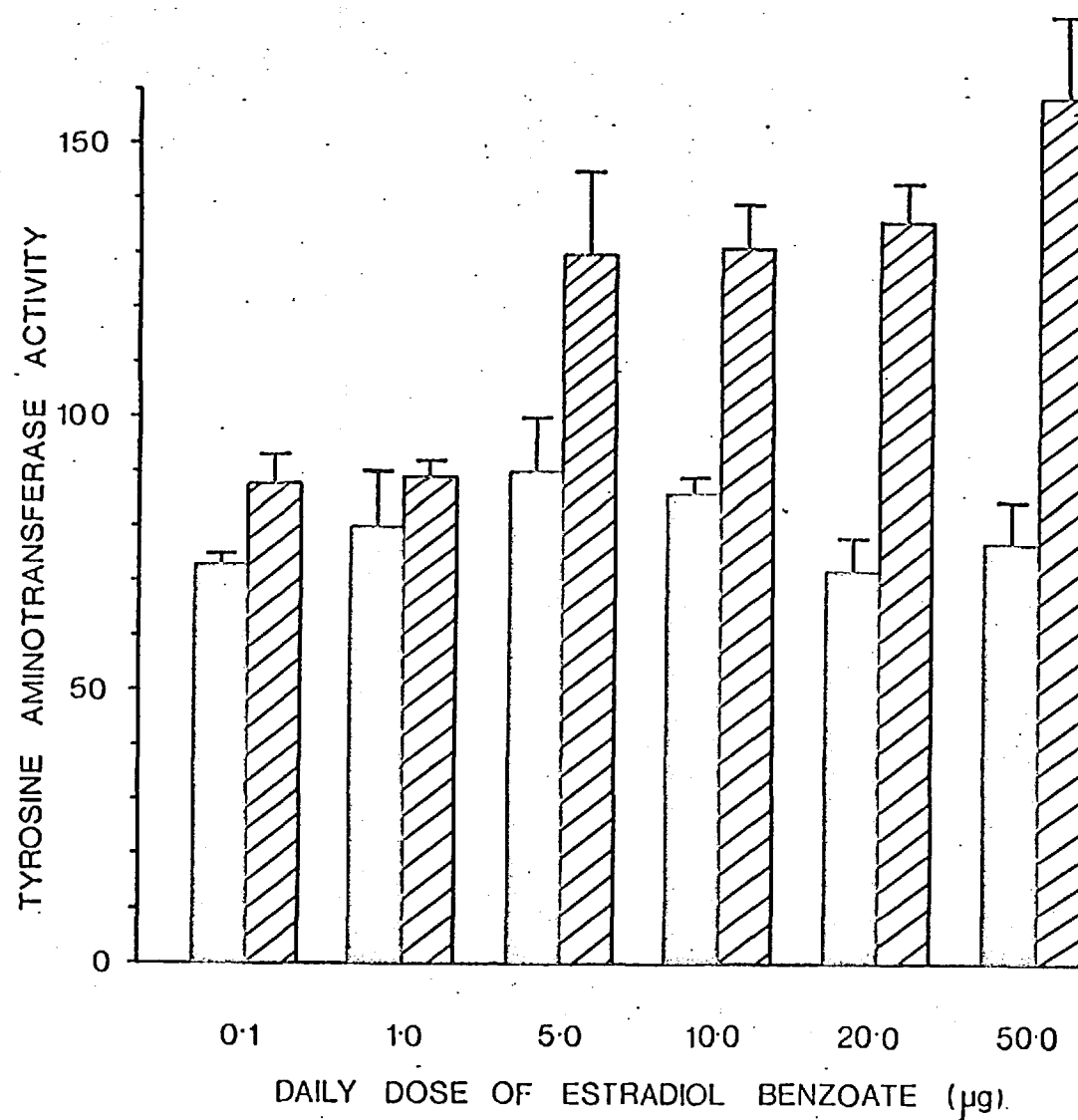


Fig. 24. Effect of Various
Doses of Oestradiol
Benzoate on Liver
Alanine Amino-
transferase in
Ovariectomised
Rats.

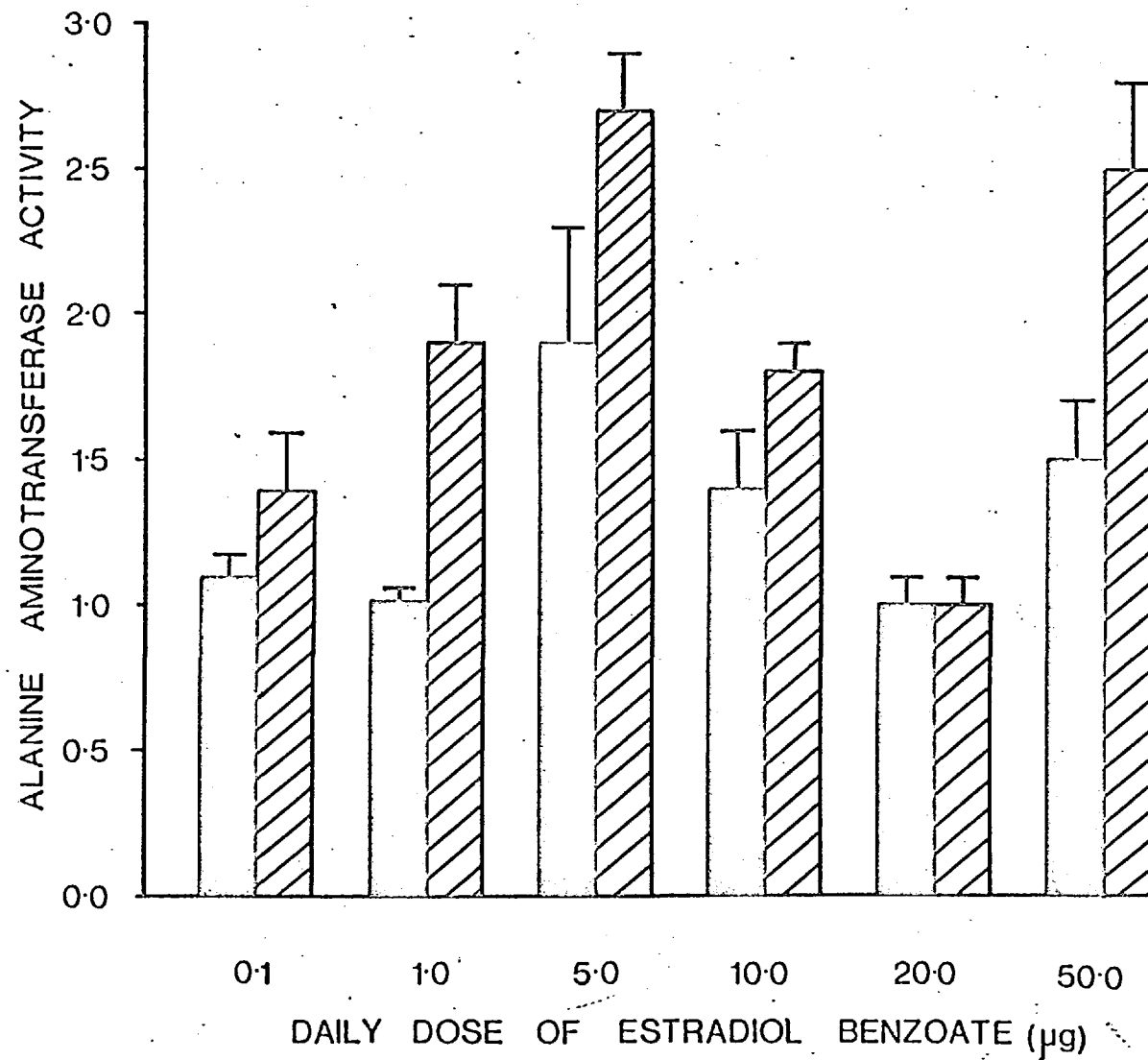
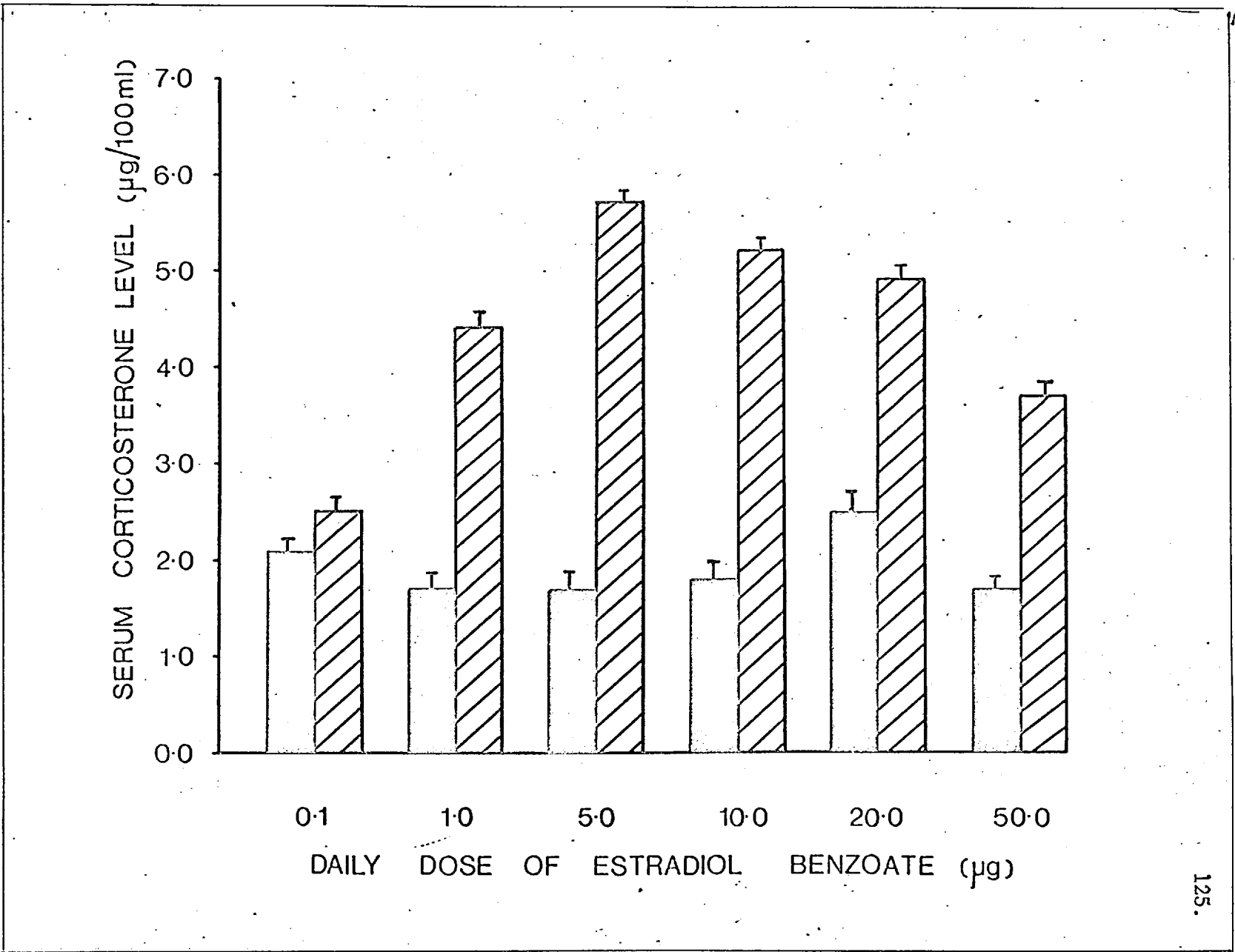


Fig. 25. Effect of Various
Doses of Oestradiol
Benzoate on Serum
Corticosterone
Levels in
Ovariectomised
Rats.

Mean values and SEM were obtained after $\log_{10}$ transformation. Control and test groups for 0.1 μg dose each consisted of 10 rats and control group for 1 μg dose consisted of 5 rats.



- (1) The effect of oestrogens at different dosages on tryptophan oxygenase, tyrosine aminotransferase and alanine aminotransferase in relation to serum corticosterone levels in ovariectomised female rats.

Procedure

The object of this study was to examine the effects of various doses of oestradiol benzoate on TO, TAT and AAT activities in relation to serum corticosterone concentrations. For this purpose, the effect of endogenous oestrogens was removed by carrying out the study on ovariectomised rats.

Keller et al (1969) observed increased AAT activity in male rats after injecting them with 10 µg oestradiol benzoate daily for 14 days. Therefore in the present study, ovariectomised rats were injected with the following doses of oestradiol benzoate over a 14 day period:-

0.1 µg, 1.0 µg, 5.0 µg, 10.0 µg, 20.0 µg 50.0 µg.

Each group was matched with an appropriate control group injected with a corresponding volume of corn oil. A group of corn oil-treated intact females has been included in Table 12, for the purposes of comparison, although they were not part of these experiments.

Results

The results for oestrogen treatment of ovariectomised rats are summarised on Table 12. Ovariectomy decreased both TO activity and serum corticosterone levels in all corn oil-treated females compared with the similarly treated group of intact females ($p < 0.001$). There was a slight decrease in TAT activity, but this was of marginal statistical significance ($p = 0.05$). When the results for 81 intact female controls and 34 ovariectomised rats studied throughout the investigations on which this thesis is based were pooled into 2 groups, and examined by Student's *t* test the difference did not achieve a significant level (intact rats 91 ± 20 ; ovariectomised rats 83 ± 20 $t = 1.971$; $0.1 > p > 0.05$). AAT activity was not modified by ovariectomy.

TO activity was unaffected by 0.1 μ g oestradiol benzoate for 14 days, but was increased by the complete range of higher doses (Fig. 22). TAT activity was unaltered by either 0.1 μ g or 1.0 μ g oestradiol benzoate. 5 μ g and 10 μ g elevated TAT compared with the respective control groups and activity was raised still further by the 20 μ g and 50 μ g doses (Fig. 23; Table 13).

The response of AAT activity was more complex since although the level of activity was increased by daily injections of 1 μ g and 5 μ g oestradiol benzoate, 10 μ g and 20 μ g doses were without significant effect. In contrast, the 50 μ g dose of oestrogen elevated AAT activity (Fig. 24; Table 13).

Serum corticosterone levels were elevated by 1 μ g oestradiol benzoate and by higher doses. Table 14 summarises these results both before and after skew correction and demonstrates that, in general, the significance levels of the observed differences are greater after elimination of the influence of a skew distribution. (Fig. 2

Adrenal weights were unchanged by the administration of any dose of oestradiol benzoate.

- (2) The effects of oestradiol benzoate administration to intact female rats for different periods of time on liver tryptophan oxygenase, tyrosine aminotransferase and alanine aminotransferase, in relation to serum corticosterone levels.

Procedure

Increased AAT activity, after administration of 10 μ g oestradiol benzoate for 14 days, was observed by Keller et al (1969). In addition, results obtained from the experiments described in the previous section, show that TO and TAT activities were increased in ovariectomised female rats treated with this dosage of oestrogen. Therefore, 10 μ g oestradiol benzoate was administered to intact females for the following time intervals; 3 days, 7 days, 10 days, 14 days, 21 days, 35 days.

Fig. 26. Time Course for
Alanine Amino-
transferase Activity
in Intact Female Rats
Given 10 μ g Oestradiol
Benzoate Daily.

In Figs. 26, 27, 28 and 29, each point represents the mean value for the numbers of rats shown in parentheses. Vertical lines represent SEM.

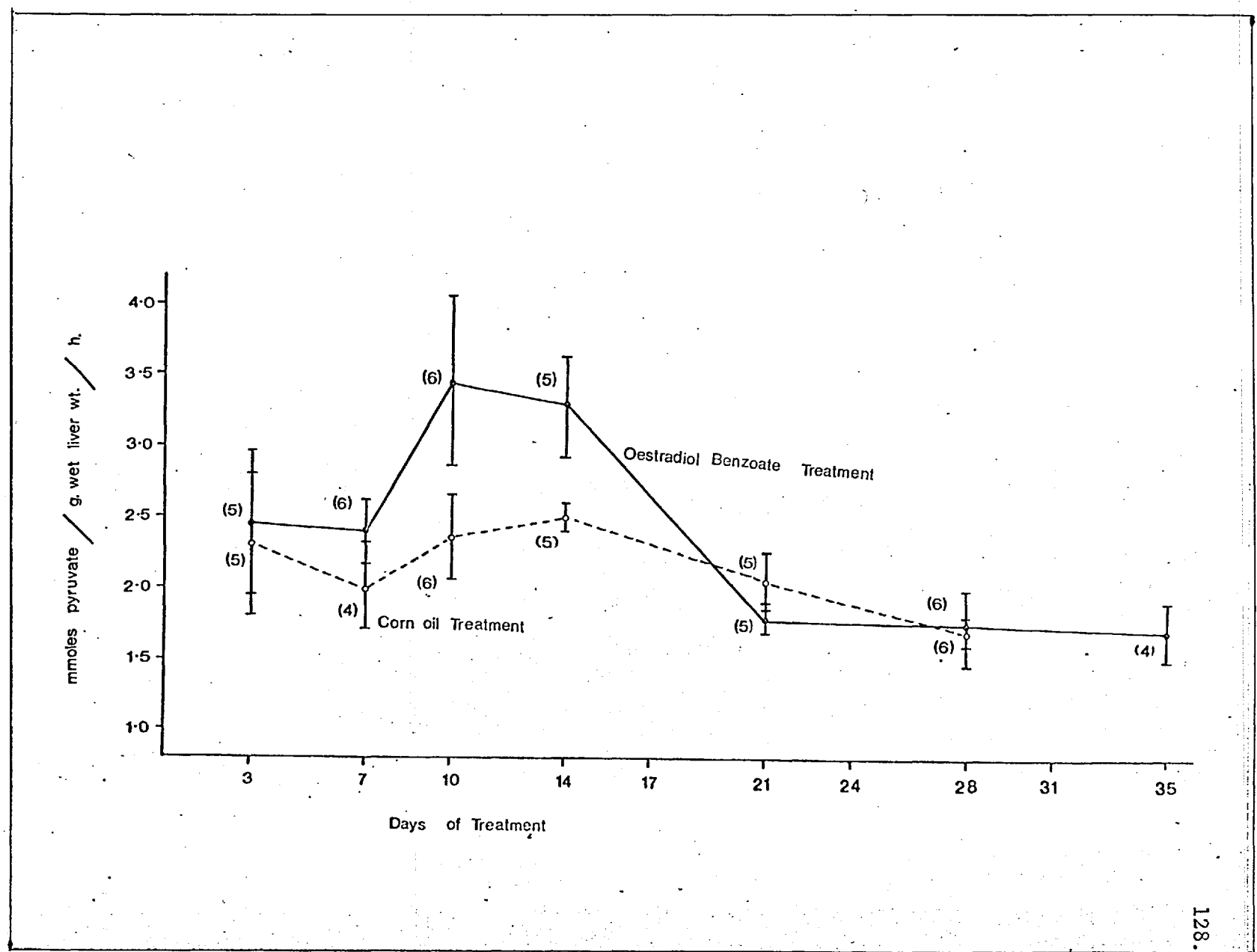


Fig. 27. Time Course for
Tyrosine Amino-
transferase Activity
In Intact Female Rats
Given 10 μ g Oestradiol
Benzoate Daily.

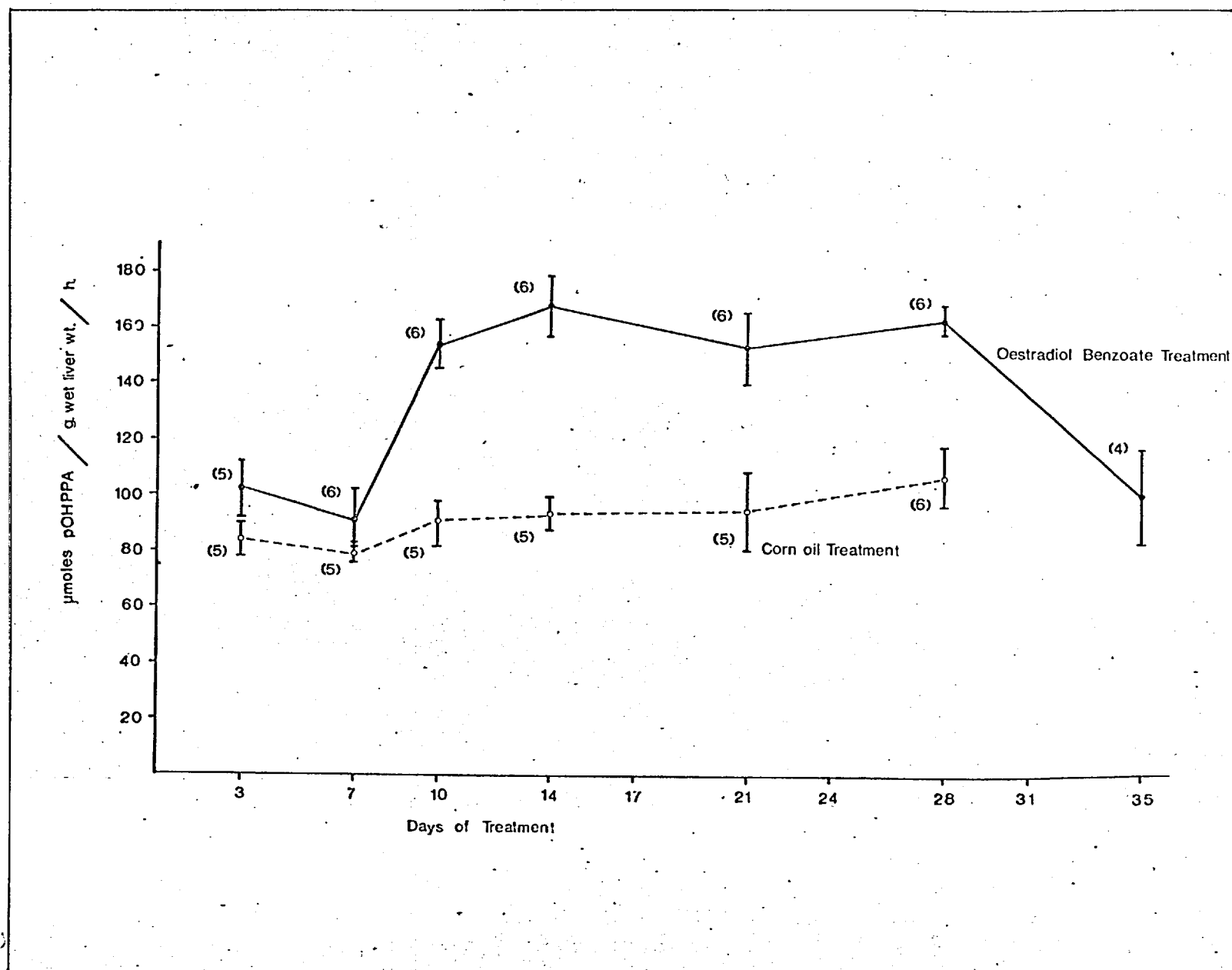


Fig. 28. Time Course for
Tryptophan Oxygen-
ase Activity In
Intact Female Rats
Given 10 μ g
Oestradiol Benzoate
Daily.

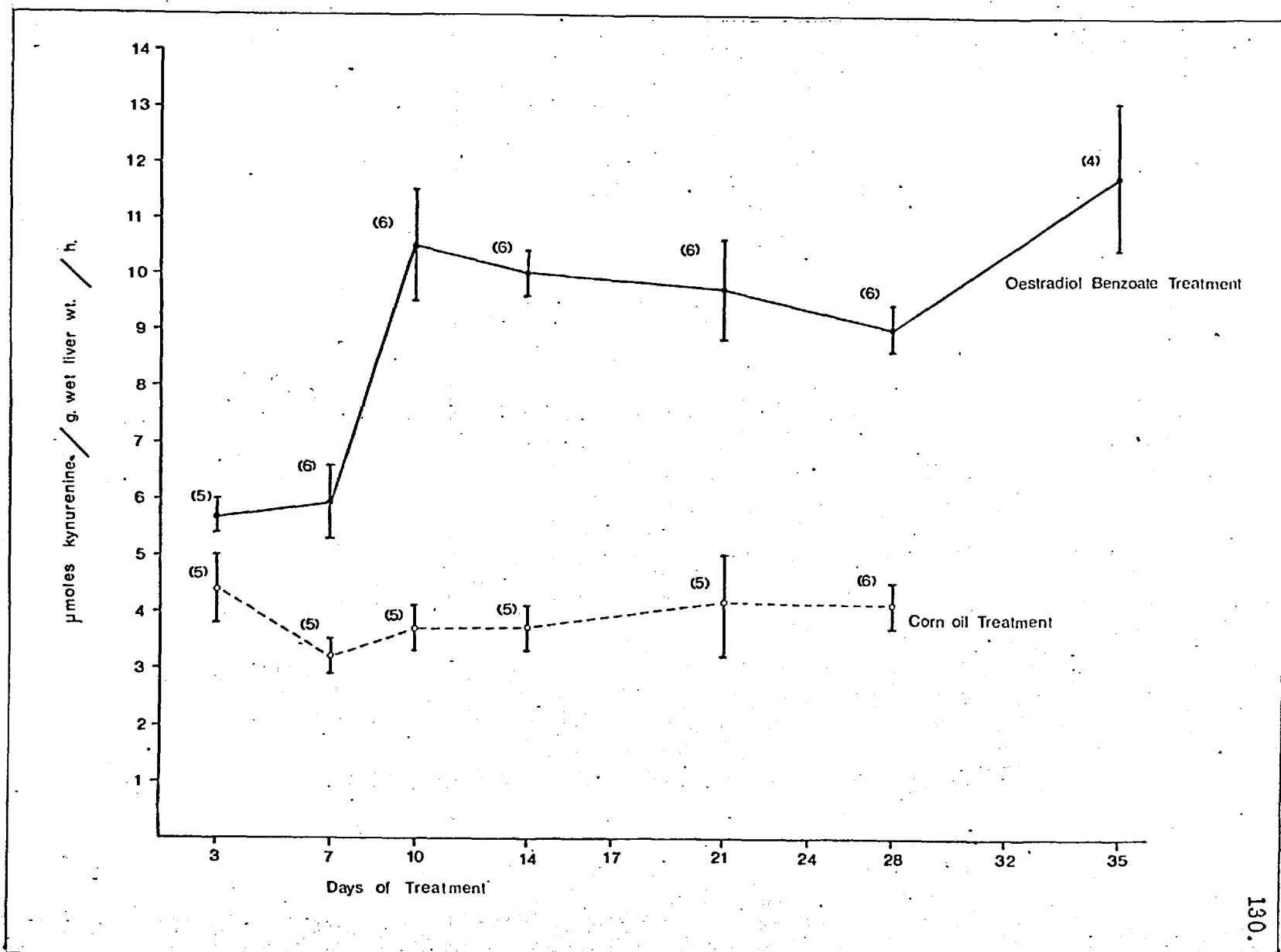


Fig. 29. Time Course For
Serum Corticosterone
Levels in Intact
Female Rats Given
10 μ g Oestradiol
Benzoate Daily.

Means and SEM were obtained
after $\log_{10}$ transformation.

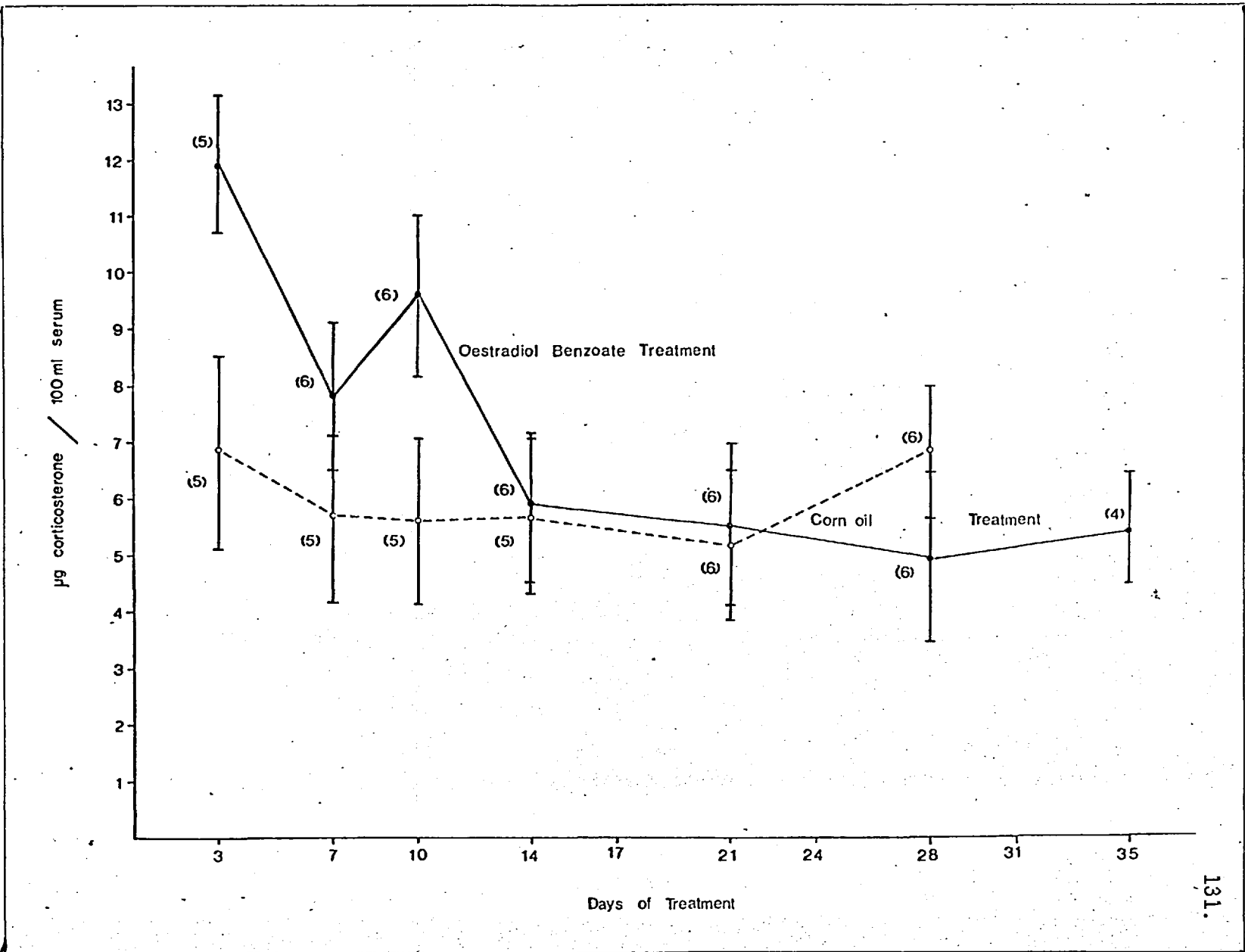


Table 15. The Response of Tryptophan Oxygenase, Tyrosine Aminotransferase, Alanine Aminotransferase to 10 µg Oestradiol Benzoate (Oest Benz) Given for Varying Lengths of Time to Intact Female Rats.

<u>Duration of Treatment</u>	<u>Nature of Treatment</u>	<u>Tryptophan Oxygenase</u> <u>µmoles kynurenine/g/h.</u>	<u>Tyrosine Aminotransferase</u> <u>µmoles OHPPA/g/h.</u>	<u>Alanine Aminotransferase</u> <u>mmoles pyruvate/g/h.</u>
3 days	10 µg Oest Benz	(5) 5.7 ± 0.3	(5) 102 ± 10	(5) 2.45 ± 0.5
	0.1 ml corn oil	(5) 4.4 ± 0.6	(5) 84 ± 6	(5) 2.3 ± 0.5
7 days	10 µg Oest Benz	(6) 5.9 ± 0.7	(6) 91 ± 12	(6) 2.4 ± 0.2
	0.1 ml corn oil	(5) 3.2 ± 0.3	(5) 79 ± 4	(4) 2.0 ± 0.3
10 days	10 µg Oest Benz	(6) 10.5 ± 1.0*	(6) 154 ± 9*	(5) 3.5 ± 0.5†
	0.1 ml corn oil	(5) 3.7 ± 0.4	(5) 91 ± 8	(6) 2.3 ± 0.3
14 days	10 µg Oest Benz	(6) 10.0 ± 0.4*	(6) 168 ± 11*	(5) 3.3 ± 0.5†
	0.1 ml corn oil	(5) 3.7 ± 0.4	(5) 94 ± 6	(5) 2.5 ± 0.1
21 days	10 µg Oest Benz	(6) 9.7 ± 0.9*	(6) 153 ± 13*	(5) 1.8 ± 0.1
	0.1 ml corn oil	(5) 4.1 ± 1.0	(5) 95 ± 14	(5) 2.05 ± 0.2
28 days	10 µg Oest Benz	(6) 9.0 ± 0.5*	(6) 164 ± 5*	(6) 1.80 ± 0.3
	0.1 ml corn oil	(6) 4.1 ± 0.4	(6) 107 ± 12	(6) 1.90 ± 0.1
35 days	10 µg Oest Benz	(4) 11.7 ± 1.3*	(4) 101 ± 17‡	(4) 1.7 ± 0.2

10 µg oestradiol benzoate was given daily for the periods indicated.

* p < 0.001 compared with corresponding controls.

† p = 0.02 compared with corresponding controls and rats treated with 10 µg oestradiol benzoate for 7 and 21 days.

‡ p < 0.001 compared with rats treated with 10 µg oestradiol benzoate for 28 days.

Table 16. Effect of 10 µg Oestradiol Benzoate for Varying Lengths of Time on Serum Corticosterone Levels in Intact Female Rats.

<u>Treatment</u>		<u>Serum Corticosterone</u> <u>µg/100 ml</u>		<u>Adrenal Weights</u> <u>mg</u>	
<u>Duration of Treatment</u>	<u>Nature of Injection</u>	<u>Uncorrected for skew</u>	<u>Corrected for skew</u>		
3 days	10 µg Oest Benz	(5) 16.0 ± 1.4	(5) 11.9 ± 1.7	(5)	95 ± 20
	0.1 ml corn oil	(5) 7.9 ± 7.03	(5) 6.8 ± 3.2	(5)	73 ± 15
7 days	10 µg Oest Benz	(6) 8.1 ± 6.8	(6) 5.7 ± 2.6	(6)	84 ± 12
	0.1 ml corn oil	(5) 7.3 ± 4.8	(5) 7.8 ± 1.8	(5)	86 ± 10
10 days	10 µg Oest Benz	(6) 9.4 ± 4.6	(6) 9.6 ± 2.4	(6)	97 ± 12
	0.1 ml corn oil	(5) 7.3 ± 5.4	(5) 5.6 ± 2.4	(5)	78 ± 10
14 days	10 µg Oest Benz	(6) 6.9 ± 3.4	(6) 5.9 ± 2.0	(6)	73 ± 25
	0.1 ml corn oil	(5) 7.5 ± 5.8	(5) 5.7 ± 2.3	(5)	82 ± 26
21 days	10 µg Oest Benz	(6) 7.6 ± 6.6	(6) 5.5 ± 2.4	(6)	98 ± 14
	0.1 ml corn oil	(5) 6.3 ± 3.4	(5) 5.2 ± 1.9	(5)	70 ± 16
28 days	10 µg Oest Benz	(6) 7.6 ± 5.2	(6) 4.9 ± 2.4	(6)	79 ± 9
	0.1 ml corn oil	(6) 7.4 ± 3.2	(6) 6.8 ± 1.6	(6)	84 ± 15
35 days	10 µg Oest Benz	(4) 5.1 ± 1.2	(4) 5.4 ± 0.1	(4)	76 ± 8

Each test group, except the rats treated for 35 days, was matched against an appropriate group of controls treated with 0.1 ml corn oil.

Results

During the first 7 days of oestradiol benzoate administration, the activity of all three enzymes remained unaltered, but was increased after 10 days of treatment. After the 14th day of oestradiol benzoate administration, there was a fall in AAT activity which remained at this reduced level for the remainder of the injection schedule (Fig. 26). TAT activity was decreased after 28 days of treatment (Table 15; Fig. 27), although TO activity remained elevated for the duration of the experiment (Table 15; Fig. 28). Serum corticosterone levels and adrenal weights remained unchanged (Table 16; Fig. 29).

- (3) Effect of the administration of 20 µg oestradiol benzoate for 28 days on tryptophan oxygenase in intact female rats.

Introduction

From the results described in the previous section, it seems that treatment with a daily dose of 10 µg oestradiol benzoate for prolonged periods does not produce increases in either AAT or TAT activities, although TO activity still remains raised throughout the 35 days of treatment. In order to investigate the effects of higher doses of oestrogen administered over an extended period on TO activity, 20 µg oestradiol benzoate were administered daily for 28 days to intact female rats. Thus, the following experimental groups were included in the study:-

- (a) Intact females treated with 0.2 ml corn oil, daily for 28 days.
- (b) Intact females treated with 20 µg oestradiol benzoate daily for 28 days.

Table 17. Effect of a Daily Dose of 20 μ g Oestradiol Benzoate (Oest Benz) for 28 days on Tryptophan Oxygenase.

<u>Treatment</u>	<u>Tryptophan Oxygenase</u> <u>μmoles kynurenine/g/h.</u>
0.1 ml corn oil for 28 days	(6) 3.9 ± 0.7
20 μ g Oest Benz for 28 days	(6) $6.7 \pm 1.4^*$

* $p < 0.001$ compared with the control group and $0.01 > p > 0.001$ compared with females treated for 14 days with 10 μ g oestradiol benzoate daily.

Results

TO activity was increased above that of the controls by administration of 20 μ g oestradiol benzoate for 28 days (Table 17). However, these elevated levels were significantly lower ($0.01 > p > 0.001$) than those seen in females treated with 10 μ g oestradiol benzoate daily for 14 or 28 days, reported in the previous section.

DISCUSSION

The effect of oestrogens on corticosterone secretion by the adrenals of male and female rats has been investigated extensively by Kitay (1963a, 1963b). He found a sex difference in the effect of a depot oestrogen preparation (polyoestradiol phosphate) on pituitary-adrenal function; male rats showed increased adrenal secretion of corticosterone and raised plasma corticosterone levels after oestrogen administration, whereas females had diminished adrenal corticosterone secretion and unchanged plasma corticosterone levels. Kitay, Coyne and Newsom, (1965) showed that ovariectomy decreased both plasma corticosterone concentrations and adrenal corticosteroidogenesis and treatment of spayed females with oestrogen reversed these effects.

In the present study, ovariectomy decreased serum corticosterone concentrations, which were increased on treatment of spayed females with oestradiol benzoate, but were unchanged in oestrogen-treated intact females. These observations are consistent with Kitay's work in which he postulated a relationship between the dosage of oestrogen and the adrenal response, such that small doses are stimulatory and large doses are inhibitory to corticosterone production. Thus in ovariectomised rats, oestrogen administration increased corticosterone production and in intact females, the combination of endogenous and administered oestrogen is sufficient to depress adrenal corticosteroidogenesis so that circulating levels of the hormone are not increased.

Elevated AAT activity in oestrogen-treated rats was first observed by Rosen, Roberts, Budnick and Nichol (1959) and later confirmed by the more extensive investigation of Keller, Richardson and Yates (1969). In the present study, oestradiol benzoate treatment of ovariectomised females produced increases in activity of all three enzymes, although there was no direct relationship between enzyme activity and the level of increased serum corticosterone. 1 µg oestradiol benzoate daily for 14 days restored TO activity to the level of the intact control, although corticosterone levels were below the range for the intact corn oil-treated control females in the same study. Larger doses of oestrogen do not raise the level of corticosterone above that of the intact females, despite continued increases in both TO and TAT activities. With AAT, two distinct peaks of activity were observed one with 5 µg doses and the other with 50 µg doses. No elevation in activity was found with the 10 µg and 20 µg doses, although the level of corticosterone was increased compared with ovariectomised controls.

In intact females treated with 10 µg oestradiol benzoate for 10 and 14 days the activity of all three enzymes increased, although in neither case was the level of serum corticosterone raised. These results provide further support for the conclusion that there is no direct relationship between increased enzyme activity and elevation in circulating corticosterone levels after oestrogen treatment. The dose of 10 µg oestradiol benzoate, given daily for 14 days, produced a similar high absolute level of TO activity in ovariectomised rats ($13.1 \mu\text{moles kynurenine/g/h} \pm \text{SD } 1.4$) to that reached in intact females ($10.0 \mu\text{moles kynurenine/g/h} \pm \text{SD } 0.4$), although the basal level in spayed females ($2.0 \mu\text{moles kynurenine/g/h} \pm \text{SD } 1.4$) was much lower than in intact females ($3.7 \mu\text{moles kynurenine/g/h} \pm \text{SD } 0.4$).

Keller, Richardson and Yates (1969) determined the effect of hydrocortisone or oestradiol benzoate administration on AAT in liver and pancreas of intact male rats and observed that whereas hydrocortisone induced increased enzyme activity in both tissues, the oestrogen only raised hepatic AAT activity. These observations were applied to provide experimental support for the hypothesis that plasma corticosteroid-binding globulin has a physiological function in regulating the distribution of adrenocorticoids. The postulated mechanism is that transcortin-bound corticosteroid can gain access to organs, such as liver, with a readily protein-permeable vascular bed and that dissociation within the tissue yields free, physiologically active steroid. Other organs, such as pancreas, it is suggested, have impeded access to protein-bound corticosteroid because the capillaries have a thin basement membrane. Keller et al interpreted the results of their study with AAT as indicating that elevated plasma transcortin-bound corticosterone produced by oestrogen administration can enter the hepatocyte and induce AAT, whereas this cannot occur in the pancreatic cells.

In the present investigation such a hypothesis was thought to be equally applicable to other glucocorticoid-inducible enzymes including TO and TAT. However, the results obtained in this part of the study do not support the general applicability of the experimental model used by Keller and his co-workers, since in both intact and spayed female rats treated with oestrogen there was no relation between changes in enzyme activity and in the total serum corticosterone level. Further, Gala and Westphal (1965) found that oestradiol administration does not alter corticosteroid-binding globulin levels in plasma of intact female rats. The relevance of corticosterone to the elevated enzyme activity produced by oestrogen treatment was not established in the experiments reported in this section and further investigations were made into the mechanism of these increases.

Prolonged administration of oestrogen depressed activity of all three enzymes, and both AAT and TAT activities were diminished to control levels after treatment with a daily dose of 10 μ g oestradiol benzoate for 21 and 28 days respectively. Administration of 20 μ g oestradiol benzoate for 28 days did not result in the full increases in TO activity observed with 10 μ g dose given for the same length of time. In contrast to these results, Rose and Brown (1969) showed that administration of 20 μ g oestradiol benzoate for 28 days resulted in decreased TO activity compared with levels in the control rats. It may be that in the present study, the precautions taken in the cageing and care of animals, described in Chapter 4, ensured that reproducible levels of enzyme activity were obtained, with small variations within each experimental group. The larger degree of variation in the results of Rose and Brown may have prevented observation of the increased levels of TO activity reported in the present study. However, from both investigations it appears that larger doses of oestrogen have an inhibitory effect on TO activity. It is therefore possible that a relation exists between the dose of oestrogen used and the response of enzyme activity such that small doses are stimulatory and large doses are inhibitory to the levels of activity of all three enzymes.

III. Mechanism of Increases in Tryptophan Oxygenase, Tyrosine
Aminotransferase and Alanine Aminotransferase Following Oestrogen
Treatment of Male and Female Rats.

INTRODUCTION

Oestrogen administration to male rats was first shown to increase pituitary adrenocorticotrophic hormone by Gemzell (1952) and subsequently has been widely observed to raise the level of plasma corticosterone (Kitay, 1963a; Gala and Westphal, 1965; Keller, Richardson, Sendelbeck, Moore and Yates, 1966; D'Angelo, 1968). The elevations in circulating corticosterone were attributed to raised adrenocorticotrophic hormone (Kitay, 1963a). Oestrogens also increase corticosteroidogenesis by direct action at the adrenal gland (Kitay, 1965; Ruhmann-Wenhold, Lauro and Nelson, 1970).

In the study described in the previous section, it was shown that oestrogen treatment produced increased TO, TAT and AAT activity unrelated to changes in serum corticosterone levels in both intact and ovariectomised rats. The role of corticosterone in the mechanism for increased enzyme activity following oestrogen treatment was therefore investigated by studying the effects of administration in hypophysectomised and adrenalectomised rats.

- (1) The effects of oestrogen treatment on tryptophan oxygenase and tyrosine aminotransferase in the livers of hypophysectomised rats.

Procedure

Mature female rats were hypophysectomised as described in Chapter 4.

The present study had already shown that both ovariectomised and intact female rats, treated with 10 µg oestradiol benzoate daily for 14 days, had elevated TO and TAT activities. It was decided therefore, to use this dose throughout these further investigations.

Table 18. Serum Corticosterone Levels in Corn Oil-and Oestrogen-Treated Hypophysectomised Female Rats.

<u>Days After Hypophysectomy</u>	<u>No. of Rats</u>	<u>Serum Corticosterone Levels in each Rat µg Corticosterone/100 ml</u>
28-29	5	0.8, 1.0, 1.7*, 3.9, 9.8*
30-31	6	4.2*
32-33	5	

* Hypophysectomised rats treated with 10 µg oestradiol benzoate daily for 14 days.

Absence of recorded serum corticosterone levels in this Table indicates that they were too low for detection by the protein-binding technique.

Table 19. Effect of Oestrogen (Oest Benz) Treatment on Liver Tryptophan Oxygenase and Tyrosine Aminotransferase Activities and Adrenal Weights in Hypophysectomised (Hypox) Female Rats.

<u>Treatment</u>		<u>Tryptophan Oxygenase</u> <u>μmoles kynurenine/g/h.</u>		<u>Tyrosine Aminotransferase</u> <u>μmoles p OHPPA/g/h.</u>		<u>Adrenal Weights</u> <u>mg.</u>
Intact female 0.1 ml corn oil	(8)	3.1 ± 0.9		(8)	95 ± 9	(8) 65 ± 5
Hypox female 0.1 ml corn oil	(8)	3.3 ± 0.8		(8)	154 ± 23*	(8) 38 ± 19*
Hypox female 10 μg Oest Benz	(7)	3.5 ± 0.7		(7)	137 ± 23*	(7) 22 ± 7*

Corn oil and oestradiol benzoate were administered in the doses shown daily for 14 days.

* p < 0.001 compared with the intact female controls.

The experimental groups included in this study were as follows:-

- (a) Intact female rats treated with 0.1 ml corn oil for 14 days.
- (b) Hypophysectomised female rats treated with 0.1 ml corn oil for 14 days.
- (c) Hypophysectomised female rats treated with 10 μ g oestradiol benzoate for 14 days.

TO and TAT activities, serum corticosterone levels and adrenal weights were determined.

Results

In the hypophysectomised rats killed within the first 18 days of the experiment, that is 25 days post hypophysectomy, the serum corticosterone was sufficient to allow detection by the protein binding technique used in the present study. However, any corticosterone in the serum of rats killed after this time was present in concentrations too low for detection by this method (Table 18).

Hypophysectomy did not alter TO activity, although TAT activity was elevated and adrenal weights were decreased. Treatment of hypophysectomised rats with oestrogen did not alter either TO or TAT activities from those of the corn oil-treated hypophysectomised control group. Adrenal weights were also unchanged and were below those in the intact control rats (Table 19).

- (2) The effect of adrenalectomy on tryptophan oxygenase, tyrosine aminotransferase and alanine aminotransferase activities in oestrogen-treated male and female rats.

Procedure

Corresponding experimental groups for both male and female rats were included in this study, namely:-

Table 20. The Effect of Adrenalectomy on Tryptophan Oxygenase, Tyrosine Aminotransferase and Alanine Aminotransferase in Male and Female Rats Treated with Oestradiol Benzoate (Oest Benz).

<u>Treatment</u>	<u>Tryptophan Oxygenase</u> <u>μmoles kynurenine/g/h.</u>	<u>Tyrosine Aminotransferase</u> <u>μmoles OHPPA/g/h.</u>	<u>Alanine Aminotransferase</u> <u>μmoles pyruvate/g/h.</u>
Intact female 0.1 ml corn oil	(12) 5.1 ± 0.7	(12) 107 ± 21	(12) 1.6 ± 0.4
Intact female 10 μg Oest Benz	(12) 9.7 ± 1.6*	(12) 180 ± 31*	(12) 3.3 ± 0.8*
Adx female 0.1 ml corn oil	(12) 1.6 ± 0.5*	(12) 115 ± 16	(12) 1.2 ± 0.4 † †
Adx female 10 μg Oest Benz	(11) 3.3 ± 1.1* † †	(11) 141 ± 31 † †	(11) 0.9 ± 0.5 † †
Intact Male 0.1 ml corn oil	(14) 2.0 ± 0.4*	(5) 109 ± 33	(10) 4.1 ± 0.8*
Intact Male 10 μg Oest Benz	(14) 5.4 ± 1.8* † †	(5) 136 ± 65	(8) 4.6 ± 2.7
Adx Male 0.1 ml corn oil	(6) 1.4 ± 0.3	(6) 58 ± 13	(5) 1.3 ± 0.3**
Adx Male 10 μg Oest Benz	(5) 3.1 ± 0.5 † †	(5) 81 ± 10	(6) 1.4 ± 0.2

Key and Legend to Table 20.

Oestradiol benzoate and oil were administered in the doses shown, daily for 14 days to both intact and adrenalectomised (adx) rats.

- * $p < 0.001$ compared with intact female control group.
- † $p < 0.001$ compared with adrenalectomised control group.
- ‡ $p < 0.001$ compared with intact oestrogen-treated females.
- † ‡ $p = 0.02$ compared with intact female controls.
- ** $p < 0.001$ compared with intact male controls.
- † ‡ $p < 0.001$ compared with both adrenalectomised male controls and oestrogen-treated intact males.

Table 21. Effect of Oestradiol Benzoate Treatment (Oest Benz) Treatment on Serum Corticosterone Levels and Adrenal Weights in Intact Male and Female Rats.

<u>Treatment</u>	<u>Adrenal Weights</u> <u>mg</u>	<u>Serum Corticosterone Levels</u> <u>µg corticosterone/100 ml</u>	
		<u>Uncorrected for skew</u>	<u>Corrected for skew</u>
Intact females 0.1 ml corn oil	(12) 76 ± 16	(7) $9.2 \pm 5.7^{\dagger}$	(7) $7.3 \pm 2.2^{\ddagger}$
Intact females 10 µg Oest Benz	(12) 84 ± 12	(9) 6.9 ± 2.8	(9) 6.6 ± 2.1
Intact males 0.1 ml corn oil	(6) 32 ± 7	(7) 4.9 ± 3.8	(6) 3.5 ± 2.6
Intact males 10 µg Oest Benz	(6) 41 ± 10	(7) $10.1 \pm 4.1^{\dagger}$	(6) $9.4 \pm 1.5^*$

Oestradiol benzoate was administered in the doses shown, daily for 14 days.

* $0.02 > p > 0.01$ compared with male controls.

† $0.05 > p > 0.02$ compared with male controls.

‡ $p = 0.02$ compared with male controls.

Fig. 30. Effect of Adrenalectomy (ADX) on Liver Tryptophan Oxygenase Activity In Male and Female Rats Treated with 10 μ g Oestradiol Benzoate (Oest Benz) Daily for 14 Days.

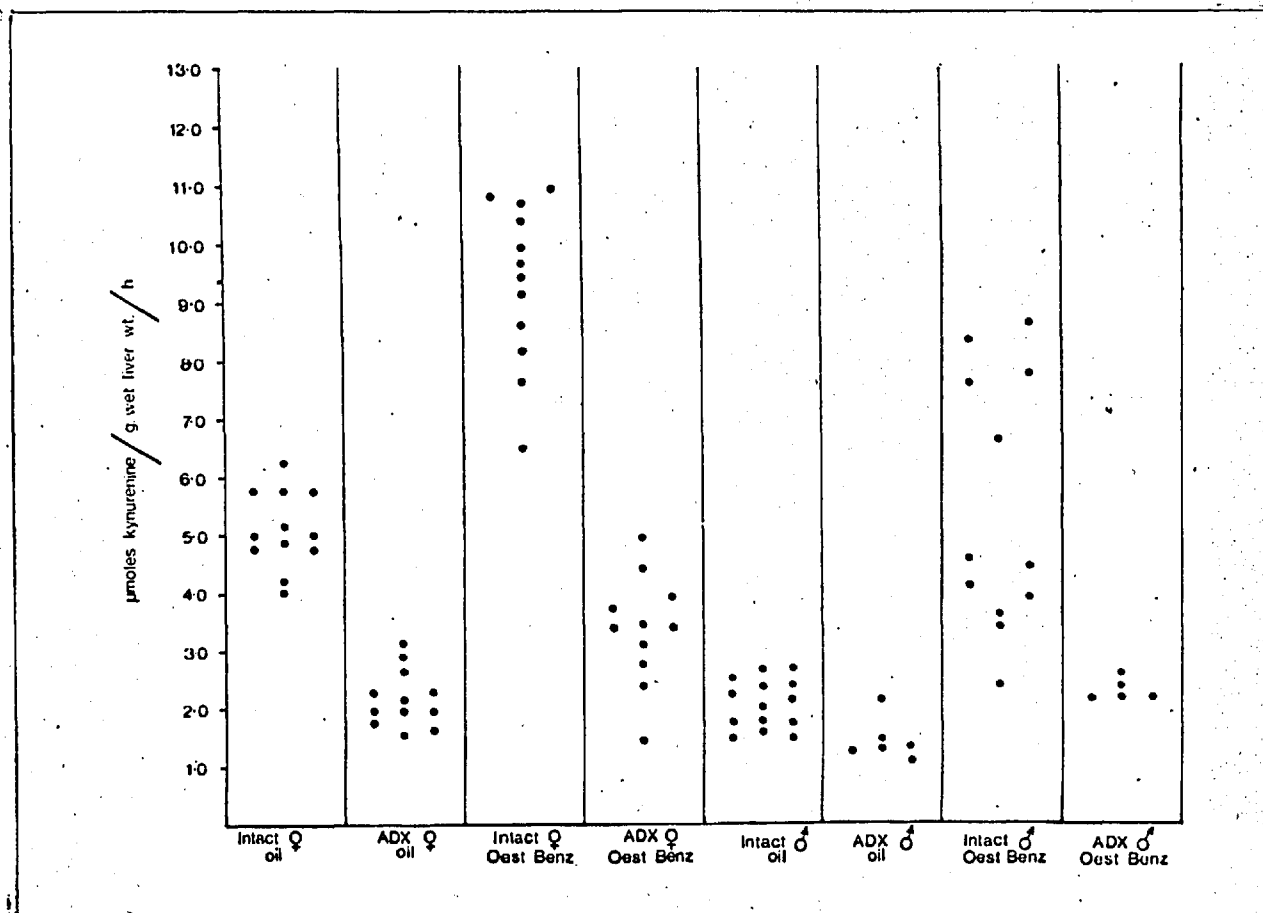


Fig. 31. Effect of Adrenalectomy (ADX) on Liver Tyrosine Aminotransferase Activity in Male and Female Rats Treated with 10 μ g Oestradiol Benzoate (Oest Benz) Daily for 14 Days.

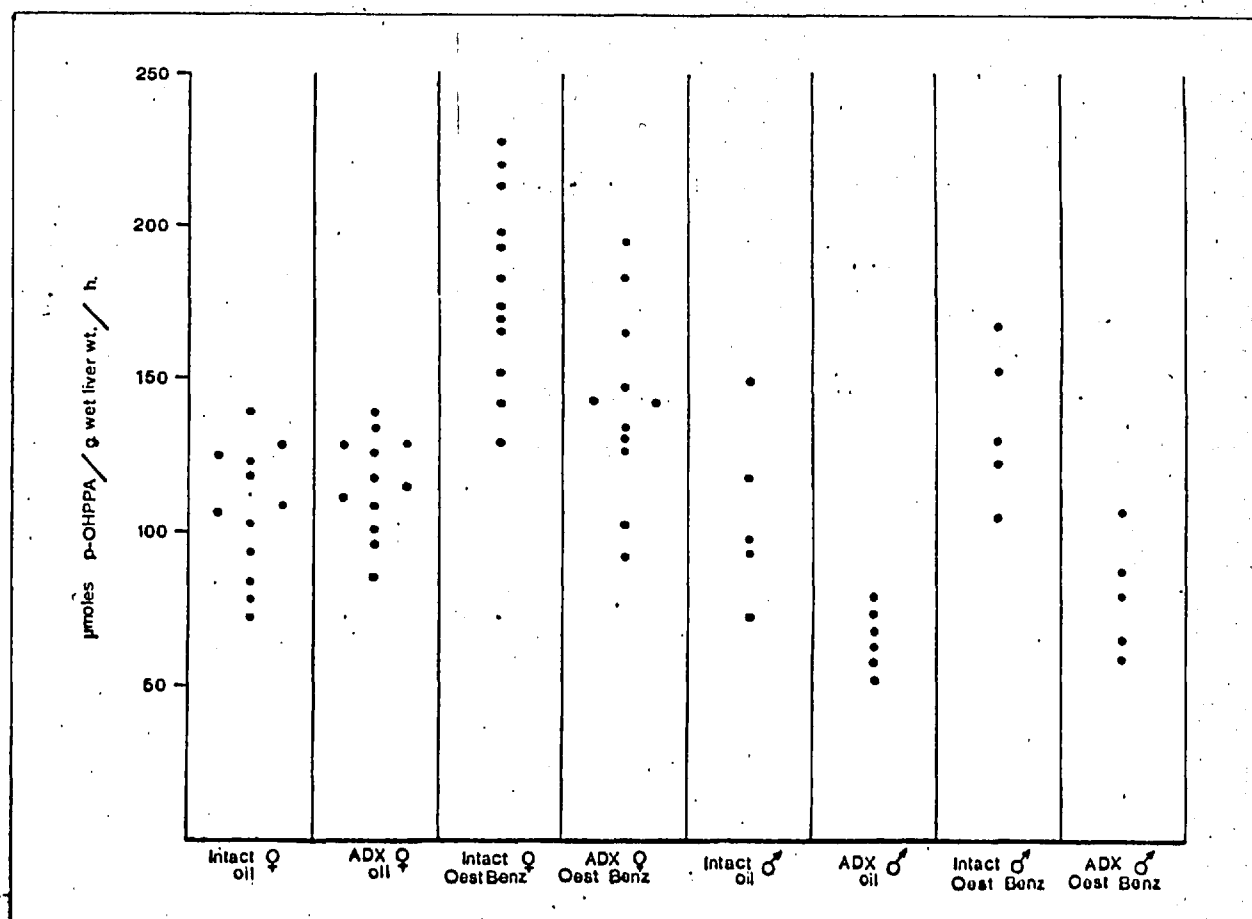


Fig. 32. Effect of Adrenalectomy (ADX) on Liver Alanine Aminotransferase Activity in Male and Female Rats Treated with 10 μ g Oestradiol Benzoate (Oest Benz) Daily for 14 Days.

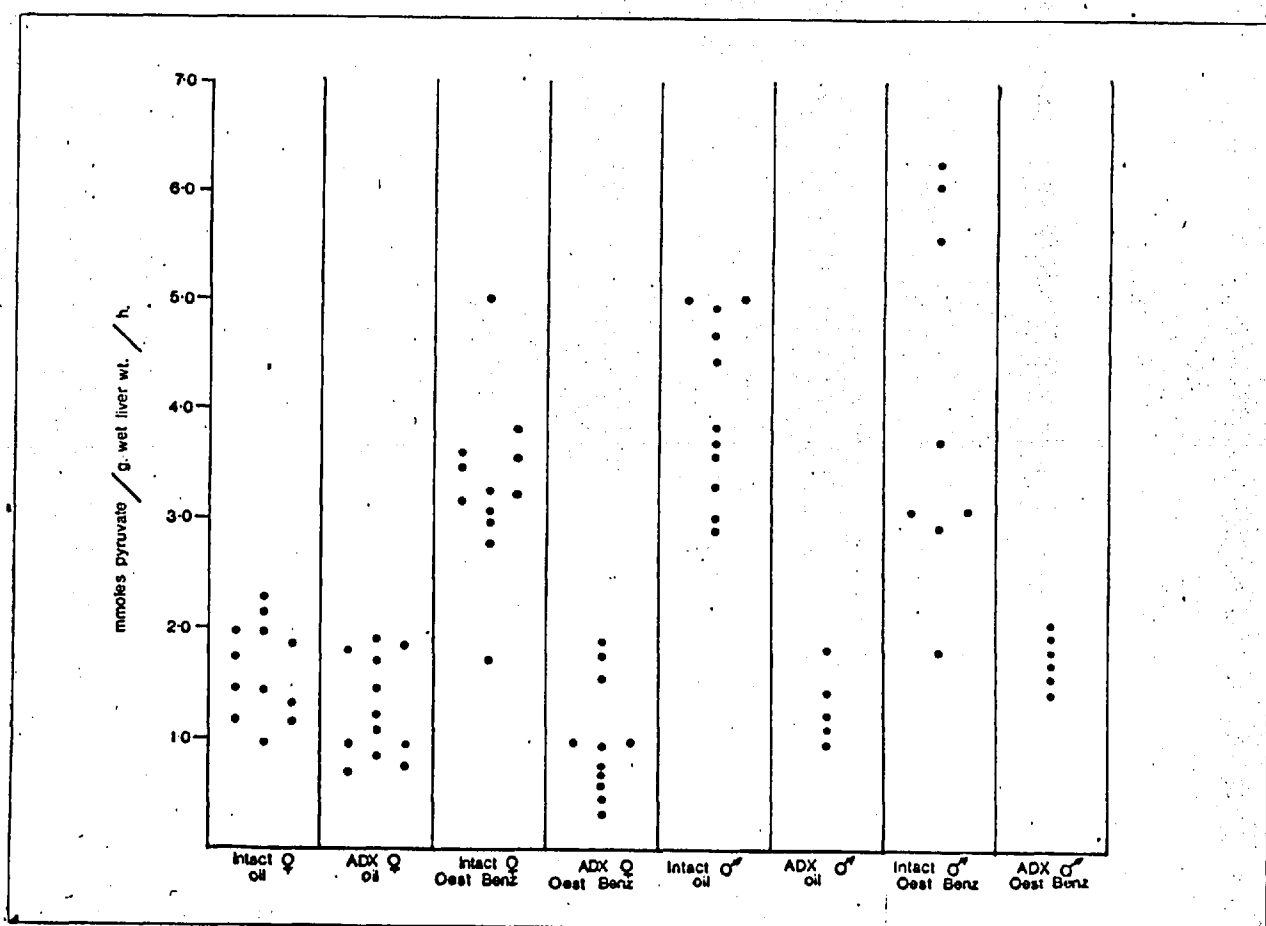
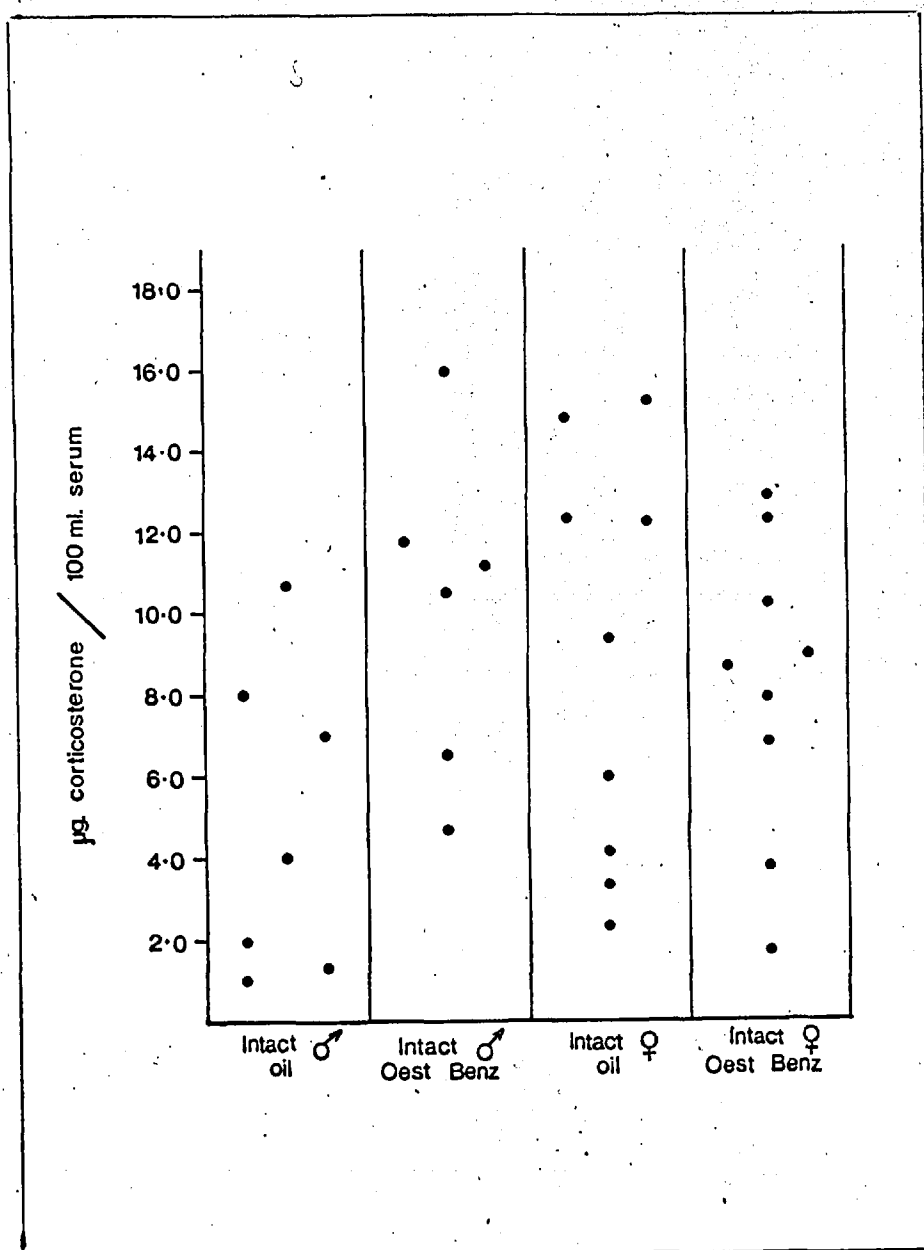


Fig. 33. Serum Corticosterone Levels in Male and Female Rats Treated with 10 μ g Oestradiol Benzoate (Oest Benz) for 14 Days.



- (a) Intact rats treated with 0.1 ml corn oil daily for 14 days.
- (b) Adrenalectomised rats treated with 0.1 ml corn oil for 14 days.
- (c) Intact rats treated with 10 µg oestradiol benzoate daily for 14 days.
- (d) Adrenalectomised rats treated with 10 µg oestradiol benzoate for 14 days.

TO, TAT and AAT activities, serum corticosterone levels and adrenal weights were measured.

Results

The results of this study are summarised on Tables 20 and 21 and Figs. 30, 31, 32 and 33. Intact corn oil-treated female rats had higher TO activity and serum corticosterone levels than did the corresponding group of males, although there was no difference in TAT activities. In male rats, AAT activity was elevated above that in females. These results confirm observations reported previously in this Chapter.

Adrenalectomy, unlike hypophysectomy, decreased TO activity in female rats, but not in male rats where the enzyme activity was unaltered. TAT activity, which was increased after hypophysectomy in females, was unchanged in adrenalectomised male and females, compared with intact control groups, although AAT activity was reduced.

Oestrogen treatment of intact female rats resulted in marked elevations in TO, TAT and AAT activities, but unchanged serum corticosterone levels and adrenal weights. In contrast, oestrogen-treated intact male rats had raised corticosterone levels and TO activity compared with the male control group although the enzyme activity was lower than in the oestrogen-treated females. AAT and TAT activities remained unaltered.

Oestrogen-treated adrenalectomised males and females had small elevations in TO activity relative to the adrenalectomised control groups of both sexes, but these levels were below those reported for oestrogen-treated intact rats. In oestrogen-

treated adrenalectomised females, AAT activity was unaltered and TAT activity was increased, compared with both intact and adrenalectomised control groups, although in similarly treated male rats TAT and AAT activities were unchanged.

There was a considerable difference in the response of male and female rats to oestrogen treatment, since female rats had marked increases in TO, TAT and AAT activities and unaltered serum corticosterone levels, whereas, in male rats only TO activity and serum corticosterone levels were elevated by oestrogen treatment. The raised TO activity in oestrogen-treated males was lower than in the corresponding group of female rats. It was possible, therefore, that endogenous androgens in the male rat were able to prevent the full increases in enzyme activity on oestrogen treatment but not in serum corticosterone levels.

In order to investigate this further the study was extended to include the effects of oestrogen treatment on enzyme activity and serum corticosterone levels in orchidectomised rats.

- (3) The effect of oestrogen treatment on tryptophan oxygenase and tyrosine aminotransferase activities and serum corticosterone levels in orchidectomised rats.

Procedure

The following groups of rats were included in these experiments:-

- (a) Orchidectomised rats treated with 0.1 ml corn oil daily for 14 days.
- (b) Orchidectomised male rats treated with 10 µg oestradiol benzoate daily for 14 days.

The following groups were included in the tables for the purposes of comparison:-

- (c) Intact male rats treated with 0.1 ml corn oil for 14 days.
- (d) Intact female rats treated with 0.1 ml corn oil for 14 days.

Table 22. Effect of Oestradiol Benzoate (Oest Benz) Treatment on Liver Tryptophan Oxygenase, Tyrosine Aminotransferase Activities and Adrenal Weights in Orchidectomised (ORCHX) Male Rats.

<u>Treatment</u>		<u>Tryptophan Oxygenase</u> <u>µmoles kynurenine/g</u> <u>wet tissue weight/h.</u>		<u>Tyrosine Aminotransferase</u> <u>µmoles p OHPPA/g. wet</u> <u>tissue weight/h.</u>		<u>Adrenal Weight</u> <u>mg.</u>
Intact Male 0.1 ml corn oil	(14)	2.0 ± 0.4	(5)	109 ± 33	(6)	32 ± 7
Intact Male 10 µg Oest Benz	(14)	5.4 ± 1.8* † ‡	(5)	136 ± 65	(6)	41 ± 10
ORCHX Male 0.1 ml corn oil	(6)	2.0 ± 0.2	(6)	86 ± 18	(6)	64 ± 8**
ORCHX Male 10 µg Oest Benz	(8)	8.7 ± 2.1*	(8)	116 ± 56	(5)	67 ± 13**
Intact Female 0.1 ml corn oil	(12)	5.1 ± 0.7	(12)	107 ± 21	(12)	76 ± 16
Intact Female 10 µg Oest Benz	(12)	9.7 ± 1.6*	(12)	180 ± 31*	(12)	84 ± 12

Oestradiol benzoate and corn oil were administered in the doses shown, daily for 14 days.

- * p < 0.001 compared with corresponding controls
- † p < 0.001 compared with intact oestrogen-treated females
- ‡ p < 0.001 compared with oestrogen-treated orchidectomised males
- ** 0.01 > p > 0.001 compared with intact corn oil-treated males.

Table 23. The Effect of Oestradiol Benzoate (Oest Benz) Treatment on Serum Corticosterone Levels in Orchidectomised (ORCHX) Male Rats.

<u>Treatment</u>	<u>Serum Corticosterone Levels</u> <u>µg corticosterone/100 ml</u>			
	<u>Uncorrected for skew</u> <u>distribution</u>		<u>Corrected for skew</u> <u>distribution</u>	
Intact Male 0.1 ml corn oil	(7)	4.9 ± 3.8	(7)	3.5 ± 2.6
Intact Male 10 µg Oest Benz	(6)	10.1 ± 4.1*	(6)	9.4 ± 1.5 [†]
ORCHX Male 0.1 ml corn oil	(6)	5.5 ± 3.7	(6)	4.0 ± 1.0
ORCHX Male 10 µg Oest Benz	(8)	13.0 ± 9.3 [†]	(8)	10.7 ± 1.9**
Intact Female 0.1 ml corn oil	(7)	9.2 ± 5.7	(7)	7.3 ± 2.2
Intact Female 10 µg Oest Benz	(9)	6.9 ± 2.8	(9)	6.6 ± 2.1

Corn oil and oestradiol benzoate were administered in the doses shown daily for 14 days.

- * 0.05 > p > 0.02 compared with intact corn oil-treated males
[†] p = 0.02 compared with intact corn oil-treated males
[‡] p = 0.05 compared with corn oil-treated orchidectomised rats
** 0.01 > p > 0.001 compared with corn oil-treated orchidectomised rats.

Fig. 34. Effect of Orchidectomy (ORCHX) on Tryptophan Oxygenase Activity in Male Rats Treated with 10 μ g Oestradiol Benzoate (Oest Benz) Daily for 14 Days. 154.

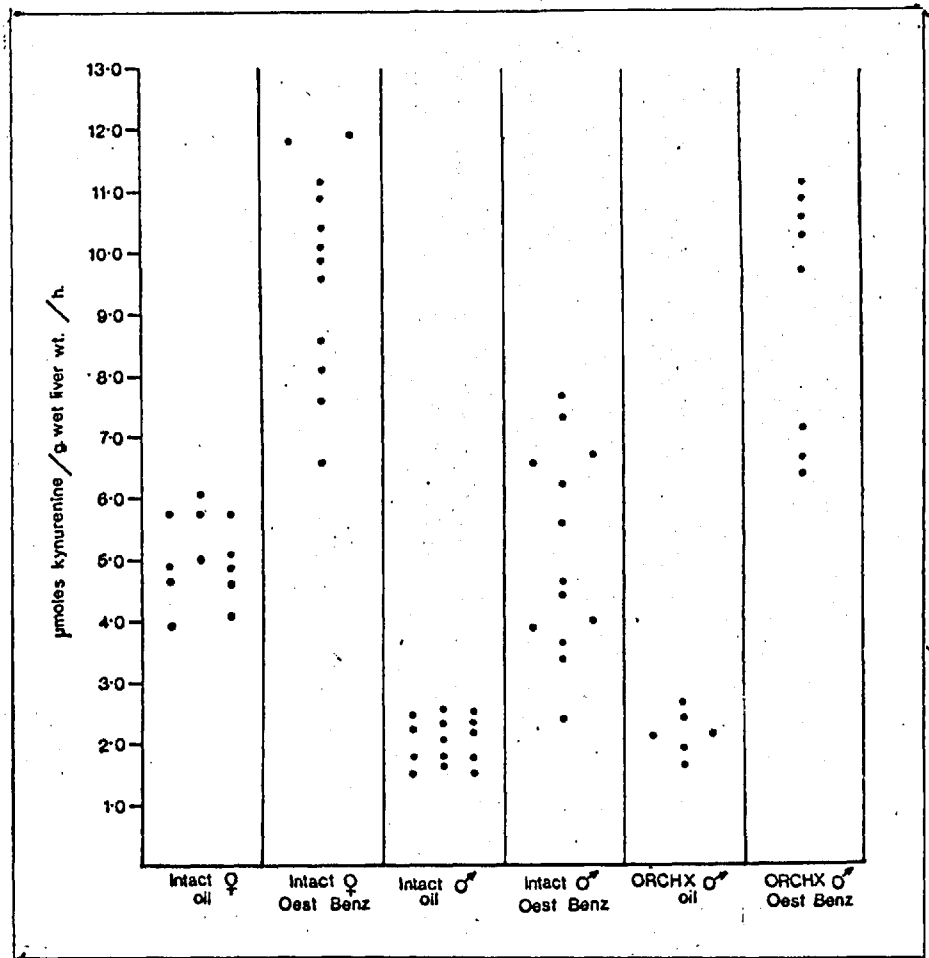
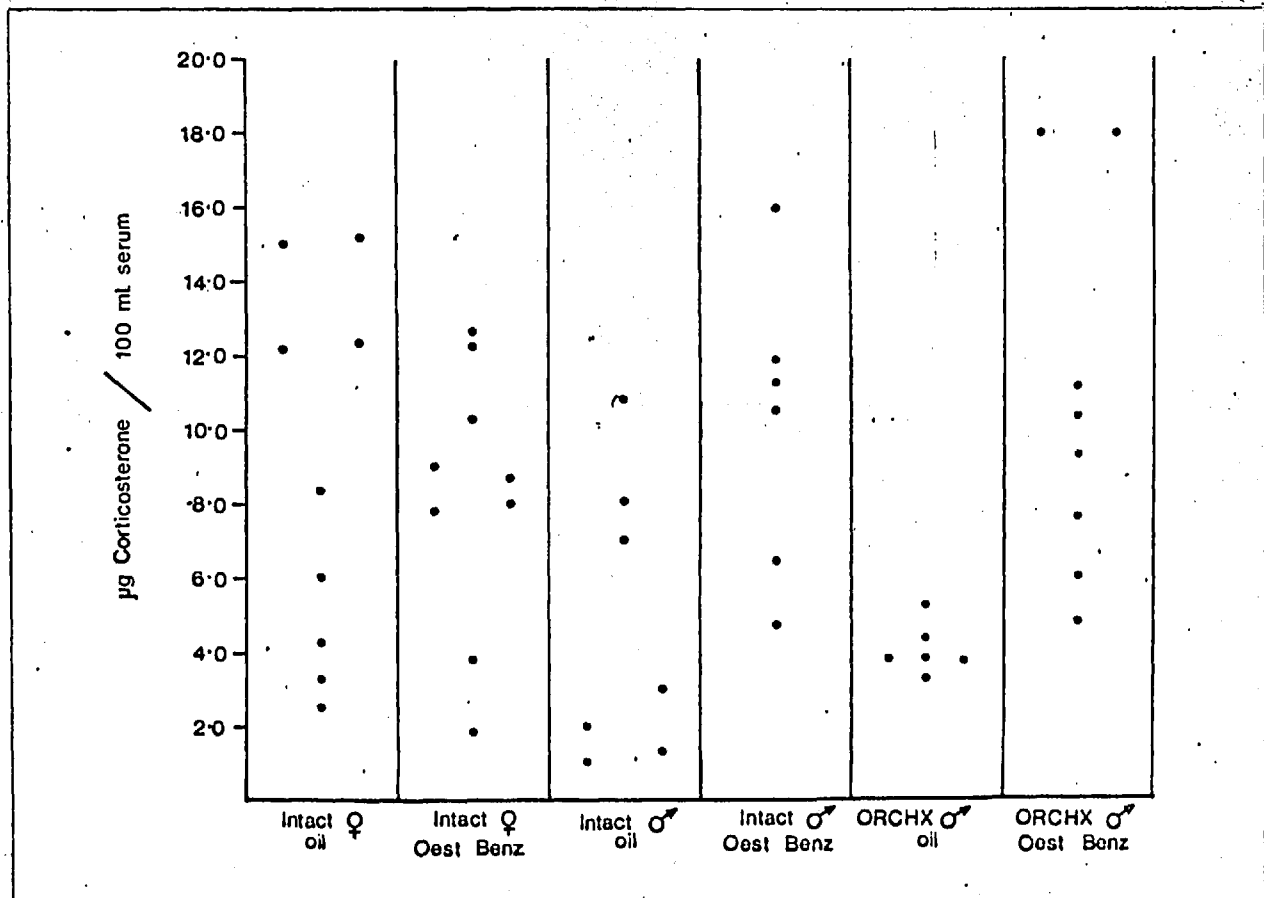


Fig. 35. Effect of Orchidectomy (ORCHX) on Serum Corticosterone Levels in Male Rats Treated with 10 μ g Oestradiol Benzoate (Oest Benz) Daily for 14 Days.



- (e) Intact male rats treated with 10 μ g oestradiol benzoate daily for 14 days.
- (f) Intact female rats treated with 10 μ g oestradiol benzoate daily for 14 days.

TO and TAT activities serum corticosterone levels and adrenal weights were recorded.

Results

TO and TAT activities, and serum corticosterone levels were unchanged by orchidectomy, although adrenal weights were increased. Oestrogen-treated orchidectomised male rats had raised TO activity compared with both control orchidectomised rats and oestrogen-treated intact males, the levels being similar to those reported for oestrogen-treated intact females. TAT activity and adrenal weights were unchanged by oestrogen treatment of orchidectomised rats, whereas serum corticosterone levels were similarly increased in both oestrogen-treated intact and castrated males (Tables 22 and 23; Figs. 34 and 35.)

DISCUSSION

Harding and Nelson (1964) found an almost total disappearance of detectable corticosterone 4 h after hypophysectomy in the rat although Shima, Matsuka and Pincus (1968) showed a limited corticosterone production from cholesterol 96 h after removal of the pituitary. Harding and Nelson (1964) measured the secretion rate of plasma corticosterone from the adrenal vein by fluorimetry, which was not sufficiently sensitive to detect the low levels reported by Shima et al (1968) who separated the hormone chromatographically after infusing labelled cholesterol through the adrenal vein.

In the present study, corticosterone was still detectable in the serum up to 25 days after hypophysectomy, by which time even the limited corticosteroidogenesis may have ceased, with the resulting disappearance of corticosterone from the serum shown in the present study. Considerable atrophy of the adrenal glands in the absence of adrenocorticotrophic hormone is indicated by the observed marked decrease of adrenal weights.

Adrenalectomy resulted in decreased TO activity in female rats. This is in agreement with the observations of Knox, Piras and Tokuyuma (1966), but contradictory to the work of both Labrie and Korner (1968) and Csanyi and Greengard (1968), who reported that both adrenalectomy and hypophysectomy had no effect on TO or TAT activities in the female rat. In the studies described by these latter authors, gland ablations were performed on immature female rats. The level of circulating corticosterone in the immature female is minimal, and rises sharply at 30 days, and then increases further until puberty (Gala and Westphal, 1965). The development of TO activity follows a similar pattern (Chytil, 1971). Thus removal of adrenal or pituitary glands before puberty may not change TO activity whereas removal of the adrenal gland from mature females may do so.

Decreased TO activity after adrenalectomy in mature females may result from unopposed growth hormone activity in the liver, since long-term growth hormone treatment of adrenalectomised rats results in a depression in the basal level of TO activity (Labrie and Korner, 1968a). Growth hormone increases liver protein synthesis which, by causing a depletion of intracellular hepatic amino acids (Geschwind and Evans, 1949) may result in a decreased capacity for TO synthesis.

In female rats, after hypophysectomy, TO activity was unchanged. The administration of adrenocorticotrophic hormone to intact rats is antagonistic to the anabolic action of growth hormone (Li, Geschwind and Evans, 1949). Since adrenocorticotrophic hormone treatment increases the level of the catabolic glucocorticoids, it is probable that these hormones are responsible for the antagonism to growth hormone. After adrenalectomy, endogenous growth hormone may increase protein synthesis, causing decreased TO activity, as suggested previously. After hypophysectomy, both growth hormone and corticosteroids are eliminated by removal of the pituitary gland, which may therefore result in the maintenance of TO activity.

TO activity in male rats was unchanged after adrenalectomy, in contrast to the effects in female rats. In subsequent studies, it was shown that ovariectomy and testosterone propionate treatment of intact female rats diminished TO activity, although androgen treatment of ovariectomised female rats and male rats did not result in further decreases. Since TO activity in the male is already below that of the female it may be impossible for any further treatment of the male rat to decrease activity of the enzyme.

TAT was unaffected by adrenalectomy in the female rat, whereas TO activity was decreased. Although both enzymes respond to glucocorticoid administration, TAT activity, unlike TO, is elevated by insulin (Reshef and Greengard, 1968) and thyroxine administration (Rivlin and Levine, 1963). Growth hormone,

which is regarded as a metabolic antagonist to insulin, is still present in the adrenalectomised rat, so that insulin action may only be permitted to maintain TAT activity at the same level as in intact rats.

In the hypophysectomised female rat, TAT activity was increased (Table 19) as both glucocorticoids and growth hormone are absent and endogenous insulin may therefore elevate the enzyme activity. Korner and Labrie (1968) and Csanyi and Greengard (1968) showed that hypophysectomy had no effect on TAT activity. However, as discussed previously for TO, these workers used immature female rats and this may account for the difference between their results and those in the present study.

Adrenalectomy decreased AAT activity in both male and female rats, in confirmation of the earlier observations of Rosen, Harding, Milholland and Nichol (1963).

In the present study, oestrogen-treated male rats had raised corticosterone levels in agreement with the results of previous workers (Keller, Sendelbeck, Richardson, Moore and Yates, 1966; D'Angelo, 1968; Gala and Westphal, 1965), although of the three enzymes only TO activity was increased. Keller et al reported elevated AAT activity in the livers of oestrogen-treated males. These authors used Holtzman strain rats, whereas in the present investigation, Sprague-Dawley rats were used, which may account for the difference between the results of the two studies.

Oestrogen treatment of female rats elevated TO, TAT and AAT activities but not serum corticosterone levels. Keller and co-workers suggested that increased plasma corticosterone concentrations, resulting from oestrogen treatment of male rats

induces liver enzymes. This may account for increased TO activity in oestrogen-treated male rats but not the results obtained with the females.

An alternative explanation for the effects of oestrogen treatment is that an intact pituitary-adrenal axis is required to maintain the presence of circulating levels of corticosterone, in order to obtain full expression of enzyme activity. The increased serum levels of corticosterone in male rats and the absence of such a change in females on oestrogen administration would therefore be irrelevant to such a mechanism. The effects of oestrogen on adrenalectomised rats reported in this study support this hypothesis and demonstrate the importance of corticosterone. Oestrogen treatment of adrenalectomised female rats did not elevate the activity of TO or TAT, as it did in intact rats, and had no effect on AAT activity. In addition, oestrogen-treated adrenalectomised male rats had lower TO activity than did similarly treated intact males.

These results were confirmed by the effects of oestrogen treatment of hypophysectomised rats, which did not change the activity of either TO or TAT, despite the persistence of detectable corticosterone levels in some animals. This emphasises the essential presence of the pituitary gland but also indicates that another factor associated with the pituitary is involved in oestrogen stimulation of enzyme activity. An action of oestrogens, independent of corticosterone is indicated by the effects of oestrogen treatment after adrenalectomy, which raised TO activity above the adrenalectomised controls but not to the same level as in the oestrogen-treated intact rats. TAT levels were similarly increased in oestrogen-treated adrenalectomised females. The results of oestrogen administration to orchidectomised rats also supports this conclusion, for although there was no difference in corticosterone levels between intact and castrated males after oestrogen

treatment, TO activity was further increased in the latter. Circulating androgens in the intact male thus prevented full expression of enzyme activity by inhibiting the independent action of oestrogens.

Oestrogens may act independently by stabilisation of enzyme structure rather than induction of enzyme synthesis and indirect support for this view is provided by the observation that TO and TAT activities, but not AAT are increased on oestrogen treatment of adrenalectomised rats, since stabilisation of activity depends on some measure of structural specificity. TO has a tetrameric quaternary structure and 4 moles of haem cofactor bind to 1 mole of purified apoenzyme (Poillon, Maeno, Koike and Feigelson, 1969). It has been suggested that TAT is also a tetramer since 4 moles of pyridoxal 5-phosphate bind to 1 mole of purified apo-tyrosine aminotransferase (Valeriote, Auricchio, Tomkins and Riley, 1969). However, only 2 moles of pyridoxal 5-phosphate cofactor bind to 1 mole of purified alanine aminotransferase (Matsuzawa and Segal, 1968) and it is therefore probable that this enzyme has a dimeric quaternary structure which may not enable any interaction with oestrogens to take place. Stabilisation of TAT by oestrogen conjugates has been reported by Singer and Mason (1967) and there may be sufficient similarity in the quaternary structures of TO and TAT to allow oestrogens to stabilise both these enzymes.

However, the absence of increased TO and TAT activities in oestrogen-treated hypophysectomised rats appears inconsistent with this hypothesis, and an alternative possibility is that oestrogens may act via another hormone. Thyroxine is a strong candidate since TAT activity increases after administration of the hormone (Rivlin and Levine, 1963) and administration of low doses of oestrogen stimulates secretion of thyroxine (Fisher and D'Angelo, 1971). Ablation of the pituitary results in the removal of thyroid stimulating hormone leading to degradation of the thyroid gland.

The involvement of thyroxine would thus explain the absence of increased liver enzyme activity after oestrogen treatment of hypophysectomised rats even in those animals with detectable levels of corticosterone.

In intact male rats, TO activity, but not TAT or AAT activities, was increased on oestrogen treatment and even in oestrogen-treated orchidectomised rats, TAT activity was not raised. Moreover, only TO activity was increased in oestrogen-treated adrenalectomised males while in similarly treated females, both TO and TAT activities were elevated. It may be that after puberty, the secretion of endogenous androgens may prevent oestrogen treatment from increasing TAT and AAT activities, even in oestrogen-treated castrated males. A further test of this hypothesis would be an investigation into the effects of oestrogen treatment on liver TAT in male rats orchidectomised before reaching puberty.

In summary, there was a sex difference in the response of enzyme activity to oestrogen administration, since the activity of all three enzymes was increased in oestrogen-treated females but only TO activity was raised in similarly treated males. The presence of circulating corticosterone and an intact pituitary adrenal axis was required for full expression of increased enzyme activity on oestrogen treatment but oestrogens also acted independently of corticosterone in increasing enzyme activity.

CHAPTER 6.

A Study of the Effects of Androgens on Liver Tryptophan
Oxygenase and Tyrosine Aminotransferase Activities in Male and
Female Rats.

- I. The Effect of Androgen Administration on Liver Tryptophan
Oxygenase and Tyrosine Aminotransferase Activities and Serum
Corticosterone Levels.
 - II. The Role of Circulating Androgens in Male Rats in Relation to
Liver Tryptophan Oxygenase and Tyrosine Aminotransferase
Activities.
- 2

I. The Effect of Androgen Administration on Liver Tryptophan Oxygenase and Tyrosine Aminotransferase Activities and Serum Corticosterone Levels.

INTRODUCTION

A sex difference in circulating corticosterone, with lower levels in male rats than in females, has been described in this study and by previous authors (Glenister and Yates, 1961; Kitay, 1961; D'Angelo, 1968). A similar situation has been reported for TO activity in Chapter 5 of this thesis, with higher levels of enzyme activity in females than in males. Kitay (1963b, 1963c) and Gala and Westphal, (1965) showed that both androgen treatment and ovariectomy diminished circulating corticosterone levels in the female rat. In the present study, ovariectomy decreased both serum corticosterone concentrations and TO activity, so it is possible that androgen treatment and ovariectomy decrease TO activity by suppressing circulating corticosterone levels.

In human subjects treated with mesterolone, a synthetic androgen, or with methyltestosterone, the response of urinary tryptophan metabolites to cortisol is diminished (McGinty and Rose, 1969). Work currently in progress in this laboratory suggests that elevated urinary tryptophan metabolite levels observed in breast cancer patients are also reduced by treatment with synthetic androgens and other anabolic steroids (Rose and Randall, 1972). An explanation for these observations is that androgenic steroids interfere with the elevation of TO activity following cortisol administration and oestrogen stimulation.

The present study was undertaken to investigate this possibility and the effects of androgens on the liver enzymes TO and TAT, with respect to serum corticosterone levels.

(1). The effect of testosterone propionate on intact males and females and ovariectomised females.

Procedure

Decreased plasma corticosterone levels in female rats were observed 14 days after a single subcutaneous injection of testosterone phenylacetate (Kitay, 1963b). It was calculated that the single injection of this slow-release depot testosterone was equivalent to a daily subcutaneous injection of 150 µg testosterone phenylacetate (Kitay, 1966). The equivalent dose of 100 µg testosterone propionate given daily for 14 days was therefore used in the present study.

Intact male and female rats and ovariectomised females were treated with oil or testosterone propionate; ovariectomy was performed as described in Chapter 4.

The following groups were included in the study:-

- (a). Intact male rats treated with 0.1 ml oil.
- (b). Intact female rats treated with 0.1 ml oil.
- (c). Ovariectomised females injected with 0.1 ml oil.
- (d). Intact male rats treated with 100 µg testosterone propionate.
- (e). Intact female rats treated with 100 µg testosterone propionate.
- (f). Ovariectomised females treated with 100 µg testosterone propionate.

Tryptophan oxygenase and tyrosine aminotransferase activities, serum corticosterone levels and adrenal weights were determined.

Results

There was no difference in TO or TAT activity, or in adrenal weights between male and female control rats, although serum corticosterone levels in the females were higher than in males. Ovariectomy diminished both TO activity and serum corticosterone levels in females, although adrenal weights and TAT activity remained unchanged. Testosterone propionate treatment of intact females decreased TO activity

Table 24. The Effect of Testosterone Propionate (test prop) Administration to Intact Male and Female Rats and Ovariectomised (Ovx) Female Rats on Liver Tryptophan Oxygenase and Tyrosine Aminotransferase Activities and Adrenal Weights.

<u>Treatment</u>	<u>Tryptophan Oxygenase</u> <u>µmoles kynurenine/g/h.</u>		<u>Tyrosine Aminotransferase</u> <u>µmoles p-OHPPA/g/h.</u>		<u>Adrenal Weight</u> <u>mg</u>	
Intact female 0.1 ml corn oil	(16)	3.2 ± 0.8	(16)	85 ± 16	(6)	58 ± 12
Intact female 100 µg test prop	(16)	2.2 ± 0.7*	(16)	93 ± 36	(5)	47 ± 5
Ovx female 0.1 ml corn oil	(7)	1.5 ± 0.3*	(7)	83 ± 22	(5)	80 ± 10
Ovx female 100 µg test prop	(7)	2.5 ± 0.3† ‡	(8)	94 ± 24	(5)	52 ± 11††
Intact male 0.1 ml corn oil	(7)	2.7 ± 1.0	(7)	89 ± 19	(5)	46 ± 9
Intact male 100 µg test prop	(10)	2.7 ± 1.0	(10)	84 ± 13	(9)	42 ± 8

Testosterone propionate and oil were administered in the doses shown above, daily for 14 days.

- * p < 0.001 compared with control intact females.
- † p < 0.02 compared with control intact females.
- ‡ p < 0.001 compared with oil-treated ovariectomised females.
- †† 0.01 > p > 0.001 compared with oil-treated ovariectomised females.

Table 25. Effect of Testosterone Propionate (test prop) administration to Intact Male and Female Rats and Ovariectomised (Ovx) Female Rats on Serum Corticosterone Levels.

<u>Treatment</u>	<u>Serum Corticosterone Levels</u> <u>µg corticosterone/100 ml</u>	
	<u>Uncorrected for skew distribution</u>	<u>Corrected for skew distribution</u>
Intact Males 0.1 ml corn oil	(7) 4.05 ± 2.9	(7) 3.2 ± 2.0
Intact Males 100 µg test prop	(9) 4.8 ± 3.2	(9) 4.1 ± 1.7
Intact Females 0.1 ml corn oil	(8) 8.3 ± 4.2	(8) 7.4 ± 1.6 †
Intact Females 100 µg test prop	(10) 4.2 ± 3.7*	(10) 2.8 ± 2.5 †
Ovx Females 0.1 ml corn oil	(6) 4.9 ± 4.9*	(6) 2.9 ± 3.3*
Ovx Females 100 µg test prop	(5) 3.1 ± 2.8	(5) 2.3 ± 2.4

Testosterone propionate and corn oil were administered in the doses shown, daily for 14 days.

Key to Table 25.

- * $0.05 > p > 0.02$ compared with corn oil-treated intact females
- † $0.02 > p > 0.01$ compared with corn oil-treated intact females
- ‡ $p = 0.05$ compared with corn oil-treated male rats.

Fig. 36. Effect of Treatment with 100 μ g Testosterone Propionate (Test Prop) Daily for 14 Days on Tryptophan Oxygenase Activity in Intact and Ovariectomised (OVX) Female and Intact Male Rats.

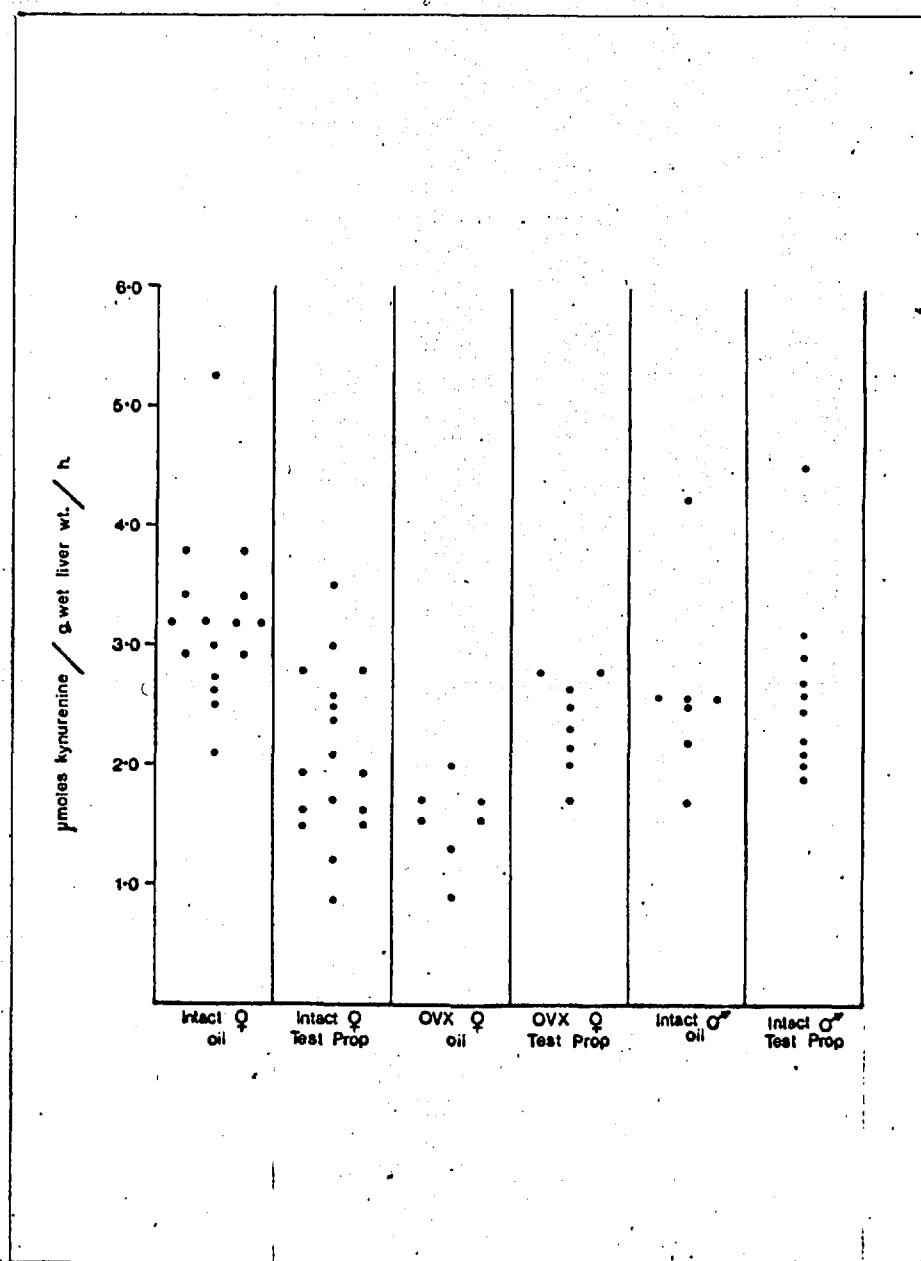
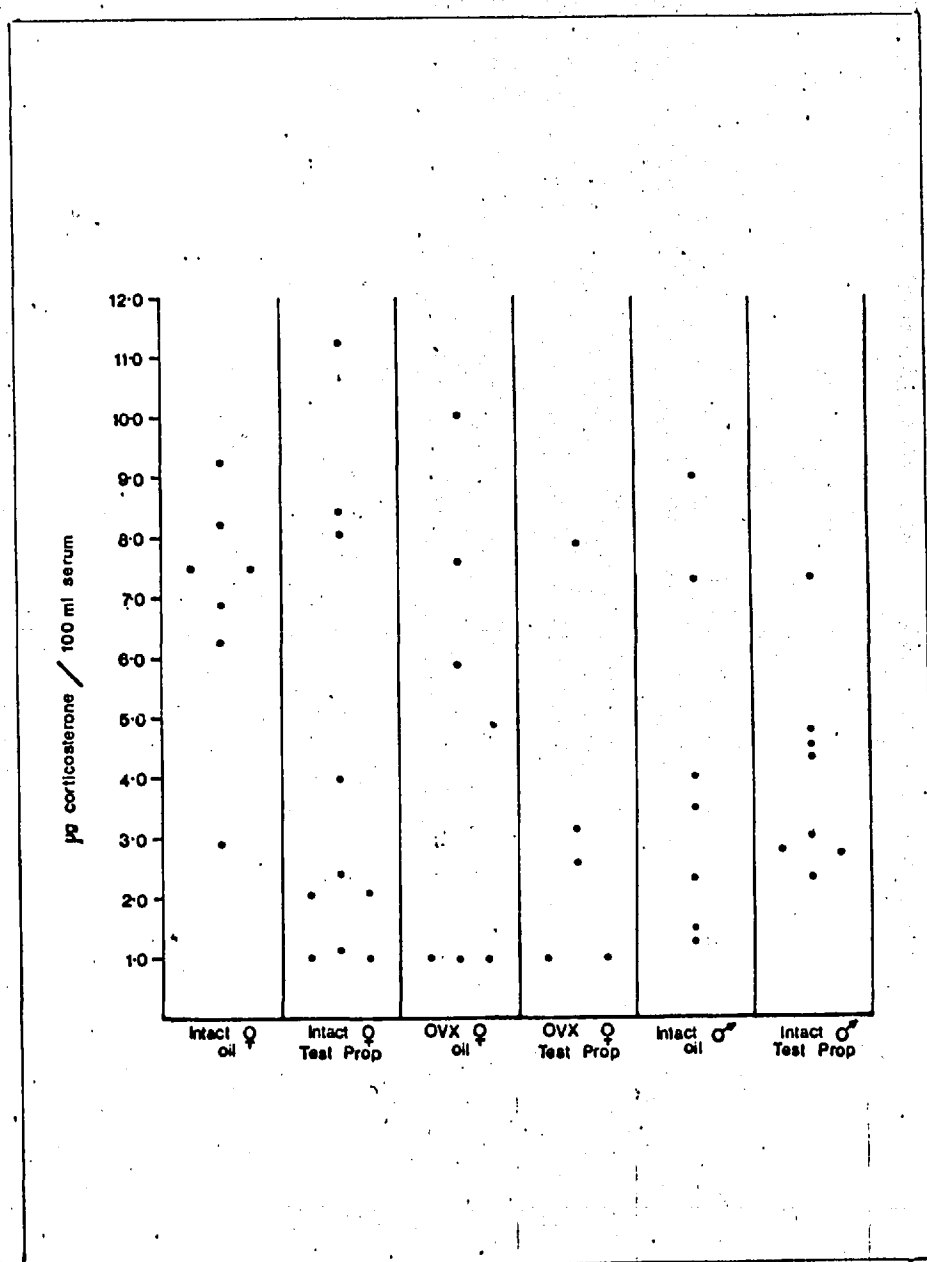


Fig. 37. Effect of Treatment with 100 μ g Testosterone Propionate (Test Prop) Daily For 14 Days on Serum Corticosterone Levels in Intact and Ovariectomised (OVX) Female and Intact Male Rats.



and serum corticosterone levels together with adrenal weights, but again TAT activity was unaltered. In contrast, TO and TAT activities, serum corticosterone concentrations, and adrenal weights were all unaffected by testosterone propionate treatment of male rats. In androgen-treated spayed females, although serum corticosterone levels remained unchanged from those in ovariectomised female controls, TO activity was elevated and adrenal weights were decreased. TAT activity remained unchanged in this group (Tables 24 and 25; Figs. 36 and 37).

(2). The effects of higher doses of testosterone propionate on intact females.

Procedure

Gala and Westphal (1965) reported that daily doses of 1 mg and 5 mg testosterone propionate injected for 10 days decreased serum corticosterone concentrations in female rats. In the experiments just described only 100 µg testosterone propionate was used, and so a further study was made into the effects of increasing the dosage of testosterone propionate on TO and TAT activities, serum corticosterone and adrenal weights.

The following groups were included:-

- (a). Intact female rats injected with 0.1 ml oil.
- (b). Intact female rats injected with 1 mg testosterone propionate.
- (c). Intact female rats injected with 0.2 ml oil
- (d). Intact female rats injected with 2 mg testosterone propionate.

Tryptophan oxygenase and tyrosine aminotransferase activities, serum corticosterone levels and adrenal weights were all determined.

Table 26. Effect of Increased Doses of Testosterone Propionate (test prop) on Tryptophan Oxygenase and Tyrosine Aminotransferase Activities and Adrenal Weights in Intact Female Rats.

<u>Treatment</u>		<u>Tryptophan Oxygenase</u> <u>µmoles kynurenine/g/h.</u>		<u>Tyrosine Aminotransferase</u> <u>µmoles p OHPPA/g/h.</u>		<u>Adrenal Weights</u> <u>mg.</u>
Intact Female 0.1 ml corn oil	(6)	2.8 ± 0.4	(6)	88 ± 12	(4)	62 ± 10
Intact Female 1 mg test prop	(6)	2.4 ± 0.6	(6)	91 ± 7	(6)	46 ± 12
Intact Female 0.2 ml corn oil	(10)	3.1 ± 0.8	(10)	95 ± 14	(4)	61 ± 5
Intact Female 2 mg test prop.	(10)	2.6 ± 0.6	(10)	105 ± 35	(4)	59 ± 14

Testosterone propionate and corn oil were administered in the doses shown daily for 14 days.

Table 27. Effect of Increased Doses of Testosterone Propionate (test prop) on Serum Corticosterone Levels in Intact Female Rats.

<u>Treatment</u>	<u>Serum Corticosterone Levels</u> <u>µg corticosterone/100 ml.</u>			
	<u>Uncorrected for skew</u> <u>distribution</u>		<u>Corrected for skew</u> <u>distribution</u>	
Intact Female 0.1 ml corn oil	(5)	6.7 ± 5.3	(5)	5.1 ± 2.1
Intact Female 1 mg test prop	(5)	7.4 ± 6.4	(5)	4.6 ± 2.8
Intact Female 0.2 ml corn oil	(5)	6.3 ± 1.0	(5)	5.9 ± 1.4
Intact Female 2 mg test prop	(5)	6.3 ± 3.6	(5)	5.2 ± 2.1

Testosterone propionate and corn oil were administered in the doses shown daily for 14 days.

Table 28. Effect of Increased Doses of Testosterone Propionate (test prop) on Uterine Weights of Female Rats.

<u>Treatment</u>	<u>Uterine Weights</u> <u>mg</u>
Intact Female 0.1 ml corn oil	(5) 0.9 ± 0.3
Intact Female 1 mg test prop	(6) $3.5 \pm 1.6^*$
Intact Female 0.2 ml corn oil	(5) 0.8 ± 0.3
Intact Female 2 mg test prop	(6) $4.4 \pm 1.3^*$

* $p < 0.001$ compared with corresponding control.

Results

Administration of 1 mg and 2 mg testosterone propionate to female rats daily for 14 days did not change the activity of either enzyme, serum corticosterone or adrenal weights (Tables 26 and 27).

After killing each rat, careful examination showed that the uteri of females treated with testosterone propionate were enlarged compared with the oil-treated controls (Table 28).

(3) The effect of 100 µg testosterone propionate, injected daily for 14 days on cortisol induction of tryptophan oxygenase and tyrosine aminotransferase in adrenalectomised male and female rats.

Procedure

Adrenalectomised rats were used in these experiments to obviate the possible conversion of testosterone to oestrogens which, Kitay has suggested, may take place in the adrenal glands (Kitay, 1963c).

Four experimental groups were used for each sex:-

- (a). Rats treated with 0.1 ml oil for 14 days, injected with saline on 15th day.
- (b). Rats treated with 0.1 ml oil for 14 days, given hydrocortisone on 15th day.
- (c). Rats injected with 100 µg testosterone propionate for 14 days, given saline on 15th day.
- (d). Rats given 100 µg testosterone propionate for 14 days, injected with hydrocortisone on 15th day.

Results

As shown in Table 29, 100 µg testosterone propionate given daily for 14 days did not change the activity of TO or TAT in the livers of adrenalectomised male and female rats. The administration of hydrocortisone to the corn oil-treated

Table 29. Effect of Testosterone Propionate (test prop) Treatment on Induction of Tryptophan Oxygenase and Tyrosine Aminotransferase by Hydrocortisone in Adrenalectomised (Adx) Male and Female Rats.

<u>Treatment</u>	<u>Tryptophan Oxygenase</u> <u>µmoles kynurenine/g/h.</u>	<u>Tyrosine Aminotransferase</u> <u>µmoles OHPPA/g/h.</u>
Adx Female; 0.1 ml corn oil Saline i.p. on 15th day.	(5) 3.0 ± 1.4	(5) 89 ± 22
Adx Female; 0.1 ml corn oil Hydrocortisone i.p. on 15th day.	(5) 13.7 ± 2.1*	(5) 274 ± 71*
Adx Female; 100 µg test prop Saline i.p. on 15th day.	(6) 2.3 ± 0.8	(6) 66 ± 19
Adx Female; 100 µg test prop Hydrocortisone i.p. on 15th day.	(7) 13.7 ± 2.3*	(7) 220 ± 96*
Adx Male; 0.1 ml corn oil Saline i.p. on 15th day	(5) 2.3 ± 1.1	(6) 99 ± 31
Adx Male; 0.1 ml corn oil Hydrocortisone i.p. on 15th day.	(5) 10.2 ± 2.0*	(6) 276 ± 91*
Adx Male; 100 µg test prop Saline i.p. on 15th day	(6) 2.5 ± 0.8	(6) 92 ± 44
Adx Male; 100 µg test prop Hydrocortisone i.p. on 15th day	(6) 10.2 ± 3.2*	(7) 259 ± 93*

Legend and Key for Table 29.

Corn oil and testosterone propionate were administered in the doses shown by daily subcutaneous injection for 14 days. Hydrocortisone and saline were given by intraperitoneal injection (i.p.) on the 15th day.

* $p < 0.001$ compared with corresponding controls.

groups of male and female rats resulted in marked elevations in TO and TAT activities, compared with the groups injected with saline. Testosterone propionate treatment did not modify the response of either enzyme to cortisol induction.

(4). The effects of fluoxymesterone on the cortisol induction of liver tryptophan oxygenase and tyrosine aminotransferase in intact female rats.

Procedure

Testosterone propionate possesses a Δ^4 -3-oxo group, which is required for aromatization of Ring A of the steroid nucleus so enabling its conversion to oestrogen. It was thought that this may have accounted for its inability to inhibit the response of liver enzymes to cortisol and therefore the effects of fluoxymesterone, a synthetic androgen possessing a 9a substituent which impedes aromatization of ring A, were investigated.

Four experimental groups were included in this study, namely:-

- (a). Intact female rats treated with 0.1 ml oil for 14 days and given saline on 15th day.
- (b). Intact female rats treated with 0.1 ml corn oil for 14 days and injected with hydrocortisone on the 15th day.
- (c). Intact female rats injected with 100 μ g fluoxymesterone for 14 days, treated with saline on the 15th day.
- (d). Intact female rats given 100 μ g fluoxymesterone for 14 days and injected with hydrocortisone on the 15th day.

Results

Treatment of intact female rats with a daily dose of 100 μ g fluoxymesterone for 14 days did not significantly affect TO or TAT activity, neither did it alter the

Table 30. Effect of 100 µg Fluoxymesterone (FM) Treatment on Induction of Tryptophan Oxygenase and Tyrosine Aminotransferase in Intact Female Rats by Hydrocortisone.

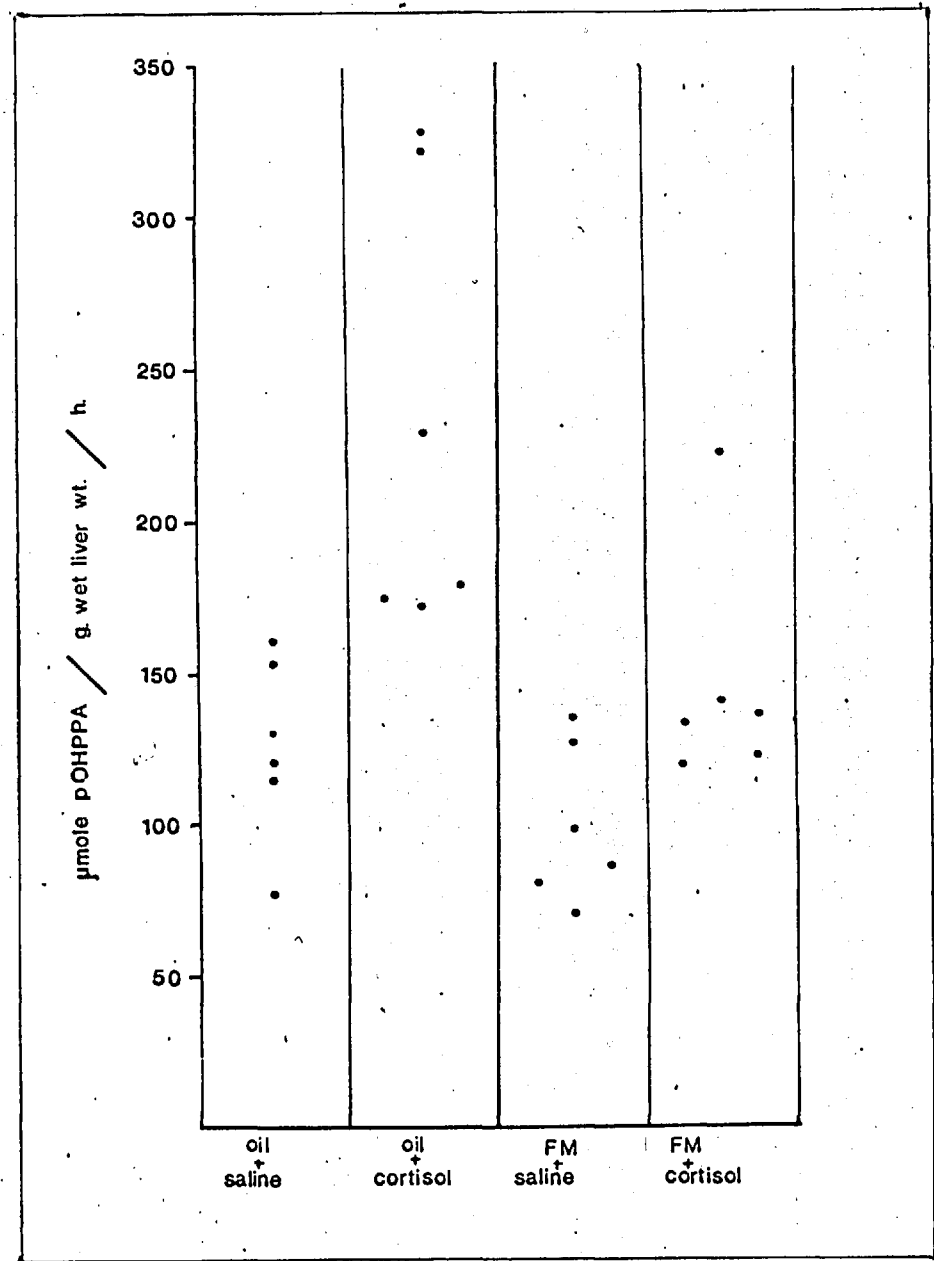
<u>Treatment</u>	<u>Tryptophan Oxygenase</u> <u>µmoles kynurenine/g/h.</u>	<u>Tyrosine Aminotransferase</u> <u>µmoles p OHPPA/g/h.</u>
Intact Female; 0.1 ml corn oil Saline i.p. on 15th day	(6) 6.6 ± 2.5	(6) 125 ± 29
Intact Female; 0.1 ml corn oil Hydrocortisone i.p. on 15th day	(6) 14.5 ± 4.4*	(6) 232 ± 75*
Intact Female; 100 µg FM Saline i.p. on 15th day	(6) 4.9 ± 1.9	(6) 98.8 ± 27
Intact Female; 100 µg FM Hydrocortisone i.p. on 15th day	(6) 11.9 ± 3.7*	(6) 156 ± 28* †

Corn oil and fluoxymesterone were administered in the doses shown by daily subcutaneous injection for 14 days. Hydrocortisone and saline were given by intraperitoneal injection (i.p.) on the 15th day.

* $p < 0.001$ compared with corresponding control group

† $0.05 > p > 0.02$ compared with oil-treated rats given hydrocortisone.

Fig. 38. Effect of Treatment with 100 μ g Fluoxymesterone (FM) Daily for 14 Days on the Cortisol Induction of Tyrosine Aminotransferase in Intact Female Rats.



response of TO to cortisol induction. However, the level of TAT activity after cortisol injection was reduced in fluoxymesterone-treated rats compared with corn oil-treated controls given cortisol (Table 30; Fig. 38).

II. The Role of Circulating Androgens in Male Rats in Relation to Liver Tryptophan Oxygenase and Tyrosine Aminotransferase Activities.

INTRODUCTION

Observations reported earlier in this thesis have shown that male rats have lower levels of TO activity in oil-treated control groups and a lower response in TO activity to oestrogen treatment than do similarly treated female rats. Oestrogen treatment of male rats increases serum corticosterone concentrations, although TAT activity is not elevated, whereas oestrogen-treated female rats have raised TAT activity although serum corticosterone levels remains unchanged. Therefore, the role of androgens in the male rat was further investigated by the administration of oestrogens and androgens to orchidectomised male rats.

It was shown previously that serum corticosterone concentrations are increased after treatment of castrated male rats with 5 mg testosterone propionate for 10 days (Gala and Westphal, 1965). However, Kitay injected orchidectomised rats with a slow-acting depot of testosterone phenylacetate, equivalent to 100 µg testosterone propionate for 14 days, and observed no effect on the level of plasma corticosterone (Kitay, 1963c). In the present investigation a daily dose of 100 µg testosterone propionate for 14 days was used.

(1). The effect of orchidectomy and testosterone propionate treatment on male rats.

Procedure

The following experimental groups were included such that group (a) were the controls for group (b) and group (c) were the controls for group (d).

- (a). Intact male rats treated with 0.1 ml oil.
- (b). Orchidectomised male rats treated with 0.1 ml oil.
- (c). Orchidectomised male rats injected with 0.1 ml oil.
- (d). Orchidectomised male rats injected with 100 µg testosterone propionate.

Table 31. Effect of Orchidectomy (ORCHX) and Testosterone Propionate (test prop) Treatment on Tryptophan Oxygenase and Tyrosine Aminotransferase Activities and Adrenal Weights in Male Rats.

<u>Treatment</u>		<u>Tryptophan Oxygenase</u> <u>μmoles kynurenine/g/h.</u>	<u>Tyrosine Aminotransferase</u> <u>μmoles OHPPA/g/h.</u>	<u>Adrenal Weight</u> <u>mg</u>
(a)	Intact Male 0.1 ml corn oil	(8) 2.5 ± 0.5	(8) 91 ± 22	(8) 47. ± 66
(b)	ORCHX Male 0.1 ml corn oil	(8) 1.3 ± 0.3*	(8) 76 ± 29	(7) 65.2 ± 14.1 †
(c)	ORCHX Male 0.1 ml corn oil	(6) 2.3 ± 0.3**	(6) 102 ± 30	(5) 63.6 ± 10.6
(d)	ORCHX Male 100 μg test prop	(7) 2.5 ± 0.5	(7) 108 ± 35	(6) 48.5 ± 3.6 ‡

Corn oil and testosterone were administered in the doses shown daily for 14 days.

* p > 0.001 compared with intact male controls.

** p > 0.001 compared with preceding group of orchidectomised males (Group b).

† 0.01 > p > 0.001 compared with corresponding intact controls (Group a).

‡ 0.01 > p > 0.001 compared with corresponding orchidectomised males (Group c).

Table 32. Effects of Orchidectomy (ORCHX) and Testosterone Propionate (test prop) Treatment on Serum Corticosterone Levels in Male Rats.

<u>Treatment</u>	<u>Serum Corticosterone</u> <u>µg corticosterone/100 ml</u>	
	<u>Uncorrected for skew</u> <u>distribution</u>	<u>Corrected for skew</u> <u>distribution</u>
Intact Males 0.1 ml corn oil	(8) 4.8 ± 4.8	(8) 3.1 ± 2.7
ORCHX Male 0.1 ml corn oil	(8) 5.7 ± 2.7	(8) 4.9 ± 1.6
ORCHX Male 0.1 ml corn oil	(6) 4.7 ± 2.2	(6) 4.4 ± 1.5
ORCHX Male 100 µg test prop	(6) 3.5 ± 2.0	(6) 3.0 ± 1.9

Testosterone propionate and corn oil were administered in the doses shown daily for 14 days.

Results

Orchidectomy resulted in decreased TO activity and heavier adrenal weights than in the intact control group, although TAT activity and serum corticosterone levels were unchanged. Testosterone propionate treatment of orchidectomised rats did not alter TO and TAT activities nor serum corticosterone concentrations and adrenal weights. The corn oil-treated orchidectomised rats in group (c) had higher TO activity than in group (b), comparable to the intact males. (Tables 31 and 32)

(2). The effect of oestrogen administration on orchidectomised rats.

Procedure

The experimental groups included in this investigation are as follows:-

- (a). Orchidectomised male rats treated with 0.1 ml corn oil for 14 days.
- (b). Orchidectomised male rats injected with 10 µg oestradiol benzoate for 14 days.

Other groups are incorporated into the tables for comparison, although they were not part of the experiments performed at the same time as (a) and (b), namely:-

- (c). Intact male rats treated with 0.1 ml corn oil for 14 days.
- (d). Intact female rats injected with 0.1 ml corn oil for 14 days.
- (e). Intact male rats treated with 10 µg oestradiol benzoate for 14 days.
- (f). Intact female rats treated with 10 µg oestradiol benzoate for 14 days.

Results

Orchidectomy did not produce any significant changes in TO and TAT activities, serum corticosterone or adrenal weights, compared with intact male rats. (Tables 33 and 34).

10 µg oestradiol benzoate administered daily for 14 days increased both TO activity and serum corticosterone levels in the castrated males. The raised corticosterone concentrations were comparable to those seen in oestrogen-treated

Table 33. Effect of Oestradiol Benzoate (Oest Benz) Treatment on Liver Tryptophan Oxygenase, Tyrosine Aminotransferase Activities and Adrenal Weights in Orchidectomised (ORCHX) Male Rats.

<u>Treatment</u>		<u>Tryptophan Oxygenase</u> <u>μmoles kynurenine/g.</u> <u>wet tissue weight/h.</u>		<u>Tyrosine Aminotransferase</u> <u>μmoles p OHPPA/g. wet</u> <u>tissue weight/h.</u>		<u>Adrenal Weight</u> <u>mg.</u>
Intact Male 0.1 ml corn oil	(14)	2.0 ± 0.4	(5)	109 ± 33	(6)	32 ± 7
Intact Male 10 μg Oest Benz	(14)	5.4 ± 1.8* † ‡	(5)	136 ± 65	(6)	41 ± 10
ORCHX Male 0.1 ml corn oil	(6)	2.0 ± 0.2	(6)	86 ± 18	(6)	64 ± 8**
ORCHX Male 10 μg Oest Benz	(8)	8.7 ± 2.1*	(8)	116 ± 56	(5)	67 ± 13**
Intact Female 0.1 ml corn oil	(12)	5.1 ± 0.7	(12)	107 ± 21	(12)	76 ± 16
Intact Female 10 μg Oest Benz	(12)	9.7 ± 1.6*	(12)	180 ± 31*	(12)	84 ± 12

Oestradiol benzoate and corn oil were administered in the doses shown, daily for 14 days.

- * $p < 0.001$ compared with corresponding controls
- † $p < 0.001$ compared with intact oestrogen-treated females
- ‡ $p < 0.001$ compared with oestrogen-treated orchidectomised males
- ** $0.01 > p > 0.001$ compared with intact corn oil-treated males.

Table 34. The Effect of Oestradiol Benzoate (Oest Benz) Treatment on Serum Corticosterone Levels in Orchidectomised (ORCHX) Male Rats.

<u>Treatment</u>	<u>Serum Corticosterone Levels</u> <u>µg corticosterone/100 ml</u>	
	<u>Uncorrected for skew</u> <u>distribution</u>	<u>Corrected for skew</u> <u>distribution</u>
Intact Male 0.1 ml corn oil	(7) 4.9 ± 3.8	(7) 3.5 ± 2.6
Intact Male 10 µg Oest Benz	(6) 10.1 ± 4.1*	(6) 9.4 ± 1.5 [†]
ORCHX Male 0.1 ml corn oil	(6) 5.5 ± 3.7	(6) 4.0 ± 1.0
ORCHX Male 10 µg Oest Benz	(8) 13.0 ± 9.3 [‡]	(8) 10.7 ± 1.9**
Intact Female 0.1 ml corn oil	(7) 9.2 ± 5.7	(7) 7.3 ± 2.2
Intact Female 10 µg Oest Benz	(9) 6.9 ± 2.8	(9) 6.6 ± 2.1

Corn oil and oestradiol benzoate were administered in the doses shown daily for 14 days.

* 0.05 > p > 0.02 compared with intact corn oil-treated males

[†] p = 0.02 compared with intact corn oil-treated males

[‡] p = 0.05 compared with corn oil-treated orchidectomised rats

** 0.01 > p > 0.001 compared with corn oil-treated orchidectomised rats.

Fig. 39. Effect of Orchidectomy (ORCHX) on Tryptophan Oxygenase Activity in Male Rats Treated with 10 μ g Oestradiol Benzoate (Oest Benz) Daily for 14 Days.

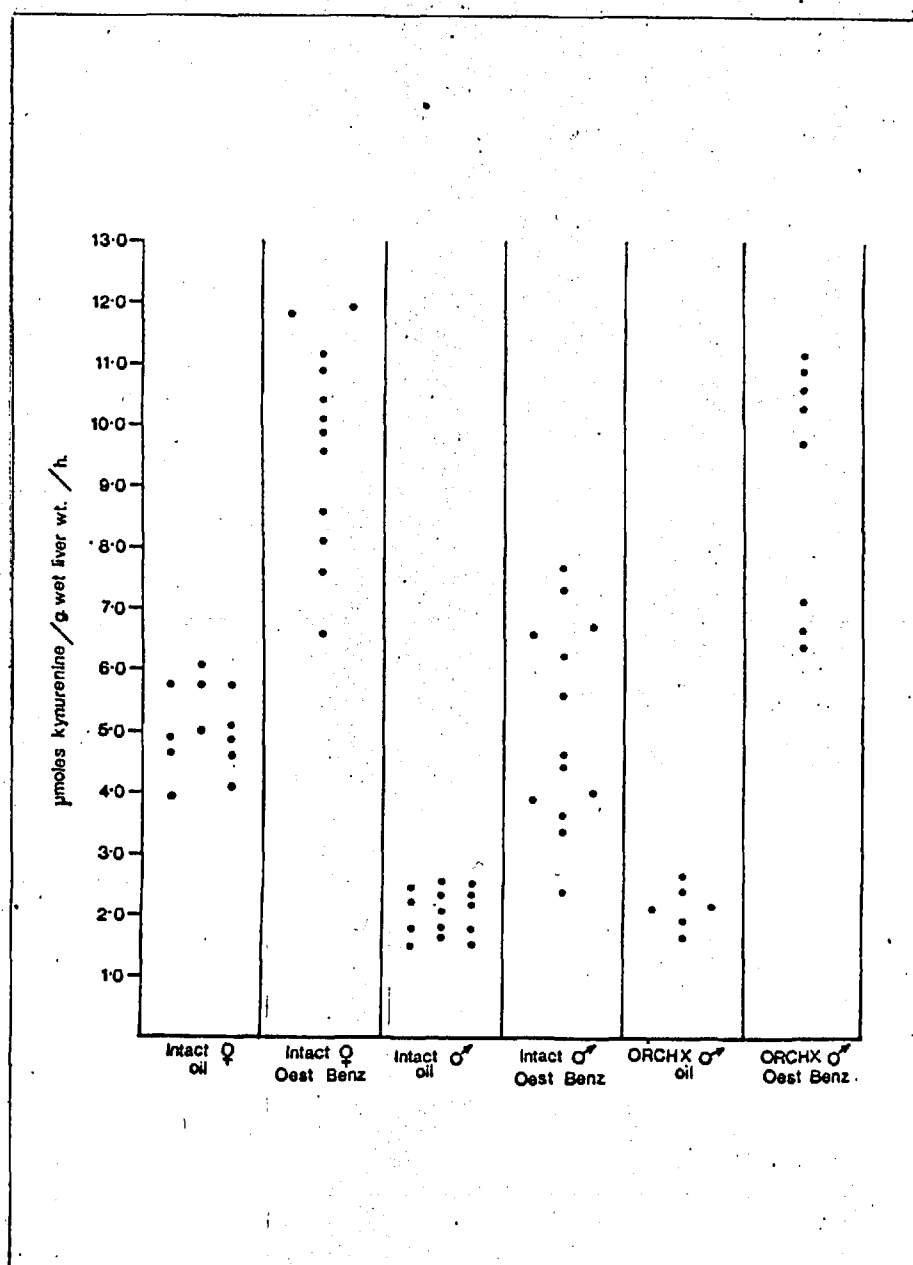


Table 35. Effect of Orchidectomy (ORCHX) Hydrocortisone Induction of Liver Tryptophan Oxygenase and Tyrosine Aminotransferase in Male Rats.

<u>Treatment</u>	<u>Tryptophan Oxygenase</u> <u>μmoles kynurenine/g/h.</u>	<u>Tyrosine Aminotransferase</u> <u>μmoles p OHPPA/g/h.</u>
Intact Male Saline i.p.	(6) 4.2 ± 2.0	(5) 118 ± 28
Intact Male Hydrocortisone i.p.	(6) 14.9 ± 4.9*	(6) 277 ± 57*
ORCHX Male Saline i.p.	(5) 5.9 ± 2.0	(5) 104 ± 27
ORCHX Male Hydrocortisone i.p.	(5) 13.4 ± 3.6*	(5) 169 ± 44†

Saline and hydrocortisone were administered by intraperitoneal (i.p.) injection.

* p < 0.001 compared with corresponding saline-treated group.

† 0.05 > p > 0.02 compared with corresponding saline-treated group.
and 0.01 > p > 0.001 compared with intact hydrocortisone-treated males.

intact males, although the increased TO activity was higher than in this group and was similar to the results for oestrogen-treated intact female rats. In both oestrogen-treated intact and orchidectomised male rats TAT activity and adrenal weights were unchanged (Tables 33 and 34; Figs. 39 and 40).

Endogenous androgens in the male rat therefore interfered with the stimulation of enzyme activity by oestrogen treatment and in order to study this further, the effect of orchidectomy on cortisol induction of TO and TAT was investigated.

(3). The effect of orchidectomy on cortisol induction of TO and TAT

Procedure

The study was comprised of four experimental groups:-

- (a). Intact male controls injected with saline.
- (b). Intact male controls injected with hydrocortisone.
- (c). Orchidectomised male rats injected with saline.
- (d). Orchidectomised male rats injected with hydrocortisone.

Results

TO and TAT were both markedly increased in intact male rats treated with cortisol. In cortisol-treated orchidectomised rats TO activity was increased to a level similar to that of cortisol-treated intact males. In contrast, although the level of TAT activity was raised in the livers of cortisol-treated castrated male rats it was significantly lower than in similarly treated intact animals (Table 35).

DISCUSSION

Endogenous oestrogens present in the female rat stimulate pituitary secretion of adrenocorticotrophic hormone (Kitay, 1963a) and also adrenal corticosteroidogenesis by direct action at the adrenal glands (Kitay, Coyne and Newsom, 1965) and are thus responsible for the higher peripheral corticosterone levels in the female rat (Kitay, 1963b). In agreement with previous observations (Kitay, 1963b; Gala and Westphal, 1965) ovariectomy produced a fall in the level of serum corticosterone (Table 25) although serum corticosterone levels were unchanged after orchidectomy, confirming the earlier work of Kitay (1963c). Adrenocorticotrophic hormone secretion is increased after orchidectomy (Kitay, 1963b) although both the half-life of peripheral corticosterone and the rate of adrenal corticosteroidogenesis are decreased (Kitay, 1963c). Stimulation of corticosterone production by increased adrenocorticotrophic hormone secretion may be offset by the other effects of orchidectomy, resulting in a maintenance of normal serum corticosterone levels. In contrast, ovariectomy depresses both adrenocorticotrophic hormone secretion and adrenal corticosteroidogenesis (Kitay, 1963b). The combination of these two effects may be sufficient to produce a net decrease in the circulating level of corticosterone despite its extended half-life observed by Kitay (1963c) in spayed females.

Adrenal weights increased following orchidectomy and decreased after androgen treatment, although in neither case were circulating corticosterone levels unchanged, in agreement with previous work (Kitay, 1963c). Adrenal weights were unaltered after ovariectomy and reduced in androgen-treated spayed females, in contrast with the results for corticosterone levels which were decreased in the former but unchanged in the latter. Therefore, changes in levels of serum corticosterone are not reflected in the changes observed in adrenal weights. Kitay (1963b) showed

that adrenocorticotrophic hormones synthesis was increased by orchidectomy in the male, decreased by treatment of castrated males with testosterone, but unchanged by ovariectomy of the female. It may be that increased adrenal weight is due to hyperplasia from the action of adrenocorticotrophic hormone and the changes in adrenal weight may reflect changes in adrenocorticotrophic hormone synthesis.

Treatment of female rats with 100 µg testosterone propionate lowered serum corticosterone concentrations in confirmation of a previous study (Kitay, 1963b). These effects may have been due to suppression of the endogenous synthesis of oestrogens in the female rat, as suggested by Kitay (1963b). Alternatively, androgen administration may have prevented the stimulation of corticosterone synthesis by the endogenous oestrogens, since androgens diminish both oestrogen stimulation of corticosteroidogenesis by an impairment of adrenal hydroxylating systems (Ruhmann-Wenhold and Nelson, 1971) and pituitary secretion of adrenocorticotrophic hormone (Kitay, 1963b). The present study did not distinguish between decreased serum corticosterone levels due to suppression of oestrogen synthesis, or inhibition of oestrogen stimulation of corticosteroid synthesis. Testosterone propionate administration to intact and orchidectomised males and to ovariectomised females, both situations in which there is minimal oestrogen synthesis, did not alter serum corticosterone levels, which indicates that testosterone may only suppress circulating levels when there is scope for significant oestrogen antagonism.

TO activity in female rats was decreased by 100 µg testosterone propionate injected daily for 14 days, for which two possible explanations are either that enzyme activity was inhibited directly, or TO activity was decreased by the fall in serum corticosterone.

High levels of testosterone propionate inhibit TO activity in vitro by competition for cofactor binding sites (Chapter 7) but it is unlikely that the androgen concentration in the liver was sufficient for such an inhibition. Moreover, TO activity was unaffected by testosterone propionate treatment of adrenalectomised rats, and spayed females injected with androgens had raised TO activity, both of which are inconsistent with direct inhibition, but indicate that corticosterone is involved in the mechanism. Treatment of intact and orchidectomised male rats with testosterone propionate did not alter either serum corticosterone levels or TO activity, although ovariectomised control rats and androgen-treated intact females had both decreased serum corticosterone concentrations and TO activity. These observations support the explanation that the decreased serum corticosterone levels are responsible for the fall in TO activity.

Treatment of ovariectomised females with testosterone propionate resulted in small but significant increases in TO activity which were not accompanied by any further change in serum corticosterone levels. It is therefore probable that these small increases are due to direct stabilisation of the enzyme.

TAT activity was unchanged by ovariectomy or androgen treatment of female rats, although serum corticosterone levels were decreased. Both TO and TAT activities are inducible by corticosteroids, but only TAT activity is increased by insulin (Reshef and Greengard, 1969) and thyroxine (Rivlin and Levine, 1963). It may be that these hormones maintain the level of TAT activity in androgen-treated and spayed females, despite the decreased concentration of peripheral corticosterone, as proposed in the previous chapter for adrenalectomised rats.

TO activity was lower in one group of orchidectomised males compared with the matched intact control group (Table 31). Decreased enzyme activity after castration was not found in any other orchidectomised group in this study. No satisfactory explanation for the diminished TO activity in this instance can be offered and it is concluded that, in general, orchidectomy does not affect TO activity.

Gala and Westphal (1965) observed decreased serum corticosterone levels after treatment of intact female rats with 1 mg and 5 mg testosterone propionate for 10 days. In the present study, 1 mg and 2 mg testosterone propionate for 14 days were ineffective in decreasing corticosterone concentrations or TO activity. Treatment of intact females with these higher doses of testosterone propionate resulted in an enlargement of the uterus which is evidence of oestrogenic activity (Spaziani and Suddick, 1967) and therefore indicated that some of the testosterone was metabolised to oestrogen. Kitay suggested that this conversion may take place in the adrenal gland (Kitay, 1963c), although more recently, several workers have described an conversion of testosterone to oestradiol-17 β in vitro by rat ovarian preparations (Mason, 1970; Kalvert and Bloch, 1968) and also of androgen metabolites to oestrogens by testicular preparations (Inaba and Nakao, 1966). The level of oestrogen produced by conversion of testosterone when administered in higher doses may be responsible for the maintenance of TO activity in the liver and for the ineffectiveness of higher testosterone propionate doses in modifying TO activity compared with the results of 100 μ g testosterone propionate given for 14 days. A similar mechanism, involving conversion of methyl testosterone to oestrogen, was suggested by McGinty and Rose to explain increased urinary tryptophan metabolite levels in human subjects treated with methyl testosterone (McGinty and Rose, 1969).

Injection of adrenalectomised male and female rats with 100 µg testosterone propionate for 14 days had no effect on TO or TAT induction by cortisol, although this dose reduced TO activity in intact female rats. In these rats, it is possible that androgens were converted to oestrogens but sufficient androgens were still present to suppress TO activity in the intact female although this was insufficient to suppress the effects of cortisol induction. Testosterone possesses a Δ^4 -3-oxo group in ring A of the steroid nucleus, which allows aromatizations of ring A and the formation of oestrogens. However, even treatment of intact female rats with 100 µg fluoxymesterone, which is not converted to oestrogens, did not affect TO induction by cortisol.

Induction of TAT activity by cortisol was decreased on fluoxymesterone treatment, for which two possible explanations are that fluoxymesterone may interfere directly with cortisol induction of enzyme activity in the liver, or alternatively that decreased enzyme levels may result from the anabolic action of the synthetic androgen. Kenney and Lee (1971) described an interference by androgenic steroids with in vitro cortisol induction of TAT in hepatoma cells in culture. Those steroids containing a 17β side chain and an 8-oxo group were the most effective inhibitors and both fluoxymesterone and testosterone propionate contain these structural features. However, the observation that fluoxymesterone has no effect on TO induction is inconsistent with such an explanation since direct interference with cortisol action should apply to enzyme induction in general.

The anabolic action of androgens will increase protein synthesis in muscle tissue (Breuer and Florini, 1965) and this may result in a depletion of hepatic amino acids similar to that discussed in the previous chapter for growth hormone.

Cortisol induction of liver enzymes has been shown to involve the action of hepatic amino acids (Labrie and Korner, 1968b; Rosen and Milholland, 1968). Rosen and Milholland concluded from their results that the response to cortisol of TAT, but not of TO, was due to increased uptake of amino acids into liver cells. Conversely, fluoxymesterone treatment, by depleting the hepatic amino acid pool size, may have resulted in a diminished response of TAT to cortisol, although TO remained unaffected.

In the previous chapter, it was suggested that oestrogen administration increased enzyme activity in both male and female rats by a mechanism independent of increased corticosterone levels, although the presence of the glucocorticoid is required for the full expression of enzyme activity. This is further supported by the study of oestrogen and cortisol treatment of orchidectomised rats. Although oestrogen-treated male rats had lower levels of TO activity than did similarly treated female rats, orchidectomy resulted in further increases in TO activity on oestrogen administration, to levels comparable with oestrogen-treated females. Serum corticosterone levels were similarly increased in both intact and orchidectomised oestrogen-treated male rats. Orchidectomy did not affect the response of TO to cortisol, so that circulating levels of androgens did not impair the effect of increased serum corticosterone levels on TO activity in the oestrogen-treated intact male. Therefore these results indicate that oestrogens have an effect independent of increased serum corticosterone concentrations, which is antagonised by androgens.

In summary, low doses of testosterone propionate or ovariectomy decreased TO activity and serum corticosterone levels in female rats. Treatment of male rats and ovariectomised females with androgens did not change either TO or serum corticosterone concentration. Orchidectomy and testosterone treatment of orchidectomised rats failed to change either TO activity or serum corticosterone concentrations. It is proposed that the decreased TO activity in androgen-treated females is due to the diminished serum corticosterone levels.

CHAPTER 7. The Effect of Steroid Hormones on Tryptophan Oxygenase in Vitro.

THE EFFECT OF STEROID HORMONES ON TRYPTOPHAN OXYGENASE IN VITRO

INTRODUCTION

TO activity has been shown to be elevated in the livers of pregnant rats (Auricchio, Rigillo and Di Toro, 1960). In order to define the hormonal basis for these increased levels of activity, Greengard, Kalinsky and Manning injected progesterone and oestrone in combination into female rats. They used doses of 1 μ g oestrone and 4 mg progesterone or 3 or 10 times these amounts, given daily for 13 days and failed to obtain any significant increases in activity (Greengard, Kalinsky and Manning, 1968). Further investigations by Rose and Brown into the effects of the female sex hormone on TO activity in the rat have shown that the administration of progesterone at doses of 4 mg and 8 mg daily for 28 days decreased TO activity (Rose and Brown, 1969b). A possible explanation of the inability of the oestrone and progesterone combination used by Greengard and co-workers to produce elevations in TO activity is that progesterone may have exerted an inhibitory effect on TO, similar to that observed by Rose and Brown, which masked the stimulatory effect of the oestrogenic component. The doses of oestrogen used by Greengard et al are similar to those which have been shown to elevate TO activity (Chapter 5).

Oestrogen conjugates are capable of inhibiting TO activity on addition to the enzyme in vitro (Oelkers and Nolton, 1964). In an earlier study, Feigelson and Greengard (1961) demonstrated the existence of a haem cofactor, loosely bound to the apo-tryptophan oxygenase to form the holoenzyme complex. Oelkers and his associates suggested that the inhibition they observed was the result of competition between haem and the steroid conjugates for binding sites on the apoprotein (Oelkers and Nolton, 1964; Oelkers and Dulce, 1964). This

raised the possibility that administration of progesterone and other steroids in vivo may decrease TO activity by direct inhibition of the enzyme and the following in vitro study was undertaken to investigate this possibility further.

METHODS

(1). Steroids

The steroids were dissolved in absolute ethanol so that the required amount for addition to the enzyme assay medium was present in 0.1 ml.

(2). Tryptophan oxygenase assay method

The same assay procedure as described previously in Chapter 4 was used, with minor modifications in the volume of water and buffer to accommodate the addition of the ethanol or steroid solution. Each incubation flask contained 2 ml liver supernatant, 5 μ M-haem, 0.8 mM-ascorbic acid, 1.66 mM-tryptophan and 0.4 ml buffer. The remaining 0.1 ml contained the ethanol or the steroid solution. Control assays were included with each experiment in the presence and absence of ethanol, so that each inhibition study had its own control flasks containing the same liver supernatant used in the flasks containing steroid. Without exception it was found, that the addition of 0.1 ml ethanol did not alter enzyme activity. (Table 36).

Table 36. The effect of water and ethanol on tryptophan oxygenase activity

<u>Volume Added</u>	<u>No. of determinations</u>	<u>TO activity</u> <u>μmoles Kynurenine/</u> <u>g/h.</u>
0.1 ml H ₂ O	15	3.7 $\pm$ 0.6**
0.1 ml EtOH	15	3.4 $\pm$ 0.8*

* Mean value $\pm$ S.D.

Fig. 41. Inhibition of Tryptophan Oxygenase In Vitro by Addition of Progesterone

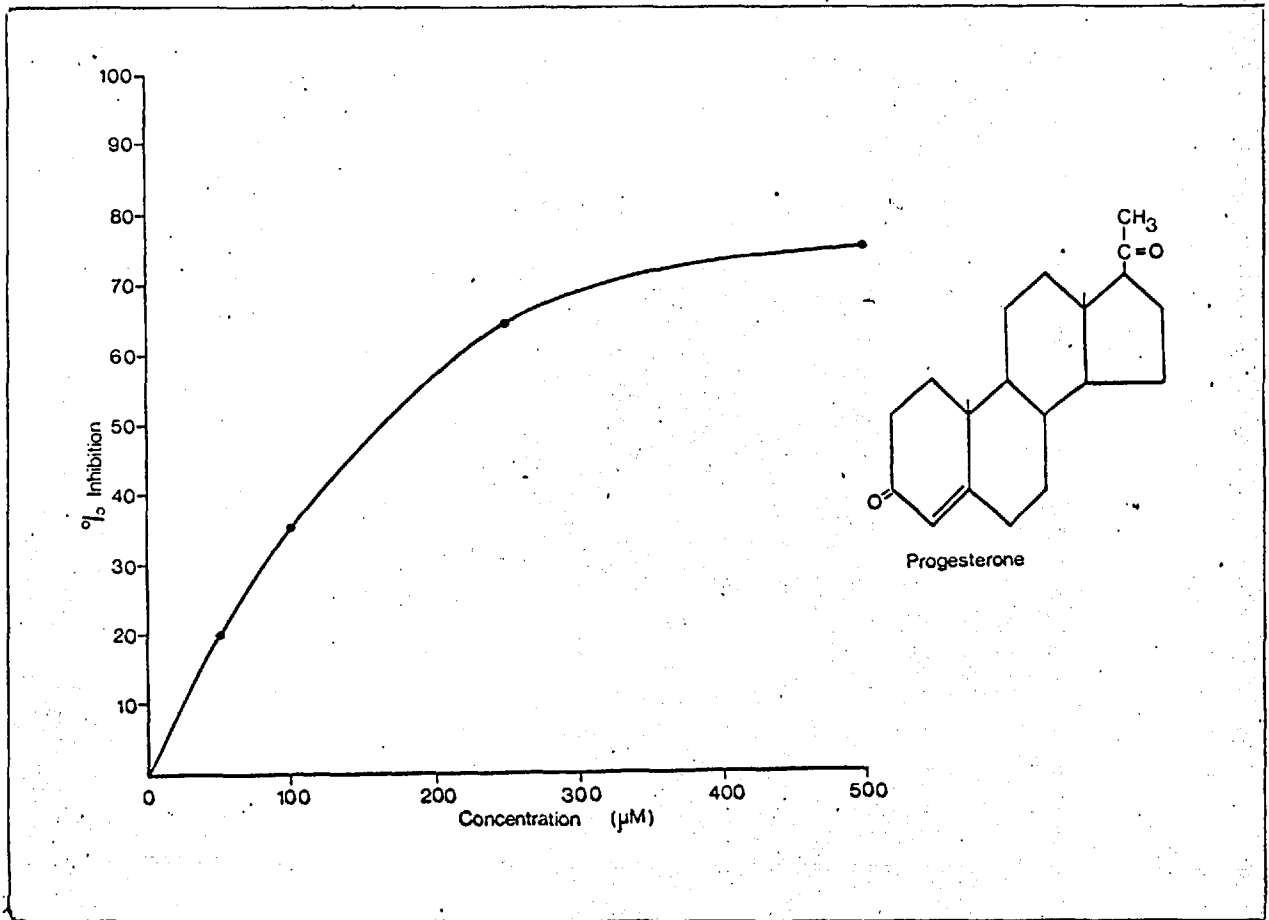


Fig. 42.

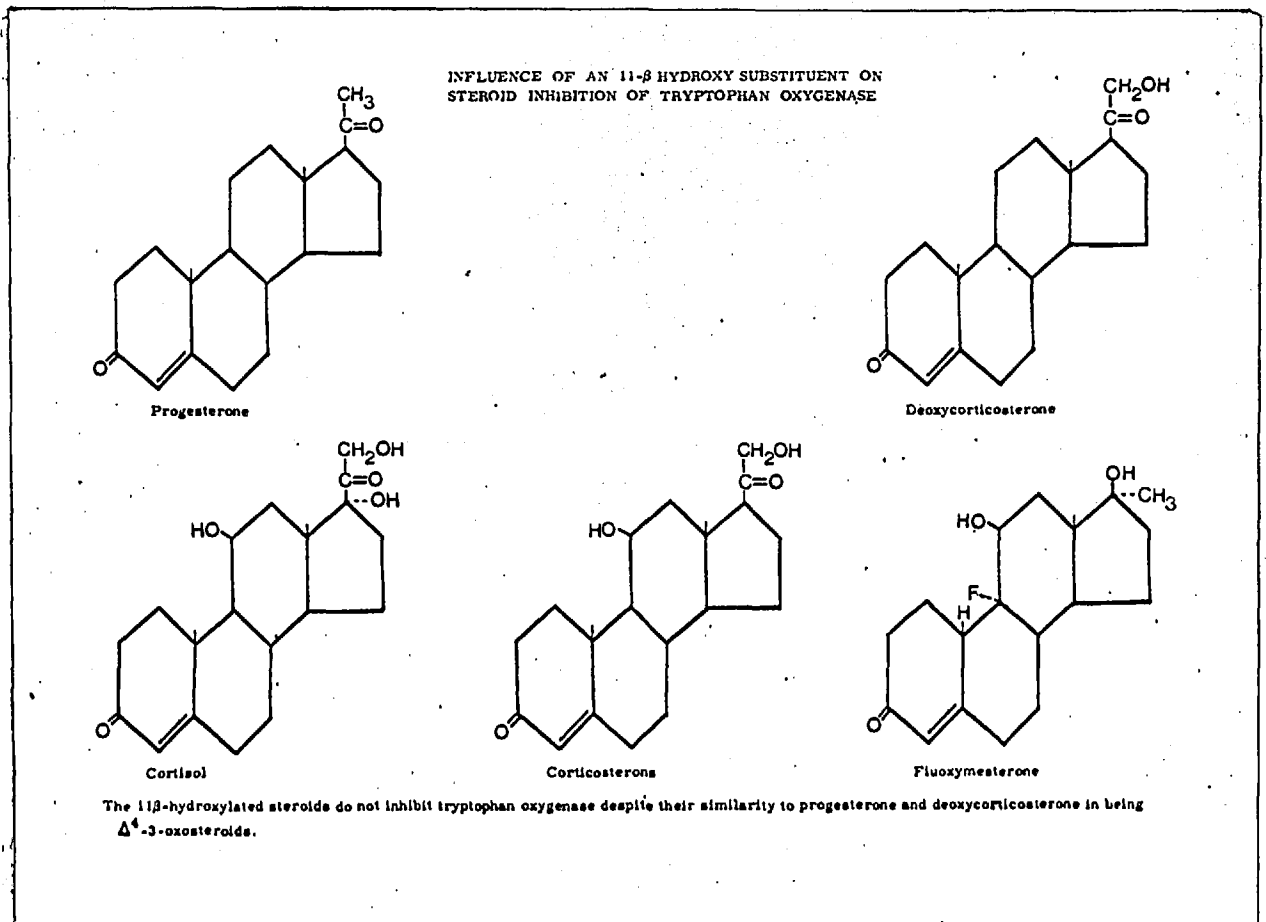


Fig. 43. Inhibition of Tryptophan Oxygenase Activity by Addition of Testosterone Propionate and Methenolone In Vitro.

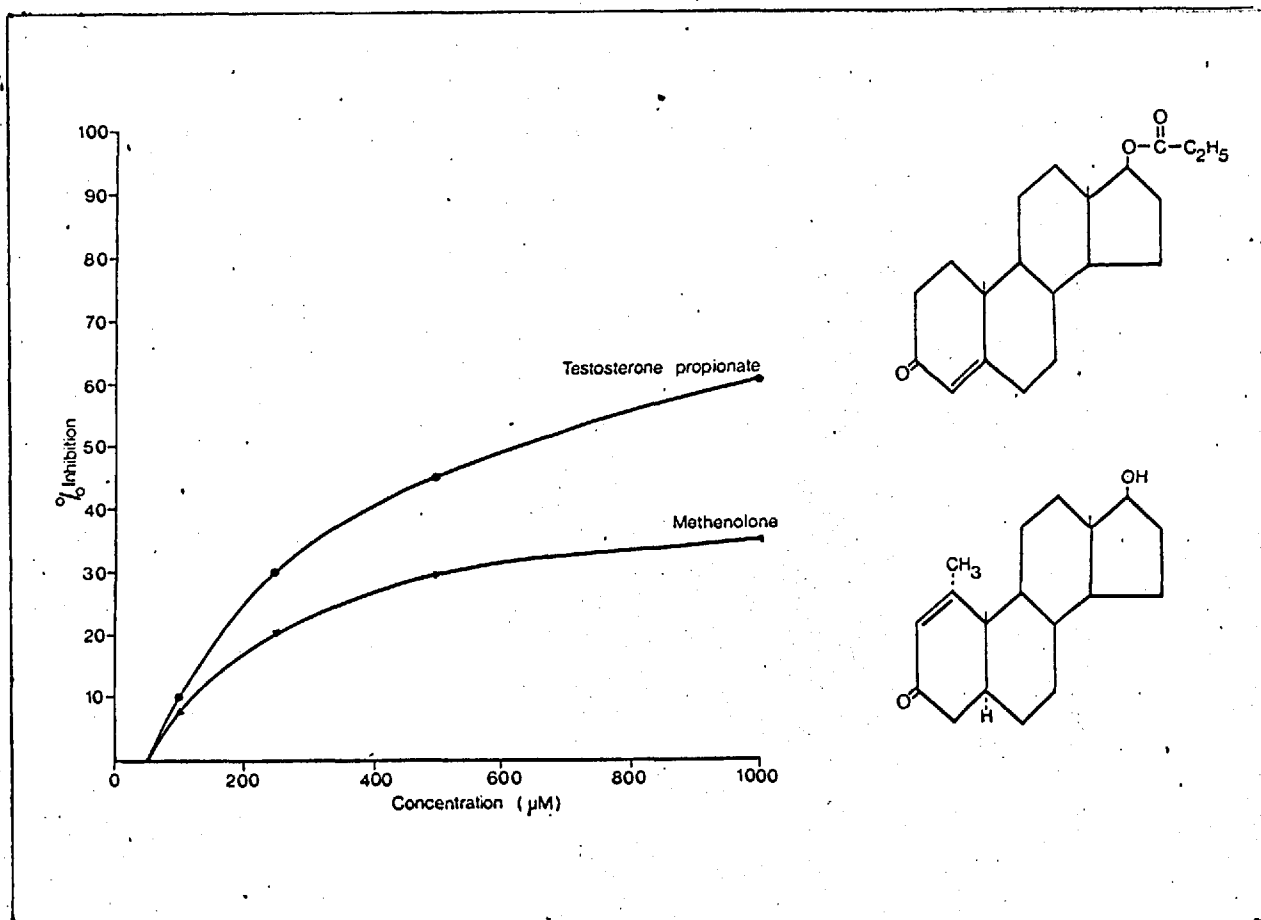
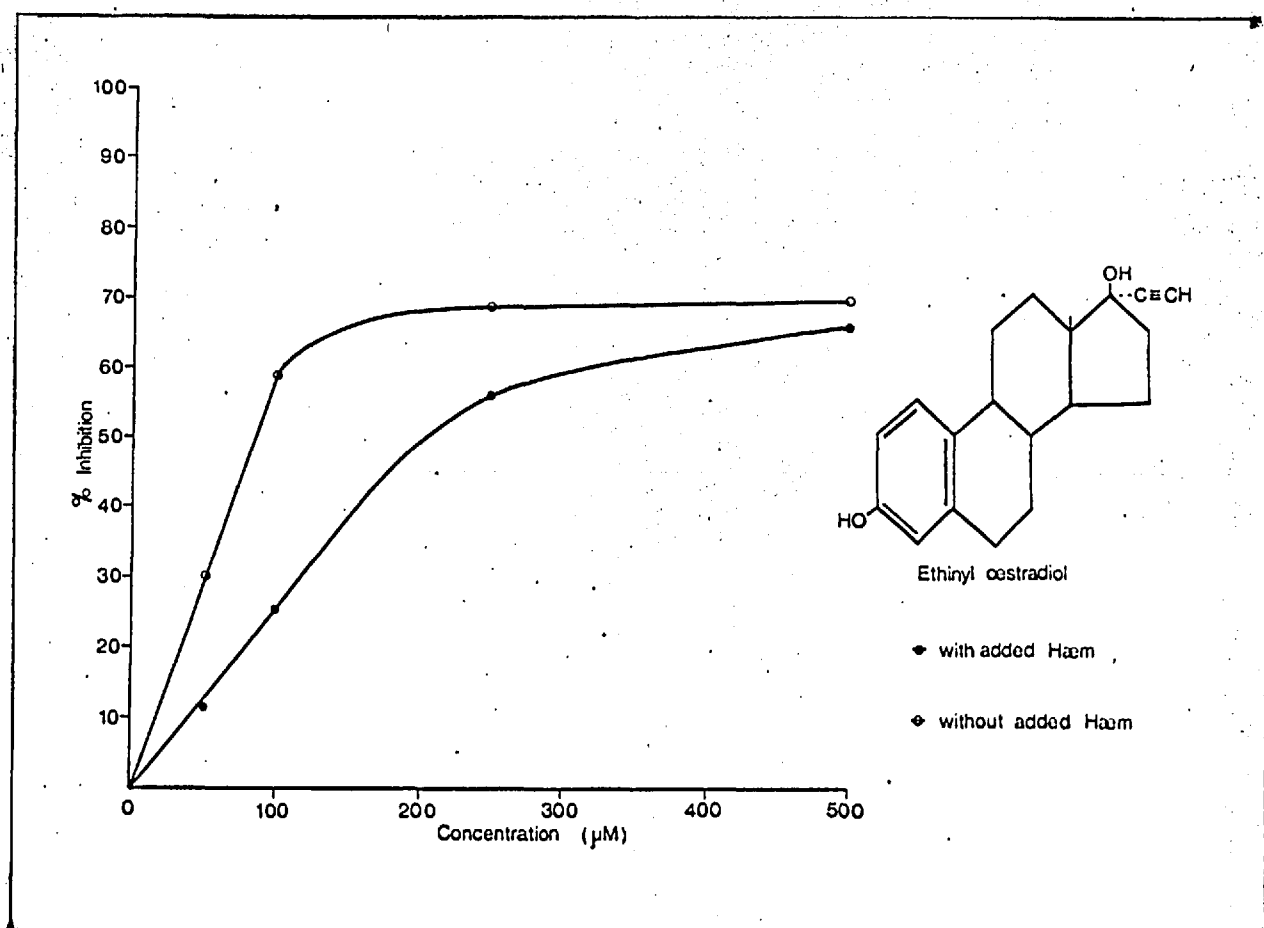


Fig. 44. Inhibition of Tryptophan Oxygenase Activity by Addition of Ethinyl Oestradiol In Vitro.



Experiment I. The Effect of some Δ^4 -3-oxosteroids on Tryptophan Oxygenase

Both progesterone and deoxycorticosterone were potent inhibitors of TO activity over the range 50-500 μ M (Table 37; Fig. 41). In both cases there was between 16-22% inhibition of activity at 50 μ M rising to a loss of 75% of the enzyme activity with 500 μ M concentration of either steroid. The structure of these steroids includes a ketone group at position 3 on ring A conjugated to a double bond between carbons 4 and 5 (Fig. 42).

Testosterone propionate and methenolone inhibited TO activity, although not to the same extent as either progesterone or deoxycorticosterone. Both testosterone propionate and methenolone failed to inhibit enzyme activity at 50 μ M concentrations and methenolone produced only a 30-40% inhibition at 500 μ M. These two steroids have similar structural features to progesterone and deoxycorticosterone, although methenolone has a oxo group at position 3 conjugated to a double bond between carbons 1 and 2 on ring A instead of a double bond between carbons 4 and 5, (Fig. 43).

The importance of the presence of a double bond in ring A conjugated to the oxo group is emphasised by the results obtained with mesterolone, another anabolic steroid. Mesterolone has an identical structure to methenolone except that the double bond is absent from ring A, and was unable to inhibit TO activity.

In contrast to the results described above, cortisol, corticosterone and fluoxymesterone failed to inhibit TO activity even when present at a concentration of 1mM. As shown in Fig. 42 these Δ^4 -3-oxo steroids all have an 11 β hydroxyl group present in their structures, which is the only point of difference between corticosterone, a non-inhibitor, and deoxycorticosterone, a potent inhibitor of TO activity.

Table 37. Inhibition of Tryptophan Oxygenase In Vitro by Steroids and Diethylstilboestrol.

<u>Compound</u>	<u>Steroid Concentration</u>					
	<u>50 μM</u>		<u>100 μM</u>		<u>500 μM</u>	
	<u>Enzyme Activity</u>	<u>Inhibition %</u>	<u>Enzyme Activity</u>	<u>Inhibition %</u>	<u>Enzyme Activity</u>	<u>Inhibition %</u>
Progesterone	2.7 ± 0.3	16-22	2.2 ± 0.5	25-36	0.9 ± 0.4	70-80
Deoxycorticosterone	2.8 ± 0.3	18-21	2.5 ± 0.2	18-26	0.8 ± 0.4	68-81
Testosterone propionate	3.4 ± 0.1	0	3.0 ± 0.1	9-13	2.0 ± 0.3	43-54
Plethenolone	3.4 ± 0.3	0	3.0 ± 0.2	10-14	2.1 ± 0.2	38-49
Oestradiol-17 β	3.1 ± 0.2	7-11	2.3 ± 0.1	16-21	1.2 ± 0.4	65-78
Ethinyl oestradiol	3.0 ± 0.1	11-19	2.8 ± 0.3	15-25	1.0 ± 0.5	73-79
Oestrone-3-sulphate	3.4 ± 0.1	0	3.1 ± 0.2	9-11	2.3 ± 0.3	44-52
Diethylstilboestrol	3.1 ± 0.2	9-11	2.6 ± 0.3	21-26	1.0 ± 0.3	71-82
Corticosterone		0		0		0
Cortisol		0		0		0
Fluoxymesterone		0		0		0
Mesterolone		0		0		0
Mestranol		0		0		0

All values for enzyme activity given in this table are mean $\pm$ SD for 5-6 assays, and the range of % inhibition are stated for each steroid. "0" denotes no detectable inhibition.

Experiment 2. The effect of oestrogenic steroids on tryptophan oxygenase

Both oestradiol-17 β and ethinyl oestradiol, a synthetic oestrogen, were shown to be inhibitors of TO activity, as potent as either progesterone or deoxycorticosterone (Table 37). Ethinyl oestradiol inhibited TO activity even when haem cofactor was omitted from the assay medium. (Fig. 44)

Oestrone-3-sulphate, a conjugated oestrogen, had an inhibitory effect but to a lesser extent than did oestradiol-17 β . The former was unable to inhibit enzyme activity as a concentration of 50 μ M and gave only between 9-11% inhibition at 100 μ M (Table 37).

Mestranol, the three methyl ether of ethinyl oestradiol, did not inhibit TO at any concentration. The experiments with both oestrone-3-sulphate and mestranol emphasise the importance of the hydroxyl group at position 3 on ring A. Replacement of the group by a sulphate radical, reduced the ability of the steroid to inhibit enzyme activity and replacement with a non polar ether group abolished inhibition. Diethyl stilboestrol, a phenol with oestrogenic properties when administered in vivo was found to be a potent inhibitor of TO in the in vitro system (Table 37).

Experiment 3. Mechanism of inhibition by steroids

Oelkers and Dulce suggested that oestrone-3-sulphate inhibited TO activity by competing with the haem cofactor for binding sites on the apoenzyme (Oelkers and Dulce, 1964). It was decided to examine this possibility with respect to the inhibition by progesterone, ethinyl oestradiol and diethylstilboestrol.

Fig. 45. Double Reciprocal Plot for Inhibition by Progesterone or Ethinyl Oestradiol to Show Competition with Haem Cofactor.

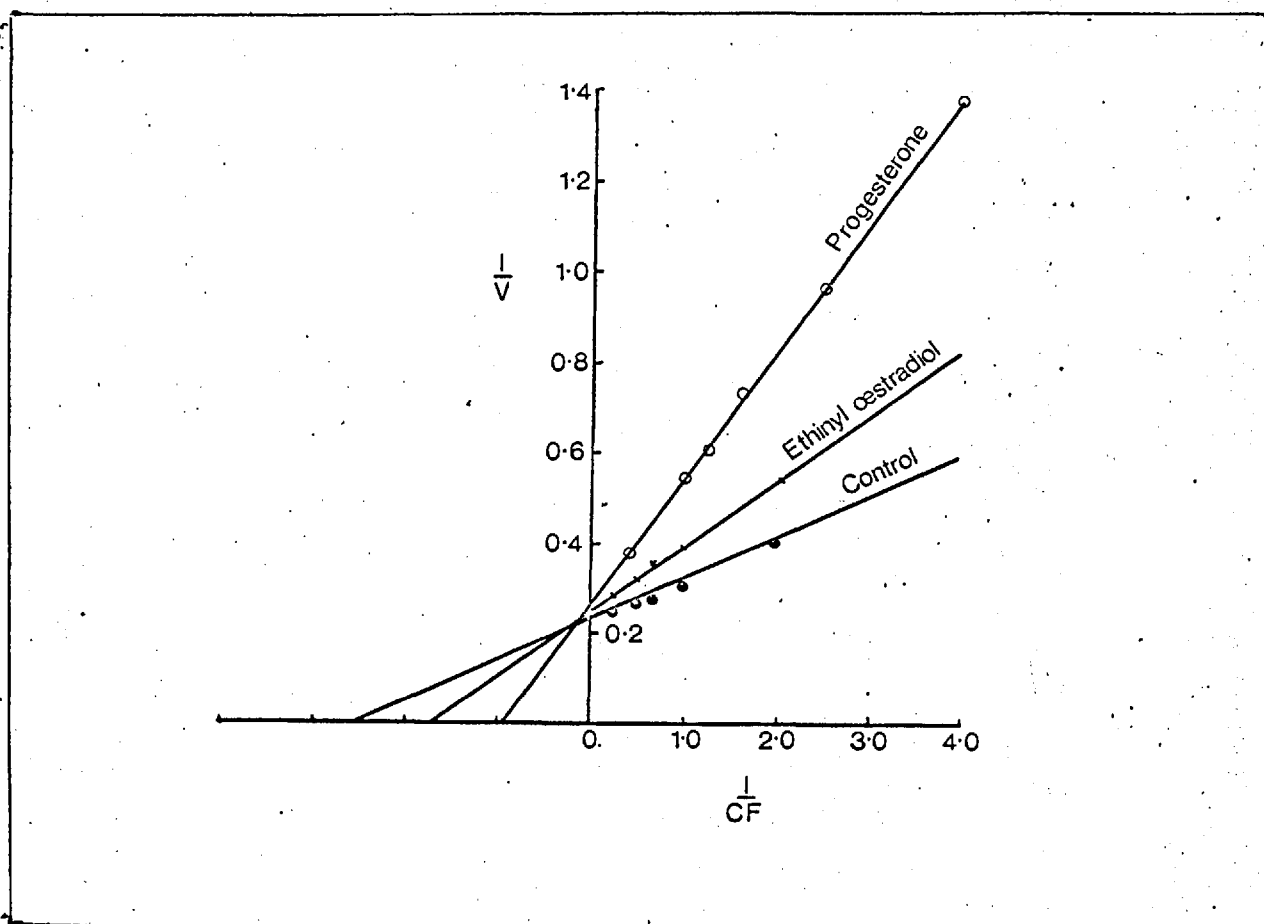
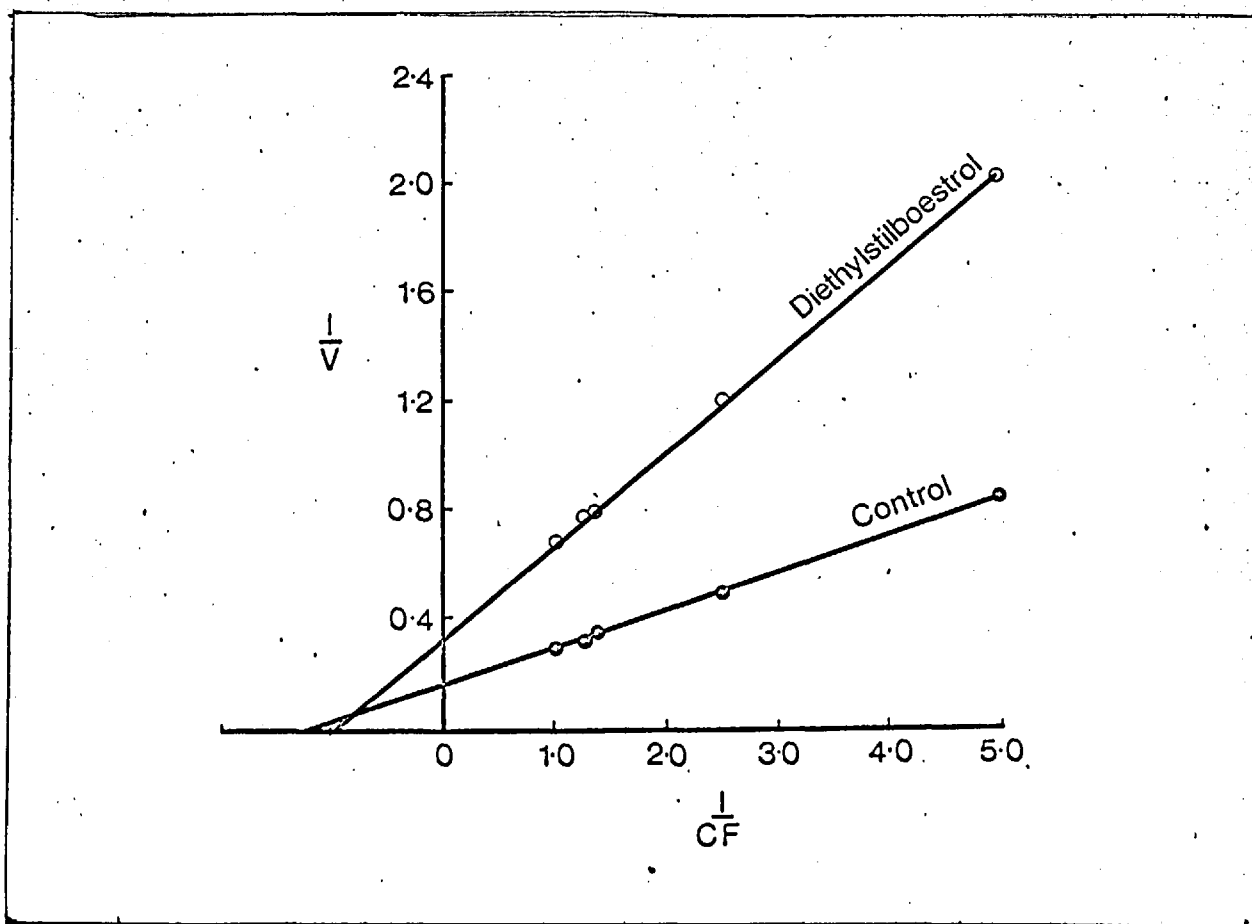


Fig. 46. Double Reciprocal Plot for Inhibition by Diethyl Stilboestrol.



$\frac{1}{V}$ = Reciprocal of Rate of Enzyme Activity. $\frac{1}{CF}$ = Reciprocal of Haem Concentration.

The procedure adopted in these experiments was to vary the concentrations of haem cofactor in the presence of the enzyme, the other constituents of the reaction mixture and a constant concentration of the steroid. With each experiment, a series of flasks with 0.1 ml ethanol in place of the steroid were included. Data from such experiments were presented as Lineweaver-Burke plots. Routinely, concentrations of 5 μ M-haem were used in the assay system, since this optimal cofactor concentration allowed saturation of the apoenzyme without inhibiting enzyme activity (Chapter 4), but for this set of experiments, it was necessary for the kinetics to be linear and first order, so that a range of haem concentrations less than 5 μ M were used. Incubations were carried out in the presence of 100 μ M concentrations of progesterone and ethinyl oestradiol. Data was calculated using the method of least squares.

Reciprocal plots for both progesterone and ethinyl oestradiol are shown on Fig. 45. The intercepts reached a focal point on the $\frac{1}{V}$ axis and thus were consistent with the general form of competitive inhibition.

A similar procedure was adopted for diethyl stilboestrol and the Lineweaver-Burke plot is shown on Fig. 46. An intercept was formed on the axis of $\frac{1}{CF}$ which denoted that inhibition was not competitive for the cofactor.

Discussion

The ability of the steroids investigated in the present study to inhibit TO in vitro depended on certain structural features. Ring A seemed to be of particular importance and the nature of the substituent at position 3 was shown to be crucial. Those compounds with a .oxo group at position 3 conjugated to a double bond between carbons 4 and 5, as in progesterone, deoxycorticosterone and testosterone

propionate, or between carbons 1 and 2 as in methenolone, were all capable of inhibiting TO in vitro. The presence of a hydroxyl group at position 3 as in the oestradiol-17 β or ethinyl oestradiol also conferred inhibitory properties to the steroids. The presence of an oxo group at position 3 with no conjugated double bond at ring A, as in mesterolone, rendered inhibition of enzyme activity impossible. This indicates that some form of enolation at position 3 is necessary for inhibition to take place. This suggestion is supported by other observations in this study; the substitution of the hydroxyl at position 3 with a sulphate ester, as in oestrone-3-sulphate, decreased the level of inhibition, and inhibition was prevented completely by the substitution of the hydroxyl group in ring A of ethinyl oestradiol by 3-methyl ether. Sulphate esterification may therefore have reduced enolation, and substitution by the non polar dimethyl ether group may have prevented enolation completely.

The presence of an 11 β -hydroxyl group in the Δ^4 -3-oxo steroids also prevented inhibition from taking place. It is possible that the inclusion of this substituent in the steroid nucleus prevented the Δ^4 -3-oxo steroids from assuming the steric confirmation necessary for inhibition.

Progesterone and ethinyl oestradiol were shown to inhibit TO activity by competition with the haem cofactor. Oestrone-3-sulphate had previously been shown to act by similar mechanism (Oelkers and Dulce, 1964). The precise way in which the steroids may effect competitive inhibition with the haem cofactor is not indicated by the scope of the present study. It is possible that the steroids compete directly with the haem cofactor for binding sites on the apo-tryptophan oxygenase.

Alternatively the steroids may modify the haem molecule in some way, rendering it incapable of performing its normal cofactor function. Perhaps it is this modified haem cofactor which competes for the binding site on the apoprotein. In order to differentiate between these two possibilities, studies with purified TO and cofactor should be made.

A similar study to that described in this thesis has been carried out by Kenney and Lee (1971) to investigate the effects of various steroids on cortisol induction of TAT in an in vitro cell culture system. Steroids with a keto group at position 3 conjugated to a double bond between carbons 4, 5 in ring A were able to induce TAT activity. Inhibition of cortisol induction was enhanced by the presence of an 11β hydroxyl group. Kenney and Lee have suggested that these structural features are important since it is proposed that the steroids interact with a receptor protein in the cell. In the present study, it is proposed that interaction between steroids and protein also occurs, and that this interaction is facilitated by the presence of certain substitutions in the steroid nucleus described above.

In contrast to the results for progesterone and ethinyl oestradiol, diethyl stilboestrol was found to inhibit TO in vitro by a mechanism which was non-competitive for the haem cofactor. The phenolic compounds, adrenaline, catechol and dihydroxyphenylalanine have previously been shown to inhibit TO activity by competition with the substrate, tryptophan (Frieden, Westmark and Schor, 1961). It is possible that diethyl stilboestrol, which is also a phenol, inhibits TO by competition for substrate binding sites rather than cofactor binding sites on the apoprotein.

An earlier study described inhibition by diethyl stilboestrol of glutamate dehydrogenase activity in vitro by an alteration of its quaternary structure, such that there was a disaggregation of the polymeric structures into its constituent monomers (Yielding and Tomkins, 1962). Tryptophan oxygenase is also comprised of four monomers (Cho Chung and Pitot, 1967), so that the effects of diethyl stilboestrol observed in this present study may be due to a mechanism similar to that described by Yielding and Tomkins for glutamate dehydrogenase, which would also account for the inhibition by diethyl stilboestrol being non-competitive for the haem cofactor. This mechanism may not be confined to TO since diethyl stilboestrol also inhibits TAT in vivo by an interaction with the quaternary enzyme structure, which fulfils a protective function, preventing disaggregation of the polymer into subunit monomers of TAT (Singer and Mason, 1967). The relevance of this work to steroid interaction with TO is a subject for further investigation with highly purified protein.

The level of steroids used in this present study is well above the physiological range. In the course of several in vivo studies, other workers have used levels of hormone administration which were pharmacological doses and it is possible that results obtained from such studies are due to a direct inhibition of enzyme activity. Puchol and Caballido, (1961) using doses as high as 25 mg testosterone propionate for 5 days, reported inhibition of alanine aminotransferase and aspartate aminotransferase. Aspartate aminotransferase was unaffected by a similar dose of testosterone acetate, which indicates that the doses were high enough for the propionate rather than the androgen to exert an inhibitory effect. Inhibition of another pyridoxal phosphate enzyme, kynurenine aminotransferase has been studied by Mason and Gullekson (1960).

Inhibition by steroids of the enzymes of the tryptophan-nicotinamide pathway is not restricted to TO, since oestradiol-17 β treatment decreased kynureninase activity, although this was increased by a combination of mestranol and norethynodrel (Rose and Brown, 1969). It is possible that with the oestrogenic steroid treatment, changes in enzyme activity are dose dependent; low doses of the same order as used in the work included in this thesis being stimulatory to activity, while high doses may diminish enzyme activity by direct inhibition.

CHAPTER 8. General Discussion

During the course of the present study, it was found that adrenalectomy reduces TO activity and serum corticosterone levels in the female, but not in the male, and both male rats and ovariectomised females have lower TO activity and circulating corticosterone concentrations than do intact females. Androgen-treated females have diminished TO activity together with serum corticosterone in contrast to similarly treated males and ovariectomised females, where neither enzyme activity or serum corticosterone levels are further reduced on administration of testosterone.

It is possible that observations made by Pitot and Cho Chung (1970) may be of relevance to these results. These authors showed that there are two isoenzymes of TO, one of which is constitutively synthesised in adrenalectomised animals and therefore may be responsible for most of the activity in rats with low corticosterone levels, such as intact males and ovariectomised females. The second isoenzyme is synthesised after treatment of the rat with glucocorticoids and therefore may be present in the intact female, together with the first isoenzyme, resulting in higher TO activity than in the male. It has been suggested earlier that treatment of intact females with androgens diminishes TO activity due to the decrease in serum corticosterone levels. In view of the work by Pitot and Cho Chung, the reduction in circulating corticosterone levels may have produced this effect by decreased synthesis of the second isoenzyme. It is also possible that adrenalectomy of male rats does not result in a further fall in TO activity since most of the enzyme activity in intact male rats may have arisen from the constitutively synthesised isoenzyme, the production of which cannot be decreased.

TAT activity is increased after hypophysectomy (Chapter 5) and this result is consistent with the effects of hypophysectomy on the activities of several other amino acid-metabolising enzymes; liver AAT (Rosen, Harding and Budnick, 1961), aspartate aminotransferase in both muscle and liver (Bartlett and Glynn, 1950; Zuchlewski and Gaebler, 1957) and liver histidase activity in male rats (Feigelson, 1971a) are all increased after removal of the pituitary gland. Serine dehydrase is reduced after hypophysectomy and adrenalectomy (Freedland and Avery, 1964), while results obtained in the present study show that TO is unchanged after removal of the pituitary gland. Although the effect of hypophysectomy is not the same for all enzymes of amino acid metabolism, it is apparent from these various observations that the secretions of the pituitary gland are important in the establishment of basal levels of activity for several enzymes of amino acid metabolism.

One of the most significant observations made during the course of the work reported here is that the administration of 10 µg oestradiol benzoate for 14 days elevates the activity of all three enzymes under investigation in the intact female rat. Keller, Richardson and Yates, (1969) suggested that the increased liver AAT activity which they observed in oestrogen-treated male rats is due to the raised level of protein-bound corticosterone, also caused by oestrogen administration. Transcortin-bound glucocorticoids were previously considered to be physiologically inert (Slaunwhite, Lockie, Back and Sandberg, 1962; Kawai and Yates, 1966), although Keller et al (1969) proposed that the vascular system of the liver is sufficiently permeable to allow the protein-bound steroid into the hepatocytes where free corticosterone is released to induce AAT synthesis. In the early stages of the present investigation, it was considered that this hypothesis provided an adequate explanation

for the increased enzyme activity following oestrogen administration, since in oestrogen-treated ovariectomised rats, enzyme activity is raised above the levels of intact control females, concomittantly with elevations in serum corticosterone concentrations. Further experiments showed that the serum corticosterone levels are not increased in intact oestrogen-treated females, although there are marked elevations in enzyme activity. Moreover, the results obtained from oestrogen administration to adrenalectomised male and female rats and to castrated males, described in Chapter 5 and 6, showed that oestrogens act independently of corticosterone to produce small elevations in TO and TAT activity. However, the large increases in enzyme activity seen in oestrogen-treated intact females is not found after oestrogen administration to both hypophysectomised or adrenalectomised rats. It was therefore concluded that oestrogens require the presence of circulating corticosterone, rather than increased levels of the glucocorticoid, in order to produce maximal increase in enzyme activity. It was further suggested that corticosterone may play a permissive role in this mechanism.

A similar hypothesis was suggested by Mavrides and Lane (1970) to explain the roles of glucocorticoids and dietary intake of amino acids in the diurnal variation of TAT activity. These authors found that in adrenalectomised rats, the circadian rhythm of enzyme activity was only partially evident, indicating that while the presence of adrenal gland secretions was necessary for full expression of diurnal variation, some other factors were involved, which operated independently of corticosterone. Dietary intake of amino acids was shown by Wurtman and Axelrod (1967) to be essential for establishment of diurnal variation and after withdrawal of protein from the diet, circadian variation of TAT activity

is completely abolished. Mavrides and Lane (1970) demonstrated that doses of cortisol, too small for TAT induction, potentiate elevations in TAT activity resulting from dietary intake of amino acids. These authors concluded from their work and from the experiments of Wurtman and Axelrod (1967) that the circadian rhythm of TAT activity is a direct result of fluctuations in the dietary intake of amino acids, although for full expression of enzyme activity, the presence of corticosterone is required.

Although lower doses of oestrogen are stimulatory to TO, TAT and AAT activities, larger doses administered for longer periods were found to depress the activity of all three enzymes (Chapter 5). The concentration of oestrogens reached in the liver after these longer periods of treatment may have been sufficient to reduce enzyme activity by direct inhibition. In the studies described in Chapter 7, high levels of steroids were shown to inhibit TO activity *in vitro* by direct competition for the cofactor binding sites on the apoenzyme. It is possible that after long periods of administration, oestrogens may produce a similar effect on all three enzymes *in vivo*. A relation may therefore exist between dosage levels and enzyme activity such that low doses are stimulatory and high doses are inhibitory to TO, TAT and AAT levels. A similar proposal was made by Kitay (1963a, 1963b) to explain the effect of oestrogen administration on corticosterone synthesis in male and female rats. Oestrogen treatment of male rats raises the level of adrenal corticosterone production to that observed in the intact female, while in oestrogen treated females, the combination of administered oestrogen and endogenous oestrogen is sufficiently high to inhibit corticosteroidogenesis.

The influence of sex hormones on histidase has been studied extensively by Feigelson (1968, 1971a, 1971b, 1971c) and the results of this investigation are comparable to the present study. Ovariectomy reduces histidase activity in female rats, which have higher activity than males (Feigelson, 1969, 1971a) and these observations are similar to those reported for TO in Chapter 5. Oestrogen treatment of ovariectomised females raises histidase activity (Feigelson, 1971a) as shown in the present study for TO, TAT and AAT. Histidase activity is increased after hypophysectomy of ovariectomised females and oestrogen treatment of these rats does not result in further increases in enzyme activity (Feigelson, 1971b) which are similar to the pattern of responses seen for TAT activity in intact females (Chapter 5). Feigelson concluded from her study that a pituitary factor is involved in the increased histidase activity caused by oestrogen treatment. However, the effects of oestrogen administration on histidase activity in adrenalectomised rats were not investigated so it is uncertain whether corticosterone has any role in the elevations of histidase activity due to oestrogen administration. Androgen treatment of female rats diminishes histidase activity (Feigelson, 1971c) as described for TO activity (Chapter 6). Although orchidectomy does not decrease either TO or TAT activity, Feigelson (1971c) showed that histidase activity is diminished in castrated male rats. In both studies, androgen treatment is most effective in decreasing the activities of TO and histidase when there is scope for antagonism to oestrogens and Feigelson suggested that androgens reduced histidase activity by competition with oestrogens either during interaction at the pituitary gland or at a hepatic site.

The effects of sex hormone administration on liver-amino acid-metabolising enzymes reported in the present study and by Feigelson (1968, 1971a, 1971b) may be relevant to an elucidation of the role played by these enzymes during pregnancy, both in the rat and in human beings. In the pregnant rat both TO (Auricchio, Rigillo and DiToro, 1961) and histidase activities (Makoff and Balridge, 1964) are increased, possibly as a result of the raised level of circulating oestrogens. Harding, Rosen and Nichol (1966) showed TAT to be unchanged on the 19th day of gestation, although the possibility that the enzyme activity is raised in the first half of pregnancy was not investigated by these authors. In the same study, AAT activity, which was observed throughout gestation, was shown to diminish after the 10th day and to remain at a reduced level throughout the rest of pregnancy. Rosen et al suggested that this was caused by a reduction in hepatic amino acid levels during pregnancy, since increased dietary intake of protein re-established the level of AAT activity. Many of the glucocorticoid-inducible enzymes of amino acid metabolism, for example, TAT, and serine dehydrase, catalyse the rate-limiting steps of degradative pathways for amino acids which are also associated with carbohydrate metabolism. Mavrides and Lane (1969) postulated a direct association between hormonal control of TAT activity and gluconeogenesis. It is possible that the increased requirement for gluconeogenesis during pregnancy results in raised turnover along the catabolic pathways of the appropriate amino acids. Such increases in degradation may be caused by raised oestrogen levels stimulating the activity of the enzymes catalysing the rate-limiting steps.

Although the kynurenine pathway is not directly connected with carbohydrate metabolism, a similar reason may also exist for the raised TO activity observed in rats after oestrogen treatment (Chapter 5) and during pregnancy (Auricchio et al, 1961). The end product of tryptophan degradation by this pathway is nicotinic acid ribonucleotide, which is required in the synthesis of NAD. The enhanced metabolic activities of pregnancy, are likely to result in an increased demand for NAD, necessitating increased nicotinic acid ribonucleotide synthesis. It is possible that enhanced turnover along the tryptophan-niacin pathway, due to elevated TO activity, may serve to meet this possible requirement. However, from work performed on both human subjects and animals, there is evidence to suggest that the production of increased NAD levels does not depend solely on raised levels of TO activity. Powanda and Wannemacher, (1971) have shown that in the mouse, hydrocortisone administration induces TO synthesis but this is not accompanied by marked increases in hepatic NAD concentrations. Inhibition of TO activity with allopurinol given alone or in combination with tryptophan does not affect NAD levels in the liver, which are raised after administration of tryptophan by intraperitoneal injection. Powanda and Wannemacher concluded that the availability of tryptophan, rather than changes in TO activity, controls NAD synthesis. A similar conclusion was reached by Kim and Miller (1969) who showed that induction of TO in rat liver after injection with hydrocortisone does not affect production of $^{14}\text{CO}_2$ from side chain-labelled tryptophan, whereas this is increased after administration of high doses of tryptophan. Earlier studies by Altmann and Greengard, (1966) demonstrated that injection of hydrocortisone to hospital patients results in both raised liver TO activity and urinary excretion of kynurenine. Although no other metabolites were measured in that study, later work

has shown that after administration of hydrocortisone to human subjects, followed by a 5g loading dose of tryptophan, urinary excretion of 3-hydroxykynurenine, xanthurenic acid and 3-hydroxyanthranilic acid as well as kynurenine are increased, but the levels of 2-pyridone, a metabolite of N'-methylnicotinamide is unaffected (Rose and McGinty, 1968). In rats treated with hydrocortisone, urinary excretion of 3-hydroxykynurenine, 3-hydroxyanthranilic acid and kynurenine are elevated but xanthurenic acid are unaltered, (Mainardi, 1947). It seems, therefore, that the increased TO activity, resulting from administration of glucocorticoids both to animal and human subjects, does not affect synthesis of N'-methylnicotinamide. However, from the urinary metabolite studies of both Altmann and Greengard, (1966) and Rose and McGinty, (1968) the raised activity of the enzyme enhances 3-hydroxyanthranilic acid synthesis.

Rose and McGinty, (1969) suggested that after the administration of a loading dose, the increased amount of tryptophan which enters the degradative pathway, is diverted from nicotinic acid ribonucleotide synthesis, under the influence of picolinic carboxylase into the sequence of reaction which yield glutaryl-CoA and ultimately carbon dioxide. Picolinic carboxylase catalyses the first stage in the degradation of α -amino β carboxymuconic- ϵ -semialdehyde, formed from 3-hydroxyanthranilic acid to produce picolinic acid and carbon dioxide.

In contrast, pregnancy results in elevated urinary excretion of 2-pyridone (Brown, Thornton and Price, 1961) and N'-methylnicotinamide (Hernandez, 1964), together with kynurenine, xanthurenic acid and 3-hydroxyanthranilic acid after a 2g loading dose of tryptophan. These results show that during pregnancy in the human, there is enhanced turnover along the tryptophan-nicotinic acid ribonucleotide pathway and similar findings by Rose, Brown and Price (1968) have also shown that this is true

for women taking oestrogen-containing oral contraceptives. Although the present study has demonstrated that increased oestrogen levels elevate TO activity, from the failure of hydrocortisone to influence the excretion of N-methylnicotinamide, discussed previously, it is clear that the raised activity of this enzyme is not solely responsible for the enhanced nicotinic acid ribonucleotide synthesis, observed in pregnancy and after oestrogen treatment of human subjects.

Picolinic carboxylase provides a further potential control point on the pathway since its activity may regulate the proportion of 3-hydroxyanthranilic acid which is metabolised to glutaryl-CoA and carbon dioxide. It may be that elevated oestrogen levels diminish picolinic carboxylase activity so that less tryptophan is metabolised by the glutarate pathway and more is metabolised to nicotinic acid ribonucleotide. The effect of oestrogens on this enzyme is a subject for urgent investigation if the complexities of the influence of hormones upon the tryptophan-nicotinic acid ribonucleotide pathway are to be successfully elucidated.

APPENDIX I

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APPENDIX II

Nomenclature of Steroids

Trivial NamesSystematic Names

Corticosterone

11 β , 21-Dihydroxy-4-pregnene-3, 20-dione

Deoxycorticosterone

21-Hydroxy-4-pregnene-3, 20-dione

Ethinyl Oestradiol

17 α -Ethinyl-1, 3, 5 (10)-estratrien-3-17 β -diol

Fluoxymesterone

11 β , 17 β -Dihydroxy-9 α fluoro-17 α -methyl-
4-androstene-3-one

Hydrocortisone, Cortisol

11 β , 17 α , 21-Trihydroxy-4-pregnene-3, 20-dione

Megesterol Acetate

17 α -Acetoxy-6-methyl pregna-4, 6-diene-
3, 20-dione

Mesterolone

17 β -Hydroxy-1 α -methyl-5 α -androstene-3-one

Mestranol

17 α -Ethinyl-1, 3, 5 (10)-estratriene-3, 17 β -
diol-3-methyl ether

Methenolone

17 β -Hydroxy-1 α -methyl-androstan-3-one

Norethynodrel

17 α -Ethinyl-17 β -hydroxy-5 (10)-estrene-3-oneOestradiol-17 β 1, 3, 5 (10)-Estratriene-3, 17 β -diol

Oestradiol Benzoate

1, 3, 5 (10)-Estratriene-3, 17 β -diol-3-benzoate

Oestrone-3-Sulphate

3-Hydroxy-1, 3, 5 (10)-estratriene-17-one-3-
sulphate

Progesterone

4-Pregnene-3, 20-dione

Testosterone propionate

17 β -Hydroxy-4-androsten-3-one propionate

APPENDIX III. Papers Published as a Result of the Present Study.

1. Braidman, I.P. and Rose, D.P. (1970). Inhibition of Tryptophan Oxygenase in Rat Liver by Steroid Hormones. *Biochem. J.* 118, 7P.
2. Braidman, I.P. and Rose, D.P. (1971). Inhibition of Tryptophan Oxygenase In Vitro by Steroid Compounds. *Biochem. Pharmacol.* 20, 973.
3. Braidman, I.P. and Rose, D.P. (1971). The Effect of Sex Hormones on the Activity of Tryptophan Oxygenase and Other Corticosteroid-Inducible Enzymes in Rat Liver. *Biochem. J.* 122, 28P.
4. Braidman, I.P. and Rose, D.P. (1971). The Effects of Sex Hormones on Three Glucocorticoid-Inducible Enzymes Concerned with Amino Acid Metabolism in Rat Liver. *Endocrinology*, 89, 1250.
5. Rose, D.P. and Braidman, I.P. (1971). Excretion of Tryptophan Metabolites as Affected by Pregnancy, Contraceptive Steroids and Steroid Hormones. *Am. J. Clin. Nutr.* 24, 673.

Inhibition of Tryptophan Oxygenase in Rat Liver By Steroid Hormones

By ISOBEL P. BRAIDMAN and D. P. ROSE. (*Alexander Simpson Laboratory for Metabolic Research, St Mary's Hospital Medical School, London W.2, U.K.*)

Pregnant women and normal human subjects treated with oestrogens show evidence of an increased capacity for the conversion of L-tryptophan into nicotinic acid ribonucleotide. This has been attributed to an increased activity of liver tryptophan oxygenase (EC 1.13.1.12) brought about either by the direct action of oestrogens, or indirectly via elevated corticosteroid concentrations (Rose, 1966, 1969). Tryptophan oxygenase activity is elevated in the liver of pregnant rats (Auricchio, Rigillo & Di Toro, 1960), but treatment with a combination of oestrone and progesterone failed to produce an increase in the activity of the enzyme (Greengard, Kalinsky & Manning, 1968). In a further study tryptophan oxygenase activity was determined in supernatant fractions prepared from the livers of rats treated with progesterone (4mg or 8mg daily), oestradiol (20 μ g daily), or a combination of the two steroids for 28 days. Progesterone caused a significant decrease in tryptophan oxygenase activity both when given alone and in combination with oestradiol (D. P. Rose & R. R. Brown, unpublished work).

In an attempt to investigate this problem further, the effect of additions of various steroids *in vitro* on tryptophan oxygenase activity in rat liver supernatant preparations was studied. Progesterone and deoxycorticosterone, at a concentration of 0.1 mM, produced a significant inhibition of enzyme activity, as did the synthetic oestrogen ethinyloestradiol. However, mestranol, the 3-methyl ether of ethinyloestradiol, did not inhibit the enzyme, even when present at a concentration of 1 mM. The 11 β -hydroxylated steroids cortisol, corticosterone and fluoxymesterone (11 β , 17 β -dihydroxy-9 α -fluoro-17 α -methylandrosta-4-en-3-one) did not inhibit tryptophan oxygenase despite their structural similarity to progesterone and deoxycorticosterone in being Δ^4 -3-oxo steroids. The importance of a double bond in ring A is indicated by the finding that methenolone (17 β -hydroxy-1 α -methyl-5 α -androsta-1-en-3-one) inhibits the enzyme, whereas mesterolone (17 β -hydroxy-1 α -methyl-5 α -androsta-3-one) is without effect. Studies with various other steroids indicated that substituents remote from ring A at C-17, C-20 and C-21 are unimportant with respect to the inhibition of tryptophan oxygenase.

The enzyme inhibition by progesterone at a concentration of 0.1 mM was reversed by increasing the concentration of haematin in the assay system, and evidence obtained from Lineweaver-Burk plots was consistent with competitive inhibition. The inhibitory mechanism may involve the haem cofactor of the enzyme either by the formation of a complex with the steroid or by competition for sites on the apoenzyme. Oelkers & Dulce (1964) have suggested a similar mechanism for the inhibition of tryptophan oxygenase *in vitro* by oestrone sulphate.

We are grateful to Dr M. H. Briggs, Schering Chemicals Ltd., for gifts of methenolone and mesterolone.

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Inhibition of tryptophan oxygenase *in vitro* by steroid compounds

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LIVER tryptophan oxygenase (TPO) is subject to induction both by its substrate and by adrenal glucocorticoids.¹ Although TPO activity has been shown to be elevated in the livers of pregnant rats^{2,3} the administration of oestrone and progesterone in combination to non-pregnant females failed to produce an increase in enzyme activity.³ Further, it was subsequently found that the subcutaneous injection of progesterone alone, in a dose of 4 or 8 mg daily for 28 days, caused a significant reduction of TPO levels in rat liver.⁴

In an attempt to investigate this problem further we have studied the effect of *in vitro* additions of progesterone and various other steroids on TPO.

Methods

Livers were obtained from adult female Sprague-Dawley rats killed by decapitation. A particle-free supernatant was prepared by centrifugation of a 10% (w/v) homogenate at 105,000 g for 60 min at 4°, and the TPO activity determined by the method of Leklem *et al.*⁵

The steroids were dissolved in 100% ethanol such that the required amount for addition to the enzyme assay medium was present in 0.1 ml. Control assays were carried out with each experiment, both in the presence and absence of ethanol, so that every inhibition study had its own control, prepared from the same liver as was used in flasks containing the steroid. The ethanol did not affect the activity of TPO. The mean control value for TPO obtained in 22 assays was 3.4 ± 0.8 μ moles kynurenine produced/g wet liver tissue/hr.

Results and discussion

Inhibition of TPO activity was obtained with a number of steroids (Table 1). Progesterone and deoxycorticosterone, and to a lesser degree testosterone propionate, were effective inhibitors, but corticosterone and cortisol did not inhibit the enzyme, even when present at a 1 mM concentration. All five steroids are Δ^4 -3-oxosteroids, but the latter two compounds have an 11β hydroxyl group and it seems likely that the presence of this substituent was responsible for the absence of an inhibitory effect.

TABLE 1. *In vitro* INHIBITION OF TRYPTOPHAN OXYGENASE BY STEROIDS AND DIETHYLSTILBOESTROL

Compound	Steroid concentration			
	50 μ M		100 μ M	
	Enzyme activity*	Inhibition (%)	Enzyme activity*	Inhibition (%)
Progesterone	2.7 ± 0.3	16-22	2.2 ± 0.5	25-36
Deoxycorticosterone	2.8 ± 0.3	18-21	2.5 ± 0.2	18-26
Testosterone propionate	3.4 ± 0.1	0	3.0 ± 0.1	9-13
Oestradiol- 17β	3.1 ± 0.2	7-11	2.3 ± 0.1	16-21
Ethinyl oestradiol	3.0 ± 0.1	11-19	2.8 ± 0.3	15-25
Oestrone 3-sulphate	3.4 ± 0.1	0	3.1 ± 0.2	9-11
Diethylstilboestrol	3.1 ± 0.2	9-11	2.6 ± 0.3	21-26

* μ mole kynurenine/g wet tissue/hr (mean $\pm$ S.D.). The percentage inhibition is given as the range of results obtained from three to four separate experiments.

Lineweaver-Burk plots for TPO activity in the presence of 100 μ M progesterone or ethinyl oestradiol and various concentrations of haematin were consistent with the general form of competitive inhibition (Fig. 1).

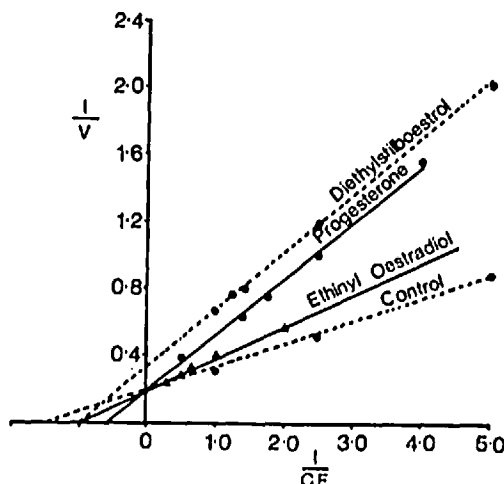


FIG. 1. Lineweaver-Burk plots for progesterone, ethinyl oestradiol and diethylstilboestrol. The steroid concentrations were 100 μ M. The ordinate is the reciprocal of the rate of TPO activity ($1/V$), and the abscissa is the reciprocal of the haem concentration ($1/CF$).

Ethinyl oestradiol inhibited TPO, but its 3-methyl ether derivative, mestranol, was without effect. Oelkers and Nolten⁶ reported that the inhibition of TPO by the unconjugated natural oestrogens oestradiol-17 β , oestrone and oestriol was greater than that obtained with the corresponding 3-sulphate esters, and this is confirmed by our results. It appears, then, that the nature of the substituent at position 3 on the steroid molecule is an important factor in determining the occurrence or not of enzyme inhibition. The 3-oxosteroids progesterone, deoxycorticosterone and testosterone propionate were TPO inhibitors, and it may be that enolation of the group at position 3 must take place to allow the steroid molecule to interact either with the haem cofactor directly, or to compete with the haem for the cofactor binding site on the apoprotein. The necessary enolation may be restricted by sulphate esterification, and prevented by the presence of an inert group such as the ether substituent of mestranol.

Diethylstilboestrol inhibited TPO, but in this instance the Lineweaver-Burk plot had the features of a non-competitive inhibition. A number of other non-steroidal phenolic compounds are also known to be non-competitive inhibitors of TPO.⁷

The levels of steroids that were found to inhibit TPO activity in this and other studies^{6,8} were well above the physiological range. However, such levels may be reached when large doses of steroids are used for *in vivo* studies of the effect of hormones upon the activity of this and other enzymes. Greengard *et al.*,³ in an attempt to define a hormonal basis for the elevated levels of liver TPO in pregnant rats, administered to non-pregnant animals a combination of 1 μ g oestrone and 4 mg progesterone, or three or 10 times these amounts of steroids daily for 13 days, and failed to obtain any significant increase in enzyme activity. Our results suggest that such large doses of progesterone may inhibit TPO by direct competition with the haem cofactor, and mask any stimulation due to the oestrogenic component. It is of interest, therefore, that we obtained significant increases in liver TPO when female rats were given 10 μ g oestradiol benzoate alone daily for 14 days,⁹ whereas a combination of 20 μ g oestradiol benzoate and 4 mg progesterone produced a reduction in enzyme activity.⁴

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Effect of cysteine on inducible synthesis of β -galactosidase in *Escherichia coli*

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EXOGENOUS cysteine inhibits the growth of *Escherichia coli* when the cells are cultured on mineral salts-glucose medium.¹⁻³ This inhibitory effect of cysteine is strongly antagonized by the simultaneous addition of leu-leu-threo-val* to the culture medium.³ It was suggested³ that cysteine inhibits the synthesis of these amino acids. This assumption is supported by the findings of other authors, according to which cysteine, *in vitro*, inhibits the activities of two enzymes (i.e. homoserine dehydrogenase⁴ and acetohydroxy acid synthetase⁵) in the biosynthetic pathways of these four amino acids.

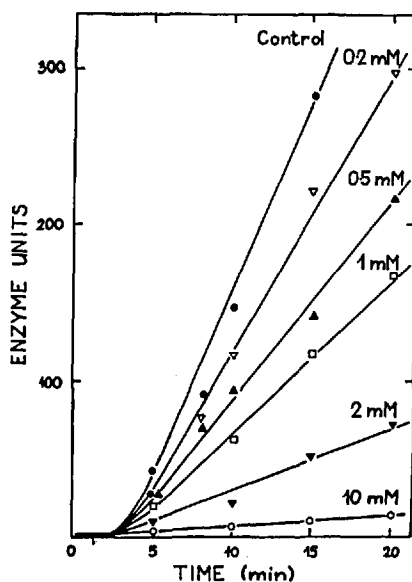


FIG. 1. Effect of cysteine on inducible synthesis of β -galactosidase in *E. coli* B.

Bacteria were grown aerobically on Roberts C mineral salts medium¹ containing 0.2% glycerol. Cells in mid-long phase were induced for β -galactosidase by addition of TMG (5×10^{-4} M). At intervals 1-ml samples were withdrawn and rapidly mixed with CM (final concentration 50 μ g/ml). The samples were then agitated for 30 min with one drop of toluene and 0.1 ml of sodium deoxycholate (final concentration 10 μ g/ml) at 37°. 1.5 ml of 0.1% ONPG in 0.066 M sodium phosphate buffer (pH 7.0) was added and incubation was continued until sufficient colour had developed. The reaction was stopped by the addition of Na_2CO_3 to a final concentration of 0.5 M.

Absorbances were read at 420 m μ wavelength against a reagent blank in "Spectromom 360" spectrophotometer. Where necessary, samples were clarified by centrifugation. A unit of activity was defined as being that amount of enzyme that hydrolyzes 1 μ mole of ONPG/hr at 37°. Enzyme content is expressed as unit per ml of culture. Cysteine was added simultaneously with the inducer at 0 min. Concentrations of the cysteine are indicated in the figure.

It was previously reported that the synthesis of RNA and protein (measured by [¹⁴C]-uracil and [¹⁴C]-proline incorporation, respectively^{2,6}) is inhibited immediately after treatment with cysteine, whereas that of the DNA proceeds for a further 20–30 min. Thus, with this type of experiments, it has not been possible to decide whether the stop of RNA or protein synthesis is due to the primary effect of cysteine. The hypothesis described above, assumes that the synthesis of protein is inhibited by cysteine, while the cessation of RNA synthesis may be the consequence of this effect.

This question was investigated by measuring the effect of cysteine on the inducible synthesis of β -galactosidase (Fig. 1 shows that it is inhibited by cysteine), since it is possible to separate the phase of enzyme induction (synthesis of mRNA) from the phase of enzyme production (translation of mRNA) by the dilution of inducer⁷ or by the removal of inducer⁸ after a pulsed induction period. We used the latter method. Thus, it was possible to measure the effect of cysteine on the process of transcription (RNA synthesis) and translation (protein synthesis) separately. The system described above was controlled by CM (50 μ g/ml; Fig. 2a), a compound which is known to inhibit directly only the protein synthesis. Figure 2b depicts the same type of experiments carried out with cysteine.

Two ml of concentrated culture was induced with TMG for 2 min at 30°, rapidly chilled by dilution with 50 ml ice-cold inducer-free medium and centrifuged at 2°. The pellet was washed once with 10 ml of iced inducer-free medium. The cells were then resuspended in 20 ml of prewarmed (30°) inducer-free glycerol-containing medium and incubated further at 30° with shaking. (The entire procedure

* Abbreviations used: leu, leucine; ileu, isoleucine; threo, threonine; val, valine; leu-ileu-threo-val, a mixture of leucine, isoleucine, threonine, valine; CM, chloramphenicol; TMG, thiomethylgalactoside; ONPG, *o*-nitrophenyl- β -D-galactopyranoside.

The Effect of Sex Hormones on the Activity of Tryptophan Oxygenase and other Corticosteroid-Inducible Enzymes in Rat Liver

By ISOBEL P. BRAIDMAN and D. P. ROSE. (*Alexander Simpson Laboratory for Metabolic Research, St Mary's Hospital Medical School, London W.2, U.K.*)

An increased capacity for the biosynthesis of nicotinic acid ribonucleotide has been observed during pregnancy (Brown, Thornton & Price, 1961) in women taking oestrogen-progestogen preparations for contraceptive purposes, and in normal human subjects receiving oestrogens alone (Rose, 1966). Women using oestrogen-progestogen types of oral contraceptive also exhibit a decrease in the concentration of plasma tyrosine (Rose & Cramp, 1970). These effects have been attributed to an increase in the activity of the corticosteroid-inducible enzymes tryptophan oxygenase (EC 1.13.1.12) and tyrosine aminotransferase (EC 2.6.1.5) with enhanced metabolism of the amino acids along degradative pathways (Rose & Braidman, 1970).

In an attempt to investigate this problem further, adult female rats were given 10 µg of oestradiol benzoate daily for 14 days by subcutaneous injection. Controls were injected with a corresponding volume of the corn-oil vehicle. Highly significant increases were observed in the activities of liver tryptophan oxygenase, tyrosine aminotransferase and alanine aminotransferase (EC 2.6.1.2), a third glucocorticoid-inducible enzyme.

The role of the adrenal gland in these changes was demonstrated by the administration of oestrogens to adrenalectomized female rats. The activities of tryptophan oxygenase and alanine aminotransferase in the adrenalectomized control animals were lower than in intact controls and tyrosine

aminotransferase activity was unchanged by adrenalectomy, as shown previously by Rosen, Harding, Milholland & Nichol (1963). After oestradiol benzoate administration, the adrenalectomized animals showed no increase in liver alanine aminotransferase and, although the activity of the other two enzymes was higher than in the controls, they were still significantly lower than in the livers of the intact female rats treated with oestrogen.

Control male rats had significantly lower tryptophan oxygenase activity than did females of comparable age and weight. Injections of 10 µg of oestradiol benzoate for 14 days produced increased activity of liver alanine aminotransferase, as described by Keller, Richardson & Yates (1969), and also increased tryptophan oxygenase activity.

The importance of sex hormones in maintaining basal tryptophan oxygenase activity was further demonstrated by the effect of ovariectomy, which significantly decreased tryptophan oxygenase activity in females. Daily subcutaneous injections of 100 µg of testosterone propionate administered for 14 days to intact female rats also resulted in decreased tryptophan oxygenase activity, not significantly different from that observed in intact male controls. Tyrosine aminotransferase activity remained unaltered.

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Effects of Sex Hormones on Three Glucocorticoid-Inducible Enzymes in Rat Liver

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ABSTRACT. The activities of three glucocorticoid-inducible enzymes, tryptophan oxygenase, tyrosine aminotransferase and alanine aminotransferase were significantly increased in the livers of female Sprague-Dawley rats by treatment with 10 µg estradiol benzoate daily for 14 days. Although estrogen-treated, adrenalectomised females did not show the same marked increases in tryptophan oxygenase and tyrosine aminotransferase, nevertheless the levels of these enzymes were higher than those of the adrenalectomised controls, whereas alanine aminotransferase was not affected by estrogens after adrenalectomy. It is suggested that the presence of adrenocorticosteroids is necessary for the full expression of the effect of estrogen upon tryptophan oxygenase, and tyrosine aminotransferase, and for the in-

duction of alanine aminotransferase. Tryptophan oxygenase was reduced by ovariectomy or testosterone propionate injection, as well as by adrenalectomy. Untreated, intact male rats had significantly lower tryptophan oxygenase levels than intact female controls, but higher activities of tyrosine aminotransferase. Estradiol benzoate-treated intact males showed proportionately similar elevations of tryptophan oxygenase to those obtained in correspondingly treated female rats. Orchidectomy did not affect tryptophan oxygenase, but the increase in enzyme activity in orchidectomised males treated with estradiol was significantly higher than the response in intact males, and similar to that observed in intact female animals. (*Endocrinology* 89: 1250, 1971)

TRYPTOPHAN oxygenase (EC 1.13.1.12) and tyrosine aminotransferase (EC 2.6.1.5) are glucocorticoid-inducible enzymes (2, 3) which catalyse the first, rate-limiting reactions of the major degradative metabolic pathways for L-tryptophan and L-tyrosine. Tryptophan oxygenase levels are elevated in the livers of pregnant rats (4, 5), and in pregnancy women show a marked alteration in the metabolism of the amino acid substrate (6). The administration of hydrocortisone to human subjects increases the urinary excretion of intermediate metabolites of the tryptophan-nicotinic acid pathway, and also reduces the level of plasma tyrosine; both effects have been attributed to enzyme induction (7, 8). The urinary excretions of kynurenine and other trypto-

phan metabolites are also elevated in estrogen-treated human subjects (9), and plasma tyrosine levels are reduced in women taking estrogen-progestogen preparations for contraceptive purposes (10). It has been suggested that these changes are due to an increase in the activities of tryptophan oxygenase and tyrosine aminotransferase, perhaps mediated by way of increased adrenocorticosteroid activity (9, 10). In contrast to the effects of estrogens, the administration of synthetic androgenic steroids diminishes the urinary excretion of tryptophan metabolites by normal male subjects (11).

In order to elucidate further the mechanisms by which sex hormones influence amino acid metabolism, a study has been made of the effects of estrogen or androgen administration upon the activities of tryptophan oxygenase, tyrosine aminotransferase, and a third glucocorticoid-inducible enzyme, alanine aminotransferase (EC 2.6.1.2), in the livers of male and female rats.

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A preliminary report of this study was made to a meeting of the Biochemical Society (1).

Materials and Methods

Male and female Sprague-Dawley rats weighing 170-250 g were kept under conditions of standardised temperature (21°C), a 12 hr light-dark cycle, and a freely available diet of Oxoid 41 pellets and water.

Endocrine surgery was performed under ether anaesthesia. Bilateral ovariectomies were carried out on weanling females which were then kept for 2-3 months before they were used for the experiment. During this time the completeness of the ovariectomies was assessed by the examination of vaginal smears. Male rats were orchidectomised when aged 60 days, and kept for 7-10 days before commencing the injection schedule. Bilateral adrenalectomised rats were kept for 4-5 days before commencing an injection schedule. They were given 1% saline to drink, and the complete removal of the adrenal glands was checked by post mortem examination.

Estradiol benzoate (Nutritional Biochemicals Corp.) and testosterone propionate (Sigma Chemical Co.) were dissolved in corn oil for the subcutaneous injections. These were given daily between 09.00 and 10.00 hr, and the rats were killed between 14.00 and 15.00 hr. Hormone-treated animals were always matched with a group of controls that were injected on the same days with a corresponding volume (0.1 ml) of corn oil. In this way misinterpretation of any effect due to the stress of injection, or to seasonal fluctuations in the enzyme activities was avoided.

Enzyme assays. Rats were killed by decapitation; the livers were quickly removed, chilled, and homogenised in a solution containing 0.02 M NaH_2PO_4 , 0.25 M sucrose, and 0.14 M KCl at pH 7.0 so that the final concentration of liver tissue was 10% (w/v). All enzyme preparations were handled at 0-4°C. Tryptophan oxygenase was assayed in the microsome-free supernatant. The reaction mixture consisted of 1.6 mM L-tryptophan, 0.8 mM ascorbic acid, 5 μM heme, 2 ml supernatant, and glass-distilled water to a final volume of 3 ml. Incubations were carried out in stoppered Erlenmeyer flasks, with air as the gas phase, in a shaking water bath at 37°C. Duplicate flasks were incubated for 60 and 90 min, and a zero time flask was included in each assay. The reaction was stopped by the addition of 10 ml 5% trichloroacetic acid,

the precipitate removed by centrifugation, and the kynurenine determined in the protein-free supernatant by the method of Brown and Price (12) modified for the use of an Autoanalyser technique. Preliminary experiments showed that the production of kynurenine proceeded linearly with time for at least 90 min. The results have been expressed as μmole kynurenine produced/g wet wt tissue/hr. The range of control values obtained for intact female rats injected with corn oil did not differ significantly from that of Greengard, Baker and Friedell (13), and the method used in the present study also gave similar levels of activity following maximal cortisol induction of tryptophan oxygenase to those reported by Csanyi and Greengard (14), using the method of Knox, Piras and Tokuyama (15).

Tyrosine aminotransferase was assayed in whole liver homogenate, in the presence of 0.95 mM pyridoxal phosphate, by the method of Rosen *et al.* (16) as modified by Greengard and Gordon (17) except that the incubations were carried out at 0, 5 and 15 min. It had been previously established that enzyme activity was linear throughout this period. The results are given as μmole p-hydroxyphenylpyruvic acid produced/g wet wt tissue/hr.

Alanine aminotransferase activity was determined in whole homogenate according to Roseh, Roberts and Nichol (18), except that as the apoenzyme in the homogenate has been shown to be fully saturated with cofactor (19) pyridoxal phosphate was not added to the incubation medium. Enzyme activity has been expressed as nmole pyruvate produced/g wet wt tissue/hr.

Levels of statistical significance were determined by Student's *t* test; differences with $p > 0.1$ were not regarded as being significant.

Results

The administration of 10 μg estradiol benzoate to female rats daily by subcutaneous injection for 14 days produced significant increases in the activities of tryptophan oxygenase, tyrosine aminotransferase and alanine aminotransferase (Table 1). The levels of tryptophan oxygenase and alanine aminotransferase were diminished in adrenalectomised female rats, but there was no change in tyrosine aminotransferase

TABLE 1. Effects of estrogen administration and adrenalectomy on the activities of female rat hepatic enzymes

Treatment	Tryptophan oxygenase	Tyrosine amino-transferase	Alanine amino-transferase
Female controls (12)	5.1 $\pm$ 0.7	107 $\pm$ 21	1.6 $\pm$ 0.4
Estradiol benzoate (12)	9.7 $\pm$ 1.6†	180 $\pm$ 31†	3.3 $\pm$ 0.7†
Adrenalectomy (12)	1.6 $\pm$ 0.5†	115 $\pm$ 16	1.3 $\pm$ 0.5†
Adrenalectomy + estradiol benzoate (11)	3.3 $\pm$ 1.1*	141 $\pm$ 31**	0.9 $\pm$ 0.5

The units of enzyme activity are as defined in the text. The results are given as the mean $\pm$ standard deviation, and the number of rats included in each group is indicated in parenthesis.

† $p < 0.001$ and ‡ $p = 0.05$ as compared to intact control groups.

* $p < 0.001$ and ** $p < 0.02$ as compared to adrenalectomy group.

activity. Treatment of adrenalectomised animals with the estrogen resulted in higher levels of tryptophan oxygenase and tyrosine aminotransferase than those of the adrenalectomised controls, but the enzyme activities remained below those of the intact estrogen-treated females.

Alanine aminotransferase was not affected by estrogen treatment following adrenalectomy (Table 1).

The tryptophan oxygenase levels in ovariectomised rats were significantly lower than those in intact, uninjected controls, and treatment of intact females with 100 μ g testosterone propionate daily for 14 days also produced significant reductions in tryptophan oxygenase activity (Table 2).

Intact male rats had lower tryptophan oxygenase levels than did intact female

controls, whereas tyrosine aminotransferase activity was higher in the male animals (Table 3). The administration of estradiol benzoate, 10 μ g daily for 14 days, increased tryptophan oxygenase activity in male rats (Table 3), and although the absolute level of enzyme activity was significantly less than that of similarly treated intact females ($p < 0.001$) the increases relative to the corresponding controls were of the same order of magnitude in both cases. Orchidectomy had no effect on tryptophan oxygenase, but treatment of orchidectomised males with estradiol benzoate produced an increase in

TABLE 3. Sex difference in tryptophan oxygenase and tyrosine aminotransferase activity, and the effect of estrogen administration upon tryptophan oxygenase activity in the liver of intact and orchidectomised male rats

Treatment	Tryptophan oxygenase	Tyrosine amino-transferase
Female controls (10)	3.1 $\pm$ 0.4	75 $\pm$ 16
Intact male controls (9)	2.0 $\pm$ 0.4†	91 $\pm$ 16**
Orchidectomised males (6)	2.0 $\pm$ 0.3	—
Estradiol benzoate-treated intact males (10)	4.9 $\pm$ 1.8*	—
Estradiol benzoate-treated orchidectomised males (8)	8.7 $\pm$ 2.1‡	—

† $p < 0.001$ as compared to female controls;

* $p < 0.001$ as compared to male controls; ** $p < 0.05$ as compared to female control; ‡ $p < 0.001$ as compared to both orchidectomised males and estradiol benzoate-treated intact males.

TABLE 2. Effect of ovariectomy or testosterone propionate administration on tryptophan oxygenase activity in female rat liver

Treatment	Tryptophan oxygenase
Uninjected intact female controls (17)	3.5 $\pm$ 0.8
Ovariectomised females (10)	1.8 $\pm$ 0.6†
Corn oil-injected intact female controls (11)	3.2 $\pm$ 0.4
Testosterone propionate injected females (12)	2.5 $\pm$ 0.6*

† $p < 0.001$ as compared to uninjected intact female control group.

* $p < 0.02$ as compared to corn oil-injected intact female controls.

this enzyme which was similar to that obtained in estrogen-treated intact females, and significantly higher than the levels produced in intact, estrogen-treated, males (Table 3).

Discussion

There have been previous reports of increased alanine aminotransferase activity in the livers of estrogen-treated rats (19, 20). Keller, Richardson and Yates (19) found that when the level of plasma corticosteroids is increased in the male rat by treatment with estrogens there is an elevation in the activity of hepatic, but not pancreatic alanine aminotransferase. They suggested that this is because transcortin-bound corticosterone can gain access to the enzyme-synthesising systems within the hepatic cells by pinocytosis, and dissociate there to yield the free steroid which induces increased alanine aminotransferase production. The thick basement membrane of the pancreatic cells prevents the passage of protein-bound steroids, and so enzyme induction does not take place within pancreatic tissue. Plasma corticosteroids are elevated in estrogen-treated women (21), and a similar mechanism to that conceived by Keller and coworkers has been proposed for tryptophan oxygenase induction in order to explain the abnormal tryptophan metabolism that occurs in estrogen-treated human subjects and women using oral contraceptives (22).

In this study, intact female rats treated with estradiol benzoate, in the same dosage and for the same duration as that used by Keller *et al.* (19), showed elevated levels of tryptophan oxygenase and tyrosine aminotransferase activity, as well as alanine aminotransferase. The observed low tryptophan oxygenase levels in adrenalectomised rats have been described by previous workers (15). Rosen *et al.* (16) have reported reduced activity of alanine aminotransferase in adrenalectomised rats, but although there was a slight reduction in the level of this enzyme in the present study it was of marginal statistical significance ($p = 0.05$). The

tyrosine aminotransferase activity was unaltered by adrenalectomy, as noted by others (16). Administration of estradiol benzoate to adrenalectomised female rats did not raise the level of tryptophan oxygenase above that of the intact female controls, nor did it increase the tyrosine aminotransferase levels to those of estrogen-treated intact females, but it did result in significantly higher activities of both enzymes than those seen in untreated adrenalectomised animals. It would appear from these observations that for the full expression of the effect of estradiol benzoate upon tryptophan oxygenase and tyrosine aminotransferase in female rat liver it is necessary that the adrenal glands be present, but that the estrogen itself also exerts an action upon the enzymes which is not mediated by way of the adrenocortical hormones.

Although Keller *et al.* (19) considered that the increases of hepatic alanine aminotransferase activity in estradiol benzoate-treated male rats were a consequence of the elevated plasma corticosterone levels, and this provides an acceptable explanation for the tryptophan oxygenase increases in males that were observed in the present study, the precise role of the adrenal glands in the estrogen-stimulated enzyme activities in female rat liver is uncertain because the plasma corticosterone is not raised by the administration of estradiol benzoate to female rats (23). One possible explanation is that the estrogen stimulates the enzyme activities, either directly or via a third hormone such as thyroid hormone, but that the presence of adrenocorticosteroids is required to play a permissive role. In such a case, it may be that the maintenance of a certain level, rather than an increased quantity of corticosterone is essential for the maximal stimulation of the enzymes. Support for this hypothesis would be obtained if it could be shown that tryptophan oxygenase activity in adrenalectomised rats treated with a replacement dose of corticosterone gives a similar response to estrogen administration to that seen in intact

animals. This is a subject for future investigation.

The question of whether or not the elevated plasma corticosteroid levels brought about by the administration of estrogens to male rats (23) and to both female and male human subjects (21, 24) has any role in the induction of hepatic tryptophan oxygenase and tyrosine aminotransferase, and the consequent modifications of amino acid metabolism, remains unanswered. However, in the rat one point against this is that the increases in tryptophan oxygenase after estradiol treatment of male and female animals were similar, relative to their corresponding controls, despite the absence of an effect upon the plasma corticosterone levels in the latter.

Ovariectomy has been shown to decrease rat plasma corticosterone (25) and, like adrenalectomy, it caused a reduction in tryptophan oxygenase activity. Testosterone administration also resulted in lowered enzyme levels in female rats, and although the mechanism for this effect of the androgen is uncertain, it is noteworthy that testosterone phenylacetate, when given in an approximately equivalent dose to that of testosterone propionate used in the present study, reduces the level of corticosterone in the plasma of female rats (26). These effects of ovariectomy and testosterone are of some clinical interest because a proportion of women who have been treated for carcinoma of the breast by bilateral oophorectomy excrete reduced quantities of tryptophan metabolites in urine (27), and so do breast cancer patients treated with synthetic androgen steroids (Rose, Randall, and Cramp; submitted for publication).

A sex difference in the activity of tryptophan oxygenase has been reported previously by Pinto and Rosenthal (28), but does not appear to have been remarked upon by other workers, although the results for the levels of this enzyme published by Greengard and coworkers (13) show a higher mean value for female rats than for males. These observations are of interest in relation

to studies of human tryptophan metabolism, because normal premenopausal women excrete higher levels of tryptophan metabolites in urine collected after an oral dose of the amino acid than men, and the output of metabolites also varies with the menstrual cycle (29). Lin, Rivlin and Knox (30) reported higher activity of tyrosine aminotransferase in male than in female rats, and they suggested that testosterone is in some way responsible for this sex difference as castrated males had reduced enzyme levels, but ovariectomy did not affect the activity in female animals.

Tryptophan oxygenase activity in estradiol-treated, orchidectomised male rats was significantly higher than in intact males treated with the estrogen. This observation indicates a role for endogenously produced androgens in suppressing the response of this enzyme to estrogens. The mechanism of this action of androgens is currently under investigation.

The present study of tryptophan oxygenase extended over a period of 14 months, and during this time a variation in the control values of the enzyme for intact female rats injected with corn oil became apparent. This seems to be related to the season, such that the enzyme levels were significantly higher in rats studied during the months of April and May than September to November (5.1 ± 0.7 ; 3.3 ± 0.4 respectively; $p < 0.001$). Although this observation requires confirmation, a similar variation of tyrosine aminotransferase, which may have had a seasonal basis, has been remarked upon by others (31).

Acknowledgments

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Excretion of tryptophan metabolites as affected by pregnancy, contraceptive steroids, and steroid hormones^{1, 2}

David P. Rose, M.D., Ph.D., and Isobel P. Braidman, B.Sc.

The metabolic pathway by which L-tryptophan is converted to nicotinic acid ribonucleotide is subject to the influence of a variety of hormones. In this paper, particular attention will be paid to the effects of adrenocorticosteroids, the hormonal environment of pregnancy, estrogens, and androgens upon tryptophan metabolism, as they are reflected by the urinary excretion of intermediates along the tryptophan-nicotinic acid ribonucleotide pathway. The possible mechanisms involved will be discussed in relation to more recent studies carried out in the authors' laboratory.

Adrenocorticosteroids

Tryptophan oxygenase is the first, and almost certainly the rate-limiting enzyme of the tryptophan-nicotinic acid ribonucleotide pathway. Its induction by glucocorticoids has been extensively investigated since it was first described by Knox (1). A direct correlation between tryptophan oxygenase activity in human liver and the level of kynurenine in urine collected after an oral dose of tryptophan has been described by Altman and Greengard (2), and is to be discussed by Dr. Greengard in a later paper.

A further study (3) showed that the excretions of kynurenine, 3-hydroxykynurenine, xanthurenic acid, and 3-hydroxyanthranilic acid are all elevated following treatment with hydrocortisone. Figure 1 summarizes the results obtained from these experiments. Treatment with pyridoxine caused a reduction in the amounts of all four metabolites excreted after hydrocortisone injection to levels that were as low as, or lower than, those excreted without hydrocortisone induction of tryptophan oxygenase. This seems to indicate that the liver contains a surplus of hydroxykynureninase apoenzyme that may be activated if pyridoxal 5-phosphate coenzyme is made

available to it by the administration of vitamin B₆. These results also suggest that kynurenine 3-hydroxylase activity is adequate for the hydroxylation of the increased quantity of kynurenine that arises from the cortisol-induced increase in tryptophan oxygenase, provided that sufficient pyridoxal phosphate is available to ensure that 3-hydroxykynurenine does not accumulate, but is metabolized to 3-hydroxyanthranilic acid.

An alternative route for the metabolism of the kynurenine produced by the enhanced tryptophan oxygenase activity is removal of the alanine side chain to yield anthranilic acid. The kynureninase that catalyzes this step also requires pyridoxal phosphate, and so vitamin B₆ administration would presumably allow an increased turnover along this route. Under normal circumstances, the enzyme has been shown not to be fully activated in rat liver (4).

Pregnancy

Elevated urinary excretions of xanthurenic acid in human pregnancy, with a return to normal levels following treatment with vitamin B₆, were described independently by Sprince et al. (5) and Vandelli (6). These changes were thought to be due to a deficiency of vitamin B₆ brought about by the fetal requirements for the vitamin; support for this view was obtained by Wachstein et al. (7, 8), who showed that the plasma and leukocyte levels of pyridoxal phosphate are diminished in maternal blood at term. More recently, Hamfelt and Hahn (9) have re-

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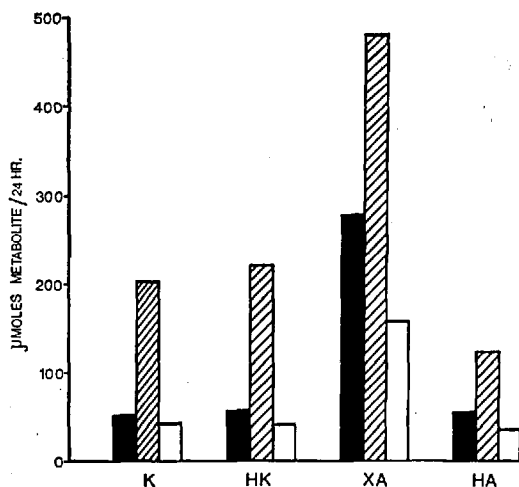


FIG. 1. Effect of hydrocortisone on tryptophan metabolism in a normal male subject. Excretions of kynurenine (K), 3-hydroxykynurenine (HK), xanthurenic acid (XA), and 3-hydroxyanthranilic acid (HA) following a 5-g L-tryptophan load are shown for: a control test (black columns); after 250 mg hydrocortisone by intramuscular injection given 5 hr before the tryptophan (cross-hatched columns); and after the hydrocortisone plus 50 mg pyridoxine on the evening before the test and again at the time of the steroid injection (open columns).

ported that early in pregnancy the excretion of xanthurenic acid is much higher for a given plasma pyridoxal phosphate level than it is for nonpregnant women with the same level of coenzyme. This indicates that the abnormal excretion of tryptophan metabolites in pregnancy is unlikely to be due solely to vitamin B₆ deficiency. In fact, previous workers had already suggested that tryptophan metabolism is modified by the altered hormonal environment of pregnancy (10).

Brown et al. (10) carried out a detailed study of tryptophan metabolism in the second or third trimesters of human pregnancy, and obtained data for the urinary excretion of both tryptophan metabolites and *N*¹-methyl-2-pyridone-5-carboxamide (2-pyridone), which is one of the two principal excretion products of nicotinic acid. Elevated levels of xanthurenic acid, 3-hydroxykynurenine, kynurenine, acetylkynurenine, and 2-pyridone were observed in urine collected after a 2-g oral dose of L-tryptophan. Treatment with pyridoxine produced a reduction in the excretion of most metabolites; although the

levels of 2-pyridone, kynurenine, 3-hydroxykynurenine, and xanthurenic acid remained above the normal range, the amount of vitamin given was less than that administered by Wachstein and Gudaitis, who obtained a complete reversion to within normal limits for xanthurenic acid excretion (11). Brown and his co-workers concluded that, in addition to vitamin B₆ deficiency, the hormonal changes of pregnancy alter tryptophan metabolism so that there is an enhanced capacity for the biosynthesis of nicotinic acid ribonucleotide from the amino acid. The report (12) that *N*¹-methylnicotinamide excretion is elevated in pregnancy is consistent with this view.

The published data would seem to indicate that at least two separate factors are responsible for the altered tryptophan metabolism of pregnancy: 1) towards term a true deficiency of vitamin B₆ develops that is due to the fetal demands for the vitamin with a transplacental loss, and 2) throughout pregnancy the endocrine changes are responsible for promoting the metabolism of tryptophan along the pathway leading to nicotinic acid ribonucleotide synthesis.

Estrogens and contraceptive steroids

The influence of female sex hormones upon tryptophan metabolism is not restricted to the gross changes of pregnancy, but is also apparent in the fluctuations that occur at different phases of the menstrual cycle. Elevated excretions of 3-hydroxykynurenine, xanthurenic acid, and 3-hydroxyanthranilic acid were found at the time of ovulation, when estrogenic activity reaches a peak, as compared with the levels immediately after menstruation (13). Further, a sex difference in the urinary excretion of metabolites following the administration of a tryptophan load has been described by several groups of workers. Michael et al. (14) reported that adult females excrete greater amounts of kynurenine, 3-hydroxykynurenine, xanthurenic acid, kynurenic acid, and 3-hydroxyanthranilic acid than adult males after a tryptophan load given in a dose of 100 mg/kg body wt, whereas Mainardi and Tenconi (15), and Rose (13) have also demonstrated a sex difference in tryptophan metabolism with

a standard 5-g dose of L-tryptophan. This sex difference was not evident when the loading dose was 2 g (16).

The role of estrogens in influencing the biosynthesis of nicotinic acid ribonucleotide was emphasized when Rose reported gross elevations of xanthurenic acid excretion by women taking estrogen-progestogen preparations for contraceptive purposes (17). These changes were observed only when urine was collected after a tryptophan load, and were reversed by the administration of large doses of pyridoxine. Further studies confirmed this observation, and showed that women using these contraceptive steroids excrete increased amounts of kynurenine, 3-hydroxykynurenine, 3-hydroxyanthranilic acid, kynurenic acid, and acetylkynurenine, as well as xanthurenic acid, in urine collected after a 5- or 2-g dose of L-tryptophan (18, 19). Treatment with pyridoxine reduced the urinary excretion of metabolites, including 3-hydroxyanthranilic acid, to normal limits. In addition to the changes in tryptophan metabolite levels women using Enovid-E (mestranol 0.1 mg, combined with norethynodrel 2.5 mg; Searle and Co.) were found to have elevated amounts of *N*¹-methylnicotinamide, but not 2-pyridone, both in urine collected without prior loading, and after a 2-g dose of tryptophan (20).

When they are given alone, estrogens produce a change in tryptophan metabolism identical to that which occurs in women taking the estrogen-progestogen type of oral contraceptives (Fig. 2), with the same response to large doses of pyridoxine (18, 21). When an adult male was given 0.1 mg ethinylestradiol daily, increased excretions of metabolites following a 5-g tryptophan load were obtained after treatment for 7 days. The excretion levels continued to rise throughout the 5 weeks of estrogen administration, and they rapidly returned to normal following cessation of treatment (18).

The progestogenic component of the estrogen-progestogen type of oral contraceptives does not appear to alter the urinary excretion of tryptophan metabolites. Figure 3 compares the excretions of 3-hydroxykynurenine, xanthurenic acid, and 3-hydroxyanthranilic acid by six young women who

were not taking an oral contraceptive with those of five women who were using the progestational oral contraceptive megestrol acetate. This steroid is used both in combination with an estrogen (Delpreglin, megestrol acetate, and mestranol, Nova Industri A/S), and

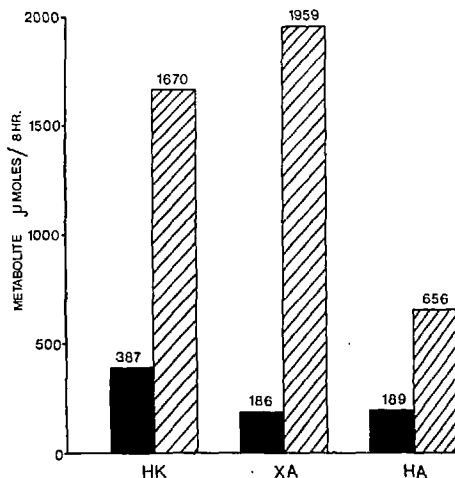


FIG. 2. Mean excretion of 3-hydroxykynurenine (HK), xanthurenic acid (XA) and 3-hydroxyanthranilic acid (HA) in 8 hr following a 5-g tryptophan load by 15 women taking an estrogen-progestogen preparation (cross-hatched columns), compared with 10 women of a similar age not taking a steroid preparation (black columns).

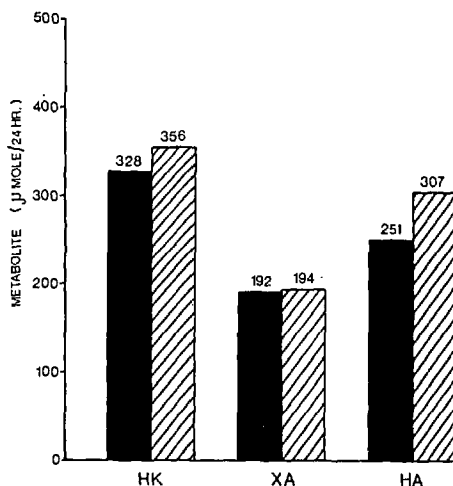


FIG. 3. Mean excretion of the same three metabolites shown in Fig. 2 by six women taking megestrol acetate (cross-hatched columns) and six control subjects (black columns). In this study urine was collected for 24 hr after the tryptophan load.

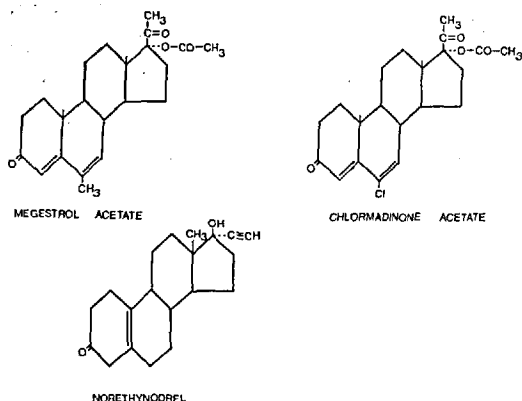


FIG. 4. Chemical structures of three progestogens.

also alone, as a purely progestogenic form of contraceptive. It is structurally similar to progesterone in being a Δ^4 -3-oxosteroid, and differs from chlormadinone acetate, the other progestational oral contraceptive, only in the substituent at position 6 of the steroid molecule (Fig. 4).

Norethynodrel, the progestogenic component of Enovid-E, has not been examined for its effect on tryptophan metabolism in man. However, this steroid may well alter the excretion of metabolites, for it is partly metabolized to estrogenic compounds in the body, and in fact, is chemically an estrane derivative: 17 α -ethinyl-17 β -hydroxy-estr-5(10)-en-3-one (Fig. 4).

Androgens

There is little published information on the effect of androgens upon tryptophan metabolism, although this is of importance in view of the changes seen in breast cancer. McGinty and Rose (22) have found that the administration of Mesterolone (1 α -methyl-5 α -androst-17 β -ol-3-one, Schering Chemicals Ltd.) reduces the excretion of tryptophan metabolites after an oral dose of the amino acid. Prior treatment with this androgen also impaired the elevation of metabolite excretions that normally occurred after the induction of tryptophan oxygenase by hydrocortisone (Fig. 5).

Discussion

The precise site(s) of action of estrogens upon the tryptophan-nicotinic acid ribo-

nucleotide pathway is not known with certainty. Rose (18) suggested that these hormones cause an increase in the activity of one or more of the enzymes concerned in this metabolic sequence, but that when the intake of the amino acid is considerably in excess of the normal dietary sources, as occurs in tryptophan loading studies, there is insufficient pyridoxal phosphate available to allow for the further metabolism of all of the tryptophan entering the pathway. This would explain why the N^1 -methylnicotinamide excretion is elevated in urine collected without prior tryptophan administration, whereas the abnormal excretion of xanthurenic acid, 3-hydroxykynurenine, and kynurenine is only

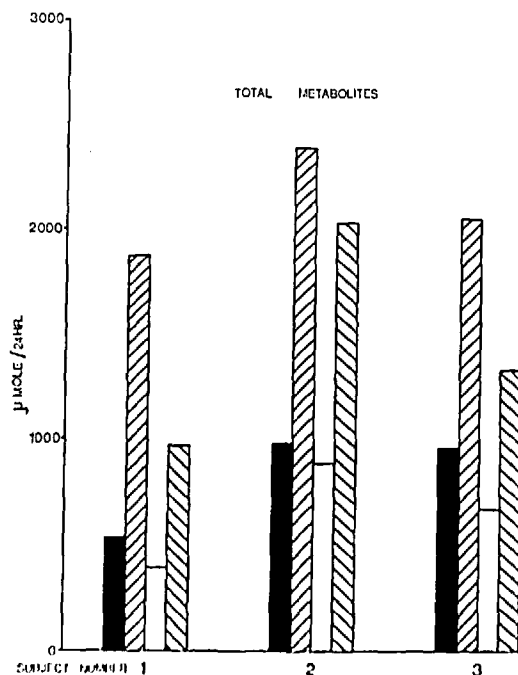


FIG. 5. Effect of an androgen of tryptophan metabolism in three normal adult male subjects. A control test was performed for which 5 g L-tryptophan was given at 2:00 PM (first column). Four days later 250 mg hydrocortisone succinate was given by intramuscular injection at 9:00 AM. An oral dose of tryptophan was given 5 hr later and urine collected for 24 hr (second column). Treatment with mesterolone, 20 mg orally three times daily, was given for 14 days. A third tryptophan test was then carried out (third column). Finally, after 3 more days of androgen treatment, another tryptophan test was carried out 5 hr after the injection of 250 mg hydrocortisone (fourth column). The values are the sums of K, HK, XA, and HA excretions.

demonstrable following a tryptophan load, and is corrected by treatment with large doses of pyridoxine.

If estrogens influence the biosynthesis of nicotinic acid ribonucleotide from L-tryptophan by producing an increase in the activity of a single enzyme, on theoretical grounds tryptophan oxygenase would seem the most likely to be concerned, as it is known to be hormone-inducible and it catalyzes the first, irreversible step on the metabolic pathway. This enzyme is increased in the liver of pregnant rats (23, 24), and also in female rats treated with a combination of mestranol, the 3-methyl ether of ethinylestradiol, and norethynodrel (D. P. Rose and R. R. Brown, unpublished observations). Recent work by the authors has substantiated the hypothesis proposed by one of us (25) that the effect of estrogens on tryptophan metabolism involves the induction of tryptophan oxygenase, and that this is mediated by way of the hypothalamo-pituitary-adrenal axis.

Pregnancy, in both rat (26) and man (27), results in a rise in the level of plasma 17-hydroxycorticosteroids. A similar increase occurs with estrogen treatment (28). Most of this increased steroid is protein-bound, and generally has been considered to be physiologically inert (29). However, Keller et al. (30) have shown that the rise in plasma corticosteroids brought about by estradiol benzoate administration is accompanied by an elevation in the activity of alanine aminotransferase, a cortisol-inducible enzyme, in rat liver. The enzyme was unaffected when the estrogen was given to adrenalectomized rats. The authors suggested that the protein-bound glucocorticoid is able to enter the hepatic cell by pinocytosis, and there dissociates from the binding globulin to yield the active free steroid, which is then responsible for induction of the enzyme.

Significant increases in alanine aminotransferase were obtained by Keller et al. (30) when estradiol benzoate was given by subcutaneous injection to adult male rats in a daily dose of 10 μ g for 14 days. Figure 6 shows that an identical regime of estradiol benzoate administration produces a highly significant increase ($P < 0.001$) in the activity of tryptophan oxygenase in the livers of intact female rats, as compared with a

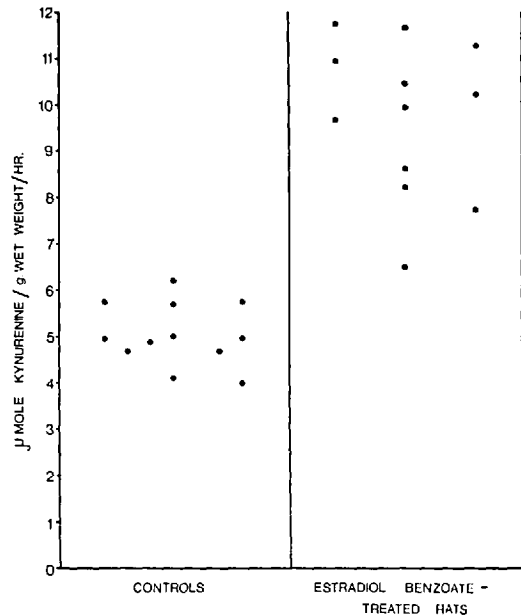


FIG. 6. Effect of estradiol benzoate on rat liver tryptophan oxygenase activity. These assays were performed in the presence of optimal amounts of heme. Control rats were injected with a volume of corn oil equal to that used as a vehicle for the estrogen.

control group injected with corn oil alone. Preliminary results indicate that the increase in tryptophan oxygenase activity does not occur in adrenalectomized animals.

The effect of estradiol on the adrenal secretion and plasma level of glucocorticoids is dose-dependent; large doses either fail to stimulate steroidogenesis (31) or actually cause inhibition (32). Consequently, the ability to demonstrate elevated tryptophan oxygenase activity in the liver of estrogen-treated rats depends upon the dose regimen employed. When intact female rats were treated with a massive 250- μ g dose of estradiol benzoate daily for 14 days there was no increase in the enzyme level (Rose, D. P., and R. R. Brown, unpublished observations).

An alternate explanation for the abnormal tryptophan metabolism produced in the human by estrogen administration or pregnancy, but one which fails to explain the elevated N^1 -methylnicotinamide excretion, is that estrogen sulfate esters, formed in the liver, inhibit kynureninase and kynurenine aminotransferase by competing with their

pyridoxal phosphate coenzyme for binding sites on the apoprotein. The effect of these steroid conjugates on the availability of pyridoxal phosphate for rat kynurenine aminotransferase is to be discussed by Dr. M. Mason in a later paper.

Estradiol disulfate has been shown recently to inhibit "hydroxykynurenine aminotransferase," the enzyme responsible for the production of xanthurenic acid from 3-hydroxykynurenine, *in vitro*, both in rat kidney and liver preparations (unpublished observations). If this inhibition takes place *in vivo*, and the very low effective concentrations suggest that it may, estrogen administration would not be expected to produce an elevated urinary excretion of xanthurenic acid. Although a high xanthurenic acid excretion is characteristic of the altered tryptophan metabolism of human pregnancy, the output of this metabolite is normal in the pregnant rat (33). There is, however, a raised kynurenine excretion, which is consistent with the known high tryptophan oxygenase activity. These findings may indicate a species difference in the behavior of kynurenine and hydroxykynurenine aminotransferase with respect to estrogen conjugates.

The clinical significance of the altered tryptophan metabolism by women using the combined estrogen-progestogen type of oral contraceptive is uncertain at the present time. However, one problem for which it may have etiological importance is depression, which is a well-recognized complication of this form of contraception.

There is considerable evidence that the level of brain 5-hydroxytryptamine is abnormally low in depressive illness (34), and three possible mechanisms exist for impaired synthesis of this neurohormone by women taking estrogen-containing oral contraceptives: 1) The amount of tryptophan available for 5-hydroxylation may be reduced, because it is being diverted into the tryptophan-nicotinic acid ribonucleotide pathway as a consequence of elevated tryptophan oxygenase activity. 2) The demand for pyridoxal phosphate coenzyme involved in the turnover of the tryptophan-nicotinic acid ribonucleotide pathway may be such that inadequate amounts are available for the decarboxylation of 5-hydroxytryptophan to yield 5-hydroxy-

tryptamine. 3) Estrogen conjugates may inhibit the decarboxylase by competing with pyridoxal phosphate for sites on the apoenzyme in the manner known to occur with a number of other enzymes (35).

These theoretical possibilities suggest that treatment with tryptophan or pyridoxine, or a combination of these two, may relieve depression due to oral contraceptives, although it must be pointed out that this occurs most frequently in those women using preparations with a high progestogen content (36), and the progestogens do not appear to modify vitamin B₆ function.

Our current concept of the changes in tryptophan metabolism that are brought about by pregnancy and estrogens, and perhaps by the abnormal hormonal environment of breast cancer, is summarized in Fig. 7, together with the possible ways in which these changes may produce diminished brain 5-hydroxytryptamine levels.

Summary

The effects of glucocorticoids, pregnancy, estrogens, contraceptive steroids, and androgens upon tryptophan metabolism in man are discussed.

Treatment with hydrocortisone produces an increase in the urinary excretion of kynurenine, 3-hydroxykynurenine, xanthurenic acid, and 3-hydroxyanthranilic acid, which is prevented by the simultaneous administration of large doses of vitamin B₆.

Pregnant women, those using estrogen-progestogen preparations for contraceptive purposes, and subjects receiving estrogens alone, all excrete elevated levels of these metabolites, although much larger quantities of xanthurenic acid are excreted than occur following single injections of hydrocortisone. The increased urinary output of *N*¹-methylnicotinamide in these situations is a reflection of the enhanced capacity for the biosynthesis of nicotinic acid ribonucleotide from L-tryptophan.

The progestogen megestrol acetate does not alter tryptophan metabolism when used alone for ovulation control.

Mesterolone, an androgenic steroid, reduces the urinary excretion of tryptophan metabolites, and impairs the response to hydrocortisone administration.

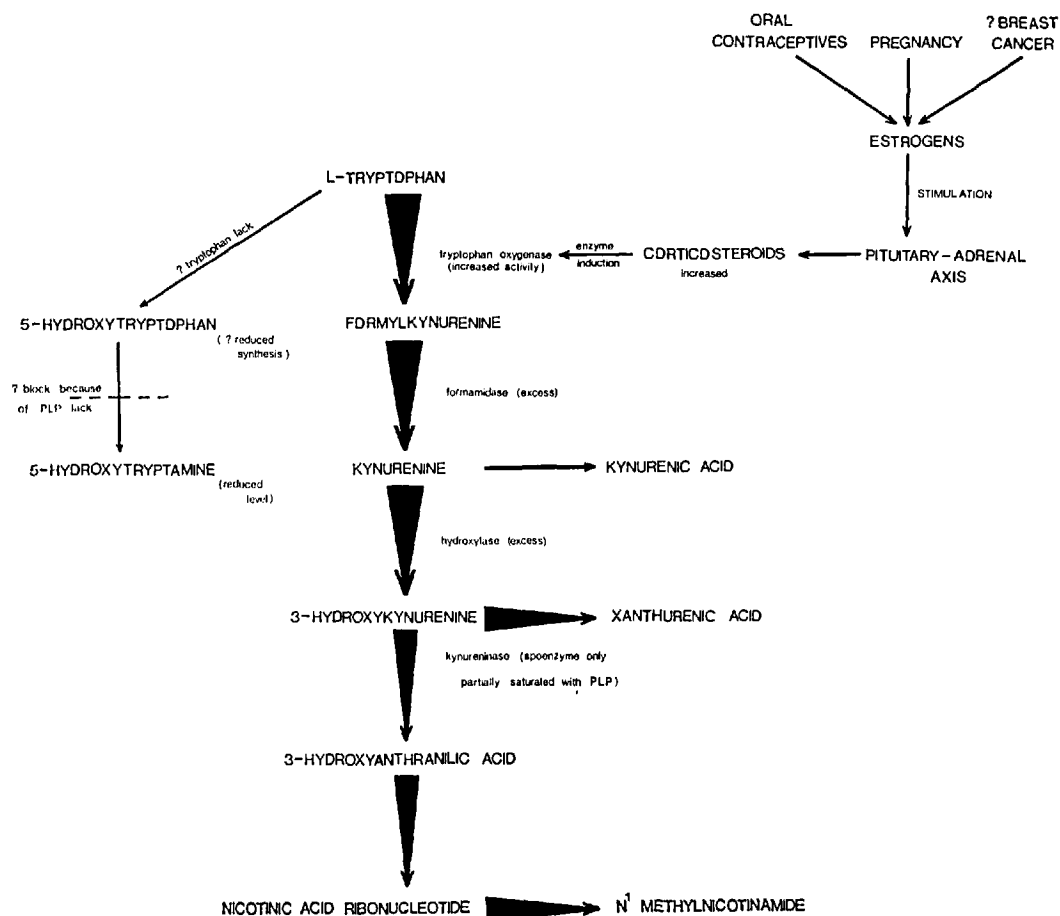


FIG. 7. Proposed scheme of the influence of estrogens and pregnancy on tryptophan metabolism.

The possible mechanisms by which pregnancy and estrogens modify tryptophan metabolism are discussed and, in the light of evidence obtained from animal experiments, it is concluded that estrogens cause an induction of tryptophan oxygenase that is mediated via the hypothalamo-pituitary-adrenal axis. Late in pregnancy, a true vitamin B₆ deficiency is superimposed upon the hormonal effects. 🌱

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Discussion

I. P. Braidman: As an extension to the study of tryptophan oxygenase, described by Dr. Rose, we have now obtained some preliminary results

for the activity of this enzyme and of alanine aminotransferase in adrenalectomized rats.

Our results confirm the observations by

Keller and co-workers (1) that the level of alanine aminotransferase as well as tryptophan oxygenase is elevated in the liver of intact female rats treated with 10 μ g estradiol benzoate daily for 14 days. Results are expressed in millimoles of pyruvate produced per gram wet liver weight per hour, and the difference from the control is highly significant ($P < 0.001$).

We have summarized the data from experiments with control and estrogen-treated adrenalectomized rats and compared these with the situation in intact controls. There was no difference in the alanine aminotransferase activity among the three groups. However, the level in tryptophan oxygenase in the control adrenalectomized rats was significantly lower than in the intact controls ($P < 0.001$).

Treatment with estradiol benzoate produced an apparent increase in tryptophan oxygenase activity as compared with controls after adrenalectomy ($0.5 < P < 0.1$), although the levels were still lower than those of the intact controls. We conclude from these results that the very high activities seen in estrogen-treated rats were a consequence of glucocorticoid induction.

An explanation for the higher level of tryptophan oxygenase activity in the estrogen-treated rats after adrenalectomy as compared with adrenalectomized controls may lie with the capacity of conjugated estrogens to stabilize cortisol-inducible enzymes. This has been reported for tyrosine aminotransferase (2) and it may be that the substantial decrease in tryptophan oxygenase activity observed in the control adrenalectomized rats is offset to a certain extent through a stabilization of the enzyme by estradiol benzoate.

R. A. Wapnir: Is there information on how much urinary tryptophan is excreted after load tests in estrogen-treated or untreated women?

D. P. Rose: In the normal subject Michael and his co-workers found, using a 100-mg/kg body wt dose of L-tryptophan, that there were just traces of the amino acid in the urine after the load. The amino acid has been fully metabolized somewhere, although clearly not in the metabolites that we determine under such conditions. I don't know of any data on the output of tryptophan in estrogen-treated women.

J. E. Leklem: In that respect, Dr. Rose, I would like to ask you why you chose a 2:00 PM time for administering your load of tryptophan?

D. P. Rose: Well, normally I do not. My regular time is 9:00 AM but in these hydrocortisone studies I wanted to allow for a period of induction after giving the hydrocortisone. So to

avoid coming in to the laboratory in the middle of the night to give the hydrocortisone, we set our study up so that we gave the hydrocortisone at 9:00 AM and then 5 hr later, at 2:00 PM, we gave the tryptophan load. This schedule was used only in this study.

F. Rosen: Is there any evidence that estradiol can induce an increase in the activity of either the kynurenine transaminase or kynureninase enzymes?

D. P. Rose: Yes, we found some data that surprise us a bit on the effect of a combination of mestranol and norethynodrel on kynureninase in rat liver and we did find there that this combination of steroids elevated the activity of kynureninase. This was distinct from what we found with the natural steroid, estradiol. Estradiol inhibited kynureninase activity in the male rat but not in the female rat. We observed a sex difference, and this has been reported previously by Dr. Mason; the male rat has a higher activity of kynureninase in the liver than the female rat. When we gave 20 μ g estradiol daily for 28 days to the male rat, the activity of the enzyme came down to the female level. When we gave the hormone to female rats, either in that or in much larger doses, there was no further reduction but we did find this difference between the natural steroid and the synthetic steroid combination.

P. Swan: Does estrogen administration change the metabolism of sulfur amino acids? This would provide evidence as to whether or not estrogen interferes generally with B₆-requiring enzymes by interfering with pyridoxine metabolism.

D. P. Rose: I know of no in vivo studies of this type. Dr. Mason, I believe, included sulfur-metabolizing enzymes in the spectrum of enzymes that he studied, and did find that conjugates inhibit these enzymes in vitro. I am speaking off the cuff, but I think I am remembering it right.

B. Clark: Do cortisol conjugates also stabilize tryptophan pyrrolase?

D. P. Rose: We haven't done any studies on this.

H. Spiera: Do you have any information as to the minimal amount of cortisone necessary to increase excretion of kynurenine?

D. P. Rose: McGinty, who is working in Sheffield, did some studies on this that haven't been published. He got down to 50 mg hydrocortisone succinate, starting at 250 mg and then going down by small increments, doing the

studies at weekly intervals and always using a 5-g load of L-tryptophan. With 50 mg hydrocortisone succinate he thought that he had just about reached the minimum quantity that would produce a response, but this work is quite preliminary.

R. R. P. Warner: Is there a significant change in urinary metabolites excreted after oral load of L-tryptophan in normal human females during different times in the menstrual cycle?

D. P. Rose: Yes, there is. Two studies, one from Dr. Brown using a 2-g load, and one from our laboratory using a 5-g load. In both studies there was a difference, with higher excretions of metabolites at the time of ovulation (about the 13th day of the cycle) than when the loading test was done immediately after the menstrual cycle. Again, I think our data were more clear-cut than Dr. Brown's because we were giving a 5-g load and this load was better when endogenous variations of hormones are studied.

G. A. Abdel-Tawab: If the concentration of ethinylestradiol is decreased, say, to 0.05 mg/pill, do you think the tryptophan metabolism will be normal under such conditions?

D. P. Rose: With 0.05 mg of ethinylestradiol, the excretions are still grossly abnormal (*Clin. Sci.* 31: 265, 1966).

N. Narasimhachari: Have serotonin or 5-hydroxyindoleacetic acid levels been done on estradiol-treated subjects?

D. P. Rose: I do not think so. I have no data and have not seen any published data either.

R. A. Toseland: We have demonstrated how one can detect spontaneous, i.e., nonloaded, levels of 3-hydroxyanthranilic acid in a female patient before taking the oral contraceptive. The significance of this is that if the patient were taking salicylates they would appear well separated from 3-hydroxyanthranilic acid. This technique of thin-layer electrophoresis on cellulose avoids the problems of paper chromatography when the metabolites clash or migrate to the same place. The determination is much faster and the extraction of the metabolites from powdered cellulose is also much faster than from paper.

G. A. Abdel-Tawab: We investigated the interrelation between female sex hormones and tryptophan metabolism along the kynurenine pathway in different age groups of women after loading with 2 g tryptophan, with and without 120-mg vitamin B₆ supplementation. The following groups were studied:

Group I. Young nonpregnant women, ages

23 to 40 years, included the following subgroups: **Subgroup A:** Fourteen cases were studied in the preovulatory phase when the prevailing female sex hormones are natural estrogens. Urine collection was started on the 9th day of the menstrual cycle. **Subgroup B:** Eight cases were studied in the postovulatory phase, starting on the 23rd day of the menstrual cycle when both natural estrogens and progestogens are produced.

Group II. Young, nonpregnant women under the effect of synthetic hormones, started early in the menstrual cycle, included three subgroups: **Subgroup A:** Eighteen cases given ethinylestradiol (0.05 mg tablets) one tablet daily from the 5th day of the menstrual cycle for 2 weeks. **Subgroup B:** Ten cases were given norethisterone (5 mg tablets) one tablet daily from the 5th day of the menstrual cycle for 2 weeks. **Subgroup C:** Twenty-two cases were studied during oral contraception with 3 mg norethisterone acetate plus 0.05 mg ethinylestradiol (the oral contraceptive commonly used in the United Arab Republic under the trade name Gyn-Anovlar), one pill daily from the 5th day of the cycle for 21 days.

Group III. Nine cases were women in the postmenopausal age (ages between 47 and 60 years).

Group IV: Fourteen nonsmoker males aged 20 to 40 years, were chosen as controls. The controls, as well as the different groups of women, were compared with respect to their ability to metabolize the tryptophan load, with and without B₆ supplementation. Subtraction of the basal level of the urinary metabolites from the levels in either the posttryptophan or the posttryptophan-pyridoxine urines gives the response of each individual to the loading dose of L-tryptophan; that is, *yield I* and *yield II*, respectively, expressed as the quantity, in milligrams/24-hr urine, excreted in excess of the basal level.

The tryptophan loading schedule, as mentioned above, was started at the anticipated time of ovulation or just prior to the time of menstruation in subgroups A, B, and C of Group II.

1) The results showed that in young, nonpregnant women in the preovulatory phase under the effect of natural estrogens there was accumulation of some bladder carcinogens, mainly 3-hydroxykynurenine and 3-hydroxyanthranilic acid, as well as some other tryptophan metabolites that included o-aminohippuric acid, acetylkynurenine, and kynurenine. This suggests that the degradation of 3-hydroxyanthranilic acid to niacin was impaired. This inhibition was corrected by vitamin B₆ supple-

mentation, indicating that the B_6 -dependent quinolinic acid decarboxylase may be the enzyme that suffers inhibition in this group.

2) In young, nonpregnant women in the postovulatory phase under the effect of both naturally occurring estrogens and progestogens, the excretion level of different metabolites was about the same as or less than that of controls. This means that the excretion pattern was corrected by progesterone, which antagonized the effect of estrogens. After a loading dose of vitamin B_6 , these women excreted more 4-pyridoxic acid than controls. This means that there was a decreased requirement for vitamin B_6 in the postovulatory phase.

3) In the postmenopausal women there was an accumulation to kynurenine, acetylkynurenine, and 3-hydroxykynurenine, and decreased production of anthranilic acid glucuronide and 3-hydroxyanthranilic acid, as well as decreased production of kynurenic acid and xanthurenic acid. This means that there was inhibition of the B_6 -dependent kynureninase and transaminases. Pyridoxine supplementation partially corrected the inhibition, especially that of 3-hydroxykynurenine transaminase.

Thus, there was functional pyridoxine deficiency in young nonpregnant women in the preovulatory phase, as well as in the women in the postmenopausal age. This may be due to the hormonal pattern prevailing. If this is the case, then one would expect that suppression of the naturally occurring ovarian estrogens will alter the excretion pattern of young, nonpregnant women in the preovulatory phase to that of the controls with normal enzymic reactions along the kynurenine pathway. Indeed, this was the case. Thus, under the effect of ethinylestradiol alone, we got a normal pattern; kynurenic acid,

acetylkynurenine, kynurenine, xanthurenic acid, and 3-hydroxyanthranilic acid were less than the corresponding values of young, nonpregnant women in the preovulatory phase. Furthermore, they excreted more 4-pyridoxic acid after vitamin B_6 supplementation than when these women were under the effect of natural ovarian estrogens, indicating that there was a decreased requirement for vitamin B_6 in the presence of the synthetic ethinylestradiol. Under the effect of norethisterone the transaminase enzymes were inhibited, as evidenced by the decreased levels of kynurenic acid and xanthurenic acid. Under the joint effect of ethinylestradiol and norethisterone, the pattern in the preovulatory phase was also corrected. This indicates that the inhibition to the transaminase induced by norethisterone was antagonized and corrected by the synthetic estrogen.

It could be concluded, therefore, that the metabolism of tryptophan is influenced by the physiological variations correlated with the menstrual cycle. Also, the use of this particular oral contraceptive did not lead to accumulation of the carcinogenic tryptophan metabolites.

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