

STUDIES ON 3-HYDROXYANTHRANILATE
OXYGENASE IN RAT LIVER AND
KIDNEY

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Dissertation submitted for the degree of Master of Science
in the University of the Witwatersrand, Johannesburg,
South Africa


1974

To the late Professor Paul Levy - my teacher and dear friend.


DECLARATION

This is to certify that the work presented in this dissertation is my own and has not been previously presented for examination.

Signed



Date



ACKNOWLEDGEMENTS

My heartfelt thanks to Professor P R Levy without whose support this thesis would not have been possible.

My sincere thanks are due to the following:

Professor Nerida Savage for her assistance with experimental techniques and her criticism of the first draft of this thesis;

Dr J J A Heffernan for his guidance in the use of statistics;

Mr A H Neely of Upjohn, who generously provided the cortisol used in these experiments;

Professor Sonia E Walker for her advice on animal feeding;
Mr W V Mc Bride for his assistance with animal housing
and the Animal Unit for their prompt supply of animals.

Mr F P Ross, Mrs G D'Or and Miss W Oliver for their technical aid.

Special thanks to Miss F Wadvalla - the illustrator, the Photo Unit, and the typist, Mrs E Towsey.

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ABBREVIATIONS

ENZYME:

3-hydroxyanthranilic acid oxygenase 3-HAAO

SUBSTRATE:

3-hydroxyanthranilic acid 3-HAA

REAGENTS

Dithiothreitol DTT

DIETARY ADDITIVES:

Dimethylaninoazobenzene DAB

RAT STRAINS:

Gilbert-Gillman GG

Long-Evans LE

Sprague-Dawley SD

NOMENCLATURE:

Enzyme Activity EA

IN ALL TABLES MEAN ENZYME ACTIVITY (MEA) IMPLIES THE

AVERAGE ACTIVITY PER GRAM WEIGHT OF WET ORGAN

Specific activity SA

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SUMMARY

1. The development of 3-Hydroxyanthranilate oxygenase in the liver and kidney of male and female rats has been studied. Cortisol does not appear to influence the post-natal development of this enzyme.
2. 3-Hydroxyanthranilate oxygenase is unstable both during purification and in storage. In vivo cortisol therapy markedly stabilizes renal 3-HAAO when stored at the homogenate stage of purification.
3. Liver and kidney 3-HAAO are heteroenzymes. They have similar catalytic and antigen sites. In neither was a 'N' terminal detected using a single technique. The molecular weight of the order of 32 000 and both enzymes show microheterogeneity.
4. Both liver and kidney enzymes are adaptable, but hepatic 3-HAAO is more readily and rapidly responsive to in vivo stimulation.

I N T R O D U C T I O N

The development of diarrhoea, dermatitis and the other manifestations of nicotinic acid deficiency in rats deprived of nicotinic acid, depends on the protein quality of their diet. Krehl et al. (1945) demonstrated that tryptophan is the amino acid which replaces this requirement for dietary nicotinic acid. In fact, high dietary tryptophan levels seem to exert more influence on pyridine nucleotide synthesis than does nicotinic acid (Feigelson et al. 1951). Such is the importance and adaptability of the tryptophan - nicotinic acid pathway in the rat.

Numerous enzymes participate in the conversion of tryptophan to nicotinic acid. The enzyme chosen for this study is 3-hydroxyanthranilic acid oxygenase (3-hydroxyanthranilate: oxygen oxidoreductase E C 1.13.1.6.). Figure 1 shows the position of 3-hydroxyanthranilic acid oxygenase (3-HAAO) in the tryptophan-nicotinic acid pathway. That 3-HAAO is indeed an enzyme in this pathway has been shown via the administration of dietary and intraperitoneal 3-hydroxyanthranilic acid resulting in increased urinary excretion of N-methylnicotinamide (Albert et al. 1948; Hanks and Urivetsky 1954 and Hanks and Henderson 1957). This enzyme is located not only in the cytoplasm of rat liver, but also in rat kidney (Cronaall 1965). A comparison of the development of certain physicochemical properties and of the adaptability of 3-HAAO within these two organs is the basis of this dissertation.

For this study purified 3-HAAO was required. Many workers have partially purified the enzyme and some of the difficulties encountered in the purification may have their explanation in the postulated role of oxygen and iron in the action of the enzyme on its substrate. Oxygen cleaves the benzene ring of 3-hydroxyanthranilic acid (Block 1960). 3-Hydroxyanthranilic

THE TRYPTOPHAN-NICOTINIC ACID PATHWAY

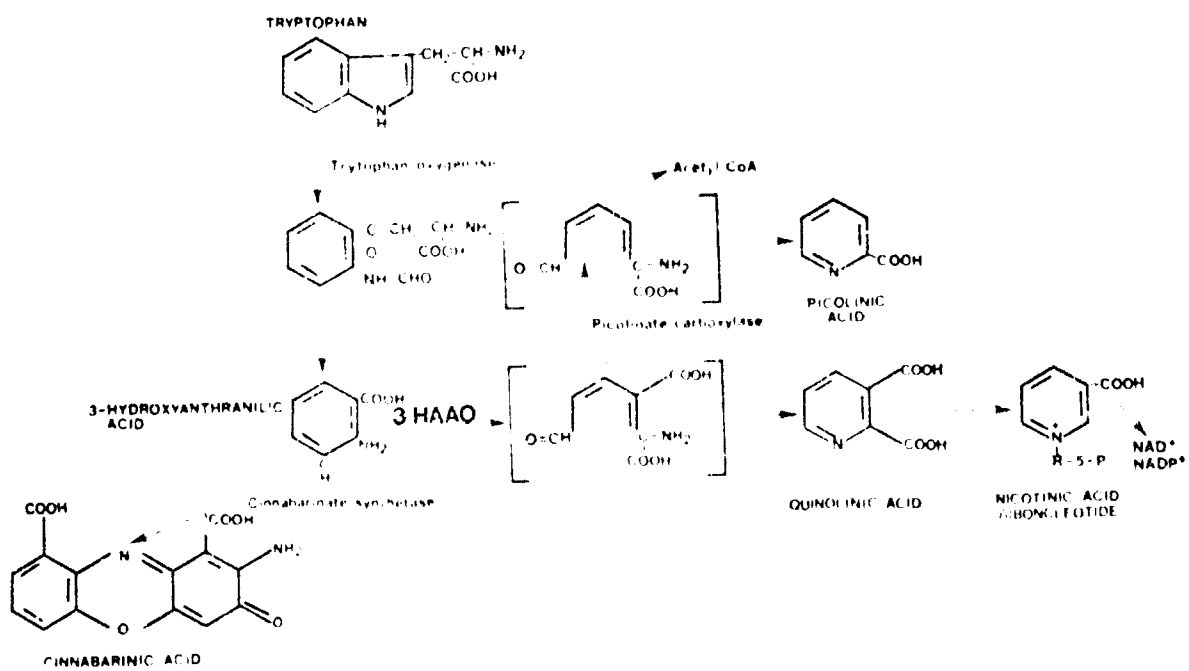


Figure 1

acid oxygenase, like tryptophan pyrrolase, falls into the group of dioxxygenases (Mason 1957) - both atoms of O_2 being incorporated into the product (Hayashi et al. 1956). Evidence points to iron being involved in the catalytic site of the dioxxygenases (Mohler and Cordes 1967). The ferrous ion, and often a second reducing agent, are employed to activate and stabilize 3-HAAO during its purification (Cox 1967; Vescia 1962; Savage et al. 1973; Stevens 1959 and Ogaswara 1966). Decker et al. (1961) consider the ferrous ion may achieve a conformational change in the enzyme. Like other phenolytic oxygenases, 3-HAAO rapidly loses its activity in the presence of oxygen. Oxidation and/or loss of bound ferrous ion account for some of the idiosyncracies encountered in the purification of this enzyme (Mitchel et al. 1963, Vescia and di Prisco 1962). The ferric ion is known to act as a non-competitive inhibitor of this system (Nishizuku et al. 1970). The instability of 3-HAAO complicates its purification and storage. Nishizuka et al. (1970) found the most stable storage of beef liver enzyme at pH 6.5. Savage et al. (1973) found that once freeze dried, monkey liver 3-HAAO was relatively stable on storage - the procedure of freeze drying however was associated with marked loss of activity. Much attention has been paid to the purification of the enzyme - comparatively little to its storage. Modes of storage, not only of the purified enzyme, but also of the crude extracts, are topics covered in this dissertation.

Results of studies carried out by other workers on purified 3-HAAO have produced the following Michaelis constants.

ORGAN STUDIED

Rat Liver	$7.0 \times 10^{-6} M$	Iaccarina <u>et al.</u> (1961)
Rat Liver	$7.4 \times 10^{-6} M$	Cox (1963)
Beef Liver	$2.1 \times 10^{-5} M$	Decker <u>et al.</u> (1961)
Beef Kidney	$2.0 \times 10^{-5} M$	Ogaswara <u>et al.</u> (1966)

In this study attention is paid to the Michaelis constant, the molecular weight, the nitrogen amino acid terminal and the antigenic site of 3-HAAO in rat liver and kidney.

The compound most commonly studied in association with hepatic enzyme development is $11\beta,17\alpha$ -21-trihydroxy- Δ^4 -pregene-3,20,-dione (cortisol). For example, tyrosine- α -oxoglutarate transaminase is partially dependent on cortisol for its post-natal development. Sereni et al. (1959) found that on administration of tyrosine the expected increase in this enzyme failed to occur in adrenalectomized rats. This response was completely restored to normal on simultaneous administration of hydrocortisone. The authors suggested that substrate availability plus adrenal secretions were important causes of the abrupt increase in activity of tyrosine- α -oxoglutarate transaminase during the first 12 hours after birth.

The pre-natal development of tryptophan pyrrolase serine dehydratase and glucose-6-phosphatase independent of cortisol (Greengard et al. 1963; Nemeth 1959; Greengard and Dewey 1967). Adult rat tryptophan pyrrolase has been shown to respond to induction by both substrate and cortisol; foetal hepatic tryptophan pyrrolase responds to neither.

Hepatic enzyme systems have been shown to develop at different rates. In the rat tyrosine transaminase and phenylalanine transaminase are present at birth, tryptophan pyrrolase is not present in significant quantities till 12 days after birth (Auerbach and Waisman, 1959). Scher and Friedman (1958) found that while tryptophan pyrrolase took 15 days to reach high levels in the rat, it took 24 hours to do this in rabbit and guinea pig.

In this study the quantitative development of 3-HAAO in rat liver and kidney has been observed in terms of the changes in

activity associated with ageing. The present author appreciates that an unchanged level may mask significant changes in overall enzyme synthesis and breakdown patterns (Recheigl 1970).

Cortisol is a well recognized hepatic enzyme inducer (Bethel 1965; Weber et al 1964); it, as yet, enjoys no such reputation in the kidney. It does however increase the free amino acid level in both organs (Noall et al 1957). The organ best adapted for free amino acid capture, after cortisol injection, is however the liver. No information is available regarding the response of 3-HAAO to cortisol in either organ.

Hepatic tryptophan oxygenase is known to be induced by cortisol (Greengard 1963; Recheigl 1971). Cortisol is known to stimulate rat hepatic ribosomal RNA synthesis. Its mode of action is disputed. Blatti et al (1970) suggested this may be achieved via regulation of the catalytic activity of RNA polymerase 1. Sajdel and Jacob (1971) suggested the hydrocortisone induces an allosteric change in the nucleolar RNA polymerase. RNA polymerase 1 is located in the nucleolus. Yu and Feigelson (1972) suggested that an increase of this polymerases activity is via an increased synthesis of a core enzyme or via synthesis of polypeptide moieties necessary in the RNA polymerase 1 system. These authors concluded (1973) that cortisol stimulation of ribosomal RNA is not via allosterically enhanced catalytic efficiency of a preformed ribosomal RNA polymerase; nor was it due to a derepression of the ribosomal RNA genome. They postulated instead that cortisol acts via an active increase in the rate of synthesis of at least one of the polypeptide components of the RNA polymerase 1 system.

In this study the level of 3-HAAO activity per gram weight of liver is found to be enhanced by cortisol administration to adult rats. The mechanism whereby cortisol achieves this is of interest

- does it act at the level of 3-hydroxyanthranilic acid oxygenases genetic apparatus, or is its primary action on the genetic apparatus which controls tryptophan pyrrolase? If the latter is true then the enhanced activity detected on testing 3-HAAO is the response to activation of the tryptophan-nicotinic acid pathway at the level of tryptophan pyrrolase. Cortisol is also capable of acting as a "ligand" and stabilizing certain proteins (Ryan 1973). Enzyme induction varies between animals of different genetic content in the same species, and also between animals of the same strain at different ages (Conney 1967). This proved true of 3-HAAO on treatment with cortisol.

Schor and Frieden (1958) postulated that one of the modes of action of cortisol on tryptophan pyrrolase was via the metabolic shift to protein catabolism resulting in the liberation of free amino acids. They felt that cortisol made an increased quantity of endogenous tryptophan available to this enzyme. This hypothesis has not been stressed in more recent papers. Exogenous administration of tryptophan has been shown to activate tryptophan pyrrolase (Kenny and Flora 1961; Greengard and Feigelson 1961). The induction of tryptophan pyrrolase by its substrate is not a linear function of dosage (Schor and Frieden, 1958). The rat has been shown capable of dispensing with nicotinic acid as an essential dietary constituent in the presence of adequate tryptophan substitution (Priest 1951). No information concerning the effect of 3-HAAO on stimulation of the tryptophan - nicotinic acid pathway by tryptophan was found.

The final in vivo stimulus imposed on this enzyme system is a pathological one in the form of a carcinogen. Shiroyama et al. (1967) showed that 3-methyl-4-dimethylaminoazobenzene (DAB) produced hepatomas when fed to rats. The concentration of hepatic 3-HAAO was impaired in both the carcinomatous tissue and

the adjacent liver cells. The effects of up to 15 weeks of dietary dimethylaminozobenzene (DAB) on crude extracts of both liver and kidney 3-HAAO have been noted.

The following aspects of 3-HAAO have been investigated in this study:-

- (a) The development of liver and kidney 3-HAAO in both sexes between 7 and 360 days of age.
- (b) The purification and storage of hepatic and renal 3-HAAO.
- (c) Investigation of certain physio-chemical properties of the enzyme. This includes properties of the catalytic site, the antigenic site, the 'N' terminal and the molecular weight of this enzyme.
- (d) The adaptability of 3-HAAO in the liver and kidney to in vivo stimulation with cortisol - a hormone, with tryptophan - an essential amino acid, and with DAB - a carcinogen.

EXPERIMENTAL

INSTRUMENTS

Tissues were homogenized in an ultraturrax (Sunke and Kunkel, Stauffen); and centrifuged in a MSE superspeed 18 refrigerated centrifuge.

The spectrophotometer used was a Beckman DU monochromator attached to a Gilford 222A photometer with an automatic cuvette positioner Model 244. The recorder was a Unicam AR45 linear/log 1,0 decade recorder, obtained from Pye Unicam, England. The power pack used for electrophoresis was a Vokan SAE 2/61 obtained from Shandon Scientific Co., England. A Universal UV Lamp (Camag Muttenz Schweiz) was used for detecting dansyl derivatives. Gel filtration was carried out in columns obtained from Weight Scientific Co., England. The fraction collector used was a LKB Ultramax(Sweden).

REAGENTS

"Analar" reagents were used in all cases unless otherwise stated. Reagents used for enzyme assay:

Dithiothreitol (DTT) - Clelands reagent, grade A obtained from Calbiochem, California. The 6-aminocaproic acid was obtained from the Koch-Light Laboratories, England; the ferrous sulphate from Protea Holdings, South Africa. The 3-hydroxyanthranilic acid was obtained from Sigma Chemical Co., U.S.A. Reagents used in purification and molecular weight estimations of 3-hydroxyanthranilic acid oxygenase (3-HAAO):

Sephadex G-100 and G-75 were obtained from Pharmacia Fine Chemicals, Sweden. Acrylamide, N,N,N',N'-tetramethylethylenediamine, and ammonium persulphate were obtained from BDH, England. Of the marker solutions used serum albumin was obtained from BDH, England, ovalbumin and trypsin inhibitor from Sigma, U.S.A., cytochrome C from Boehringer and Soehne, Mannheim, and lactic dehydrogenase and myoglobin both from Miles Serovac, Cape Town.

For dansylation 5-dimethylaminonaphthalene-1-sulphonyl chloride a Merck (Darmstadt) product was used.

Chemicals which were administered to certain groups of rats:

Solucortef supplied by Upjohn, S.A., contains hydrocortisone sodium succinate (cortisol or 11,17 α -21-trihydroxy- Δ^4 -pregenone-3,20,-dione). As this is a relatively insoluble substance it is supplied in a Mix-O-Vial containing a diluent which consists of water, sodium phosphate, sodium biphosphate and methyl- and propyl-p-hydroxybenzoate are also provided. Tryptophan was obtained from Schwartz Laboratories, New York. Actinimycin D (Cosmagen) was obtained from Merck Sharpe and Dohme. Dimethylaminoazobenzene (DAB) was obtained from BDH, England.

BUFFERS

Homogenizing Buffer: This consists of a 0,02-M tris-maleate buffer containing 0,25-M sucrose and dithiothreitol (DTT), 6-aminocaproic acid and hydrated ferrous sulphate (1-mM of each in the final concentration). The last three reagents were added just prior to use. The pH was 6.5 in those purifications in which the animals were not pre-treated. Pre-treated animals are those which were subject either to a specially prepared diet or an intraperitoneal injection. In these pre-treated animals plus those used to study the development of 3-HAAO a homogenizing buffer at pH 3.8 was used. The present author is aware that this is not a physiological pH and unusually low for enzyme work. In spite of this low extraction pH, 3-HAAO was found active when assayed at pH 7.6

Assay Buffer: A 0,05-M tris-maleate solution at pH 7.6 was used.

Eluting Buffer: A 0,03-M ammonium acetate buffer (pH 6.5) to which DTT and hydrated ferrous sulphate (1-mM of each in the final concentration) were added immediately prior to use.

Storage Buffer: This is identical with the elution buffer. All solutions were made up in doubled distilled deionized water.

METHODS

TREATMENT OF ANIMALS

The rats, caged in groups of five, were supplied with a well balanced diet (20% protein) in the form of rat pellets and water ad lib. Owing to a shortage of rats it was necessary to use different strains in these experiments - Gilbert-Gillman (GG), Long-Evans (LE) and Sprague-Dawley (SD). Rats of the GG strain were subject to abnormally high temperatures due to a mechanical fault in the temperature system controlling the battery - it was thought that this may have inhibited spermatogenesis and be the explanation for the failure of these rats to breed. (Walker 1973). The LE strain during this period also encountered breeding difficulties and this rat population consisted essentially of elderly members. This left the SD rats as the main source of animal material; just over 300 rats were available for these experiments. It was thus necessary to impose limits on the number of rats used in any single experiment - this difficulty was further accentuated by the desirability of keeping certain factors constant, eg. strain and sex, in whole groups of experiments. The effect of this limitation becomes apparent on perusal of the experimental data. The present author is aware that many more experiments could have been carried out, especially during periods of rapid change.

Rats Used to Demonstrate the Development of 3-HAAQ:-

SD strain rats of both sexes aged between 7 and 360 days were used.

Rats Used in Experiments involving Cortisol:-

(a) Studies done at the Homogenate Stage:-

(1) Two groups of male rats (SD strain), one group 15,5 weeks and the other 8 months old, were given intraperitoneal cortisol (3 mg/100 gm body weight) and killed 3 hours later. Controls were injected with an equivalent volume of saline and killed at zero time.

(2) Two groups of 8 month old rats, of SD and LE strains, were killed at 105 minutes post intraperitoneal cortisol injection (3 mg/100 gm body weight). Controls were injected with saline and killed 105 minutes later.

(3) A group of 8 month old SD strain male rats were killed at 35, 70, 105, 180, 240 and 360 minutes post cortisol injection. The dose of cortisol used was 3 mg/100 gm body weight. Owing to the shortage of rats no parallel saline control series could be run in this experiment. Results from the above experiment (2) did show cortisol to significantly elevate the level of hepatic 3-HAAO. It was thus decided to measure the 3-HAAO activity at various intervals after cortisol injection. Owing to the lack of an adequate control series it must be appreciated that the results obtained reflect both the effect of cortisol and the influence of stress on 3-HAAO.

Certain animals in the 15,5 week (SD) and 8 month (LE) groups were also subjected to an intraperitoneal injection of 0,175 mg of Actinomycin D per rat. This was either given alone or followed 1 hour later by 3 mg of cortisol per 100 gm body weight.

(a) Studies Done on the Purified Enzyme:-

Adult male rats of the GG strain were injected with cortisol in a dose of either 3 or 4,3 mg/100 gm body weight. The rats were killed at 140 minutes after injection.

Rats Used to demonstrate the effects of L-tryptophan:-

L-tryptophan (90 mg/100 gm body weight) was administered to 6 month old rats of the SD strain. The rats were killed in groups of five at 35, 70, 140, 180, 240 and 360 minutes after peritoneal injection. Tryptophan proved insoluble in saline until the pH was adjusted to 10. This resulted in subjecting the animals to intraperitoneal injections at a non-physiological pH. The only macroscopic evidence that this alkali may have damaged the peritoneal cavity was the persistence of a variable volume of fluid in the

peritoneal cavity even 6 hours post injection. This fluid was clear, watery and the volume varied even in animals in the same group. No hyperaemia of the peritoneal cavity or of the adjacent viscera was found.

Rats used to demonstrate the effect of dimethylaminoazobenzene (DAB) on 3-HAAG:-

An adult (7 month old) group of male SD rats were fed DAB. In order to obtain a homogenous exposure to the DAB it was necessary to mix a special diet for these rats. The diet had the following (w/w) composition: flour 28,5%; skimmed milk 36%; mealie meal 10,3%; brewers yeast 0,02%; spleen 8,3%; beef dripping 5,1%; NaCl 0,56%. This diet was made up to one kilogram of dry ingredients and a further 150 cc of halibut oil, 150 cc of raw linseed oil and 30 cc of corn oil were added. The control rats were fed on this diet. The experimental group had 0,66 gm of DAB added per kilogram of mixture - the dosage as recommended by Symeonidis et al. (1954) and Roberts and Warwick (1966). The DAB was dissolved in corn oil prior to inclusion in the diet. The diet was freshly prepared every 10 days.

(Optimal conditions for carcinogenesis were provided by using male rats (Morris et al. (1951) found male rats more susceptible to hepatomas than females) of the SD strain. Chauve et al. (1968) produced hepatomas in rats of the SD strain with no overt signs of adrenal insufficiency. Litwack and Morey (1970) showed adrenalectomy produced increased resistance to hepatoma induction.)

Rats were killed at 4,5 and 7,5 weeks after starting on the above diet. Cytoplasmic protein-azo dye binding was found to be significant during this period (Miller et al. 1949, Miller and Miller 1947). A further group of rats were killed after 15 weeks on this diet.

MODE OF ENZYME ASSAY:-Substrate Stock Solution:-

One ml of ethanol containing 2.8 mg of 3-hydroxyanthranilic acid was stored at -20°C.

Assay Solution:-

A 2.95 ml volume of assay buffer plus 50 μ l of substrate were pipetted into a cm cuvettes and incubated in the spectrophotometer at 29 \pm 0,5°C for 5 minutes. Enzyme extract (10 - 25 μ l) was then added.

Unit Enzyme Activity:-

One unit of enzyme is defined as the amount which increases the absorbance at 360 nm by 1,0 absorbance unit per minute at pH 7,6 and temperature 28,5°C.

Protein Measurements:-

Protein was measured by their absorbance at 280nm. The protein concentration was calculated from the following equation (Layne 1957):-

$$\text{Protein Concentration} = F (1,55 \times A_{280} - 0,76 \times A_{260}) \text{ mg/ml.}$$

F = dilution factor

A = absorbance

Specific Activity:-

This is defined as the enzyme activity in units/minutes/mg of protein.

1. PURIFICATION OF 3-HAAO:-

All procedures were carried out at 4°C unless otherwise stated. Ammonium sulphate fractionations were done under a nitrogen stream.

(a) Purification of Renal 3-HAAO:-

Stage 1:-

The Initial Extract:-

The rats were killed by cervical dislocation and the kidneys were removed and weighed. Homogenizing buffer was added to the tissue in the ratio of 2 gm tissue : 3 ml buffer. The kidneys were homogenized for 55 seconds and the homogenate was then centrifuged for 25 minutes at 30 000g. The volume of the supernatant was measured and activity and protein estimations performed.

Stage 2:-

Ammonium Sulphate Fractionation:-

Additions of ammonium sulphate were done under a nitrogen stream with constant stirring of the enzyme extract. Ammonium sulphate was slowly added to the supernatant from Stage 1 until a saturation of 30% was reached. This solution was centrifuged for 20 minutes at 32 000g. The supernatant was then brought up to 60% saturation. The solution was centrifuged as before, and the supernatant discarded.

Stage 3:-

Gel Filtration on Sephadex G-100:-

The precipitate from the previous stage was dissolved in elution buffer and placed on a Sephadex G-100 column (3,2cm a 60cm). The eluting buffer was run through the column and fractions of 6 ml or less were collected. In most cases fractions of more than 8 enzyme units per ml were pooled.

Stage 4:-

The 70% Ammonium Sulphate Fractionation:-

The pooled eluent was brought up to 70% saturation with ammonium sulphate, centrifuged for 20 minutes at 32 000g, and

the supernatant discarded. The precipitate was taken up in a minimal amount of storage buffer.

N.B. Hereafter enzyme which has completed stage 4 is referred to as "PURIFIED ENZYME".

(b) Purification of Hepatic 3-HAAO:-

Stage 1:-

Livers were homogenized for 45 seconds and centrifuged at 32 000g for 25 minutes.

Stage 2:-

Livers were handled in one of two ways -- they were subject to either ammonium sulphate fractionation, OR to an acetone fractionation.

Ammonium Sulphate Fractionation:-

The enzyme extract from stage 1 was subject to constant stirring while ammonium sulphate was added to 30%. After centrifugation (32 000g for 20 minutes) ammonium sulphate was added to the supernatant to 60% saturation.

Acetone Fractionation:-

Acetone at -15°C was rapidly stirred into the homogenate supernatant to a concentration of 42% (v/v). This solution was centrifuged at -5°C for 10 minutes reaching a maximum of 22 000g. After centrifugation, acetone was added to the supernatant to a concentration of 60% (v/v), keeping the solution at a temperature between -5° and -10°C . This was then centrifuged at -10°C for 10 minutes (maximum 10 000g). The supernatant was discarded.

Stages 3 and 4:-

These were identical with those in renal 3-HAAO purification.

2. STORAGE OF 3-HAAO:-

3-Hydroxyanthranilic acid is an unstable enzyme and storage is inevitably associated with loss of activity. Optimal storage conditions have been examined in terms of temperature, storage media and stage of enzyme purification.

(a) Temperature:-

Enzyme at the homogenate stage was stored at 4° and 37° and tested for activity over a 3 hour period.

(b) Storage Media for the purified Enzyme (liver, GG strain):-

The enzyme was stored in one of the following storage media at -20°C for 58 days:-

1. Storage buffer (0,3-M ammonium acetate with DTT and hydrated ferrous sulphate - 1-mM in the final concentration, pH 6,5.)
2. Storage buffer mixed in ration of 1 : 1 (v/v with distilled deionized water.
3. A mixture of storage buffer and glycerol (1 ; 1 v/v).
4. A mixture of storage buffer and glycerol (4 : 1 v/v).

These enzymes solutions were subject to thawing and refreezing eight times during the 58 day storage period.

5. Storage buffer followed by:-

- a. Freeze drying
- b. overnight dialysis and then freeze drying.

6. Storage diluted in water (1 : 1 v/v) followed by a. and b. above.

Aliquots of the freeze dried enzyme were taken up in storage buffer immediately prior to testing.

Stages During Purification at which the Enzyme may be Stored:-

Liver (homogenate - stage 1, and purified (stages 1 to 4) and kidney (homogenate - stage 1, after 60% ammonium sulphate fractionation - stages 1 and 2, and purified - stages 1 to 4) were stored at -20°C and assayed at intervals for up to 25 days. As the homogenate stage is convenient for storage an attempt was made to stabilize the enzyme at this stage. The following additives were used:- In Vitro: Sodium hydroxide was added to a saline solution to bring the pH to 10. This saline solution was then mixed with aliquots of both liver and kidney homogenate in the ratio of 1:3 (v:).

Tryptophan was dissolved in a similar solution, NaOH being used to adjust the pH to 10. The final concentration of tryptophan was 10 mg/ml.

Cortisol at a final concentration of 0,3 mg/ml.

Each of these substances, in the concentration shown above, was incubated with the homogenate enzyme solution at 4° and 37°C . The effects of these additives on liver and kidney stability compared with control were studied over a 3 hour period. In the case of in vitro cortisol treatment the period studied was extended to over 25 days at -20°C .

In Vivo: Cortisol in a dose of 3 mg/100 gm body weight was administered by intraperitoneal injection (p.11-12). The rats were killed at 35, 70, 105, 180, 240 and 360 minutes post injection. Homogenates (pH 3,8) were prepared (p.14-15), stored at -20°C and tested at intervals for up to 27 days.

3. ESTIMATION OF THE AVERAGE MOLECULAR WEIGHT OF 3-HAAO FROM RAT LIVER AND KIDNEY.

(a) By Gel Filtration:-

The molecular weights of 3-HAAO liver (GG strain and kidney (LE strain) have been determined using a calibrated Sephadex G-75 column (1,6 cm x 60 cm) according to the method of Andrews (1970). Proteins used as standards for molecular weight determinations were:-

Lactic dehydrogenase,
Myoglobin,
Trypsin inhibitor,
Cytochrome C,
O albumin, and
Serum albumin

The enzyme and standard protein solutions were eluted from the column using elution buffer: 3,3 ml fractions were collected. The elution volume for each protein was determined from the 280 nm peak. In the case of the enzymes from liver and kidney, both activity and absorbance at 280 nm were plotted. The molecular weight of the enzyme was determined by plotting the log of the MW of the standards against the elution volume (V_e).

(b) Polyacrylamide Gel Electrophoresis in Sodium Dodecylsulphate (SDS):-

The method of Weber and Osborn (1969) was followed except that gels were not destained electrophoretically. The enzyme was not eluted from the gels. The value of SDS is that it eliminates the effect of charge on the migration of protein, (Shapiro et al. 1967, Shapiro and Migil 1969).

The enzymes used in this experiment were the same as described in gel filtration except that liver and kidney of rats (SD strain) fed for 4½ weeks on DAB were also tested.

Two separate runs were done. In the first experiment the marker and enzyme solutions had been incubated with SDS for 2 hours at 37°C. In the second run these solutions had been incubated at 4°C for a further 36 days.

4. ELECTROPHORESIS OF ENZYME ON ACRYLAMIDE GEL:-

Polyacrylamide electrophoresis was carried out according to the method of Davis (1964). Gel concentration was 7,5%, the gel buffer consisting of 0,06-M tris /HCl (pH 8,4) containing 1-mM DTT. The running buffer was 0,05-M tris / HCl also pH 8,4 and containing 1-mM DTT. Enzyme solutions were prepared in 10% sucrose containing 0,2% bromophenol blue. A total current of 16 m Amps was used until the dye had entered the gel, thereafter the current was increased to 32 m Amps. Electrophoresis was stopped just before the dye reached the end of the gels.

Gels were stained in pairs - one for protein, the other for activity. The gel to be stained for activity was incubated in a solution of assay buffer containing substrate. Activity was detected as a dark non-fluorescent band under UV light apparatus. The protein was fixed with 20% trichloroacetic acid and stained with 2,5% Coomassie blue Methanol (CH₃OH: H₂O: Cl₃COOH - 5:4:1).

Both liver and kidney enzyme failed to migrate when the pH of the gel and running buffer were 8,4. If the pH of the running buffer was increased to 9,2, slight movement was

observed. Increased migration was observed when the pH for both the gel and running buffer was 9,2.

5. DETERMINATION OF THE 'N'- TERMINAL GROUPS BY THE DANSYL CHLORIDE METHOD:-

The procedure used was as follows:-

The purified enzyme was dialysed against a 0,3-M ammonium acetate solution. After freeze drying 10-15 mg of this protein were dissolved in a 0,5 ml 8-M urea solution in 4,2% NaCHO₃ (pH 9,5). Dansyl chloride (25 mg) in 0,5 ml of acetone were added. The solution was shaken overnight and then dialysed against distilled water for 72 - 96 hours. The precipitate was washed with water twice followed by two acetone washing and spun down by centrifugation. The protein was hydrolysed overnight in a sealed tube with 0,5 ml of 5-N HCl. The acid was removed under vacuum and the hydrolysate dissolved in either ether or water. Markers of dansyl amino acids were prepared as described by Boulton and Bush (1964). Lysozyme and ribonuclease were treated in an identical fashion as described above and also used as marker solutions. Chromatography of the dansyl amino acids was carried out on silica gel, using the following solvent systems:-

(A) Unidimensional Chromatography:-

- (a) Chloroform : methanol : acetic acid (15:4:1) (v/v).
- (b) Butanol : acetic acid : water (4:2:1) (v/v).
- (c) Methyl acetate : propanol : aqueous ammonia (1:2:4). (v/v).
- (d) Chloroform : ethyl acetate : methanol : acetic acid (30:50:20:1) (v/v).
- (e) Benzene : pyridine : acetic acid (16:4:1) (v/v)

(B) Two Dimensional Chromatography:-

Solvent (b) in the first direction and solvent (d) in the second direction.

6. THE CATALYTIC SITE:-

In the following experiments the assay conditions were identical to those described on page except for the consecutive alterations in pH, temperature and substrate concentration, as described in each section.

(a) pH Optimum:-

The enzyme was reacted at pH values varying between 5,7 and 8,5. Assay buffer was adjusted to the desired pH with NaOH.

(b) Temperature Optimum:-

At a pH of 6 the activity of 3-HAAO was assayed at temperatures ranging between 31° - 43°C.

(c) Michaelis Constant:-

This was determined at a pH of 7,6 and temperature of 29 ± 0,5°C. The enzymes tested were liver and kidney 3-HAAO of the SD strain with and without in vivo cortisol or DAB treatment.

7. THE ANTIGENIC SITE:-

Animals:-

Two adult male rabbits of the New Zealand white strain were housed in separate cages and fed ad lib on rabbit pellets and water. Both rabbits remained healthy throughout the period of the experiment. A control blood specimen was taken

from both rabbits prior to starting antibody induction. Blood was obtained from vessels in the pinna of the ears in both cases.

Purification of Antigen:-

Rat liver (GG strain) 3-HAAO was purified according to the scheme on page 16 (Stage II involved the use of ammonium sulphate). The 70% ammonium sulphate precipitate was further purified by being subjected to chromatography on a column of G-75 (1,6 cm x 60 cm). Three fractions with a mean specific activity of 27,6 (total activity = 658 units, total protein = 23,86 mg) were pooled (Fig. 2 shows the protein / activity elution profile). The combined fractions were precipitated with 70% ammonium sulphate. The enzyme was dissolved in normal saline and dialysed against saline to remove ammonium sulphate. The enzyme was then mixed in a 1 : 1 ratio with Freuds Complete Adjuvant (Williams and Chase 1967).

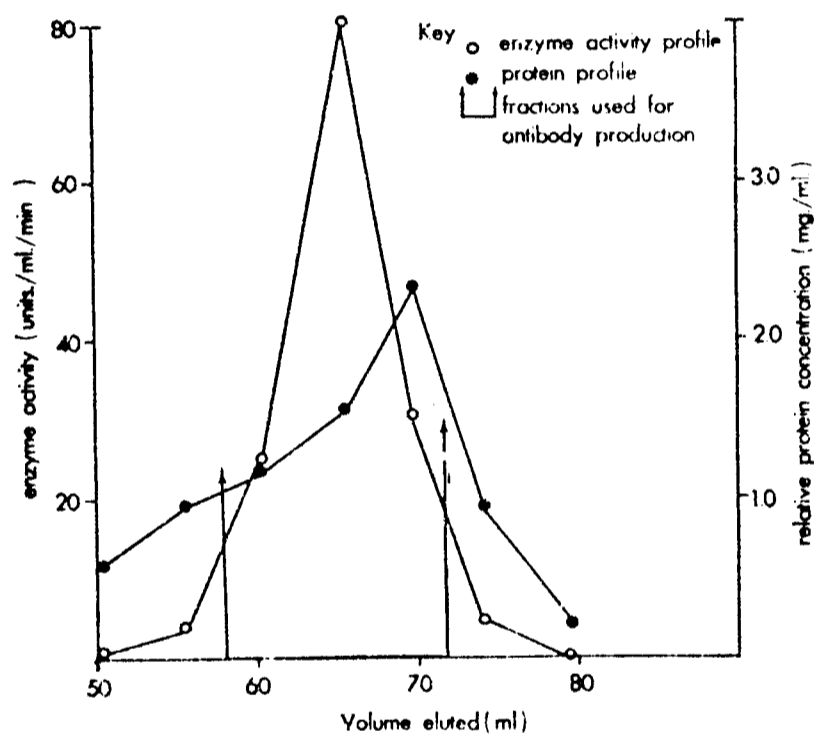
Preparation of Antibody:-

The rabbits were injected intramuscularly for the reasons outlined by Leskowitz and Waksman (1960). The experimental rabbit was injected with 3 ml of the enzyme / adjuvant mixture - this contained 3 mg / ml of enzyme protein. The control animal received an equivalent of saline / adjuvant. Volumes of not more than 0,75 ml were injected into any one site. No booster was administered.

The rabbits were bled at intervals, the blood allowed to clot and the serum was pipetted off. The serum was used as such for antibody titration experiments, was subjected to 50% ammonium sulphate fractionation prior to use in gel diffusion. The antibody precipitate was dissolved in 0,9% saline prior to use.

Figure 2

Activity and protein profiles of GG rat liver 3-hydroxyanthranilic acid oxygenase on G-75 sephadex (16.60 cm.) column used for antibody production in New Zealand white rabbit



(a) Gel Diffusion:-

The agar for the double diffusion experiment was prepared as follows:-

Bacto agar (15 gm) was soaked overnight in 1 litre of distilled water. This was then autoclaved at 20 lbs / sq. inch for 20 minutes and the following substances were added:-

KH_2PO_4	1,53 gm
Na_2HPO_4	8,47 gm
NaCl	9,0 gm
Na azide	10,0 gm

The pH of this solution was between 7,2 - 7,5 and was maintained at a temperature of 68°C. A 1 mm thick layer of agar was poured into a petri dish and allowed to set. A plastic matrix with one central and four peripheral circular projections was placed on the set layer of agar. A further layer of agar was poured into the petri dish to a depth of 3 mm, allowed to set and the matrix was then removed.

Antibody or control serum were placed in the central well while antigen was pipetted into the peripheral wells. Diffusion was carried out at 37,20 and 4°C. Staining conformed to the technique employed by Ureil (1967).

(b) Antibody Antigen Titration:-

Enzyme of both liver and kidney at 'homogenate' and 'purified' stages were incubated with antibody and allowed to react at 4°C. The enzyme activity was tested and antibody

added until no further activity could be detected. The solution was then centrifuged at 4°C and the antibody-antigen precipitate separated from the supernatant. Varying volumes of purified GG liver antigen were added to the supernatant, allowed to react with any remaining antibody, and activity was re-tested. Schematic representation of antibody antigen titration appears on Fig. 3.

The utilization of control serum did not eliminate errors due to the variable response of 3-HAAO to dilution; sometimes there resulted an enhanced activity of the enzyme, whereas at other times some enzymatic activity appeared to be lost. For instance, a three fold dilution of an enzyme solution containing 100 units of enzyme activity could result in a solution containing either more or less but seldom the same amount of enzyme activity. In other words, dilution affected the measurable enzyme activity and did so in an unpredictable manner. Other workers in this field have found similar erratic behaviour of this enzyme. Inevitable small volume losses inherent in this procedure are not necessarily equal in control and experimental specimens. The present author found that this method is both crude and inaccurate, and furthermore it consumes relatively large quantities of antibody. As a quantitative measure of enzyme present it proved a failure; qualitatively, the gel diffusion method was superior.

8. A TEST FOR DAB ENZYME COMPLEX FORMATION:-

Aliquots of formic acid were added to purified liver and kidney 3-HAAO of animals which had been subjected to 4,5 and 7,5 weeks of dietary DAB. The colour was then noted with the naked eye. This is a modification of the technique employed by Ketterer et al. (1967), based on the pink colour produced on acidification of the azo dye.

Figure 3.

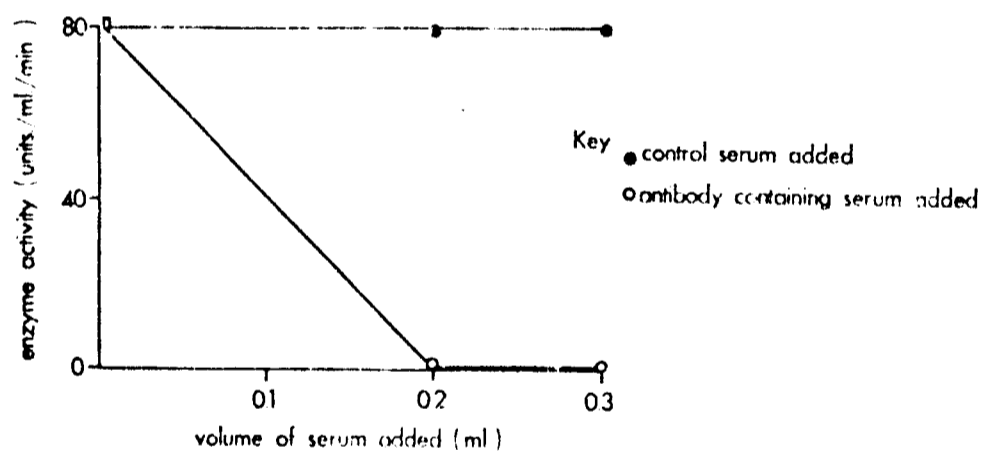
Schematic representation of neutralization of 3-hydroxyanthranilic acid oxygenase activity
via antibody-antigen precipitation

Enzyme used - liver either purified or homogenate stage
 kidney

May be cortisol treated in vivo

Strain - LE, CG or SD

Antibody - produced against GG liver antigen



R E S U L T S

(A) RESULTS OF STUDIES CONFINED TO THE HOMOGENATE STAGE:-1. THE QUANTITATIVE DEVELOPMENT OF 3-HAAO

One fifth of the total number of rats available were used in this study - in spite of this the experimental data obtained lacks sufficient detail.

(a) Tissue Investigated:-

The following organs of 7 day and 8 month old rats were examined:- Spleen, lung, heart, small intestine, liver, kidney and brain.

3-HAAO was isolated from the cytoplasm of only liver and kidney. It is present in both of these organs at 7 days (liver - $138,5 \pm 8,5$, kidney - $25,5 \pm 2,5$ both expressed as enzyme units per gram weight of organ) and at 8 months (liver - $137,5 \pm 4,8$ kidney - $124,8 \pm 5,1$ EA/gm wet wt. organ) of age.

(b) The Temporal Quantities of 3-HAAO:-

Figure 4 shows the hepatic enzyme development between 7 and 360 days in male and female SD rats. The male liver enzyme is developing rapidly and demonstrates the "overshoot" phenomenon at 59 days. By 4 months this enzyme has quantitatively stabilized. Female hepatic 3-HAAO is at a higher level at 7 days of age and no significant increase in the level of this enzyme was noted.

Figure 5 demonstrates the renal 3-HAAO development in the same animals used to study the hepatic enzyme development. The kidney enzyme is far lower at 7 days than hepatic 3-HAAO. The

*Enzymes that undergo a large increase in activity during a short period of time, frequently rise above their adult level and subsequently fall back to a relatively stable level. This is called the overshoot phenomenon. (Moog 1971).

Figure 4.

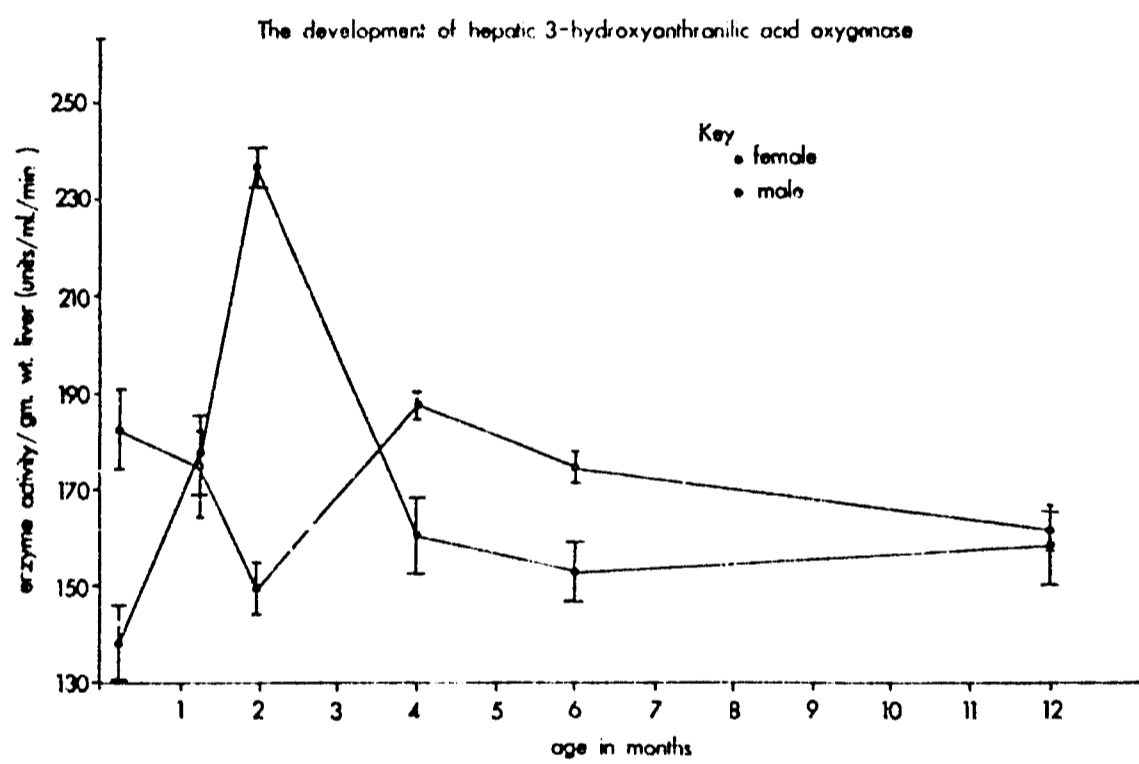
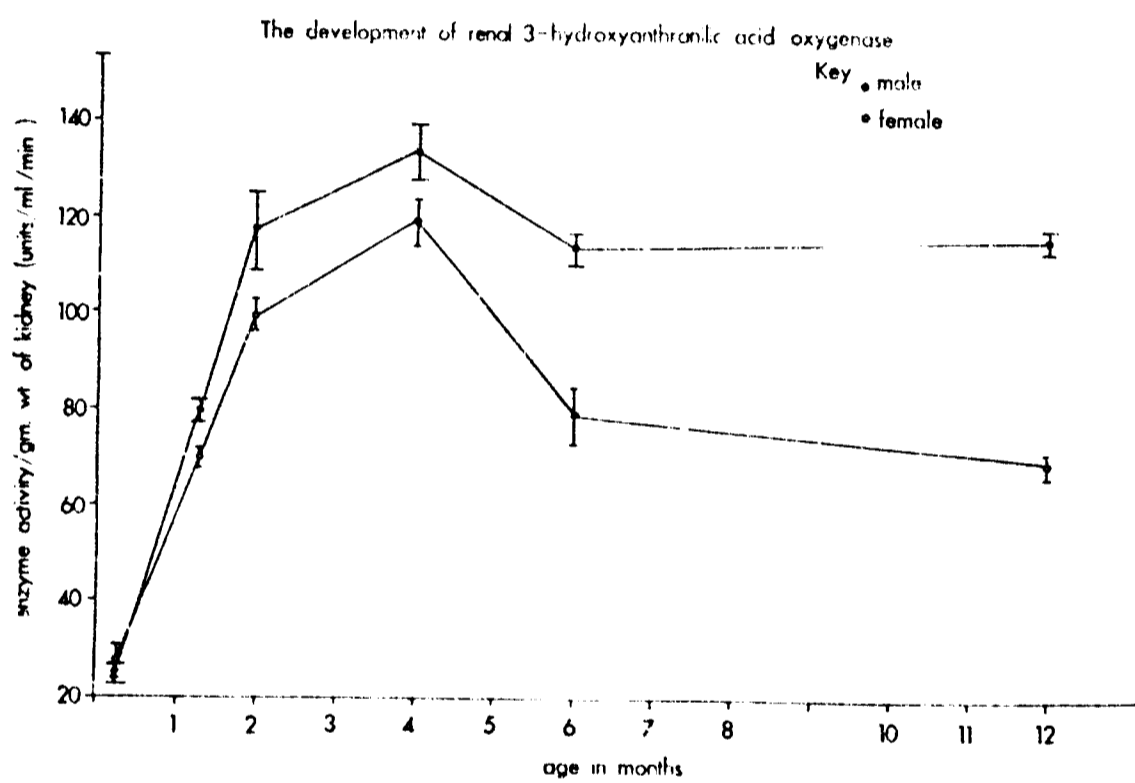


Figure 5.



development of this enzyme in the male and female tends to a similar overall pattern, but the male maintains a higher concentration the ratio of which increases with age. The female kidney enzyme demonstrates the "overshoot phenomenon" absent in the male owing to his ability to maintain the higher renal level of 3-HAAO in adult life.

Further evidence that the quantity of 3-HAAO differs in male and female kidney is found in Figure 6 which outlines the variability of specific activity associated with the development of this enzyme in the male and female kidney. The specific activity in the ratio of enzyme activity to cytoplasmic protein concentration is statistically significantly different between male and female at 59 and 183 days of age.

Figure 7 shows the changes in specific activity associated with hepatocyte development. At 39 days the level of 3-HAAO is relatively higher than the other cytoplasmic proteins - this may well imply relatively early development of this enzyme's genetic apparatus.

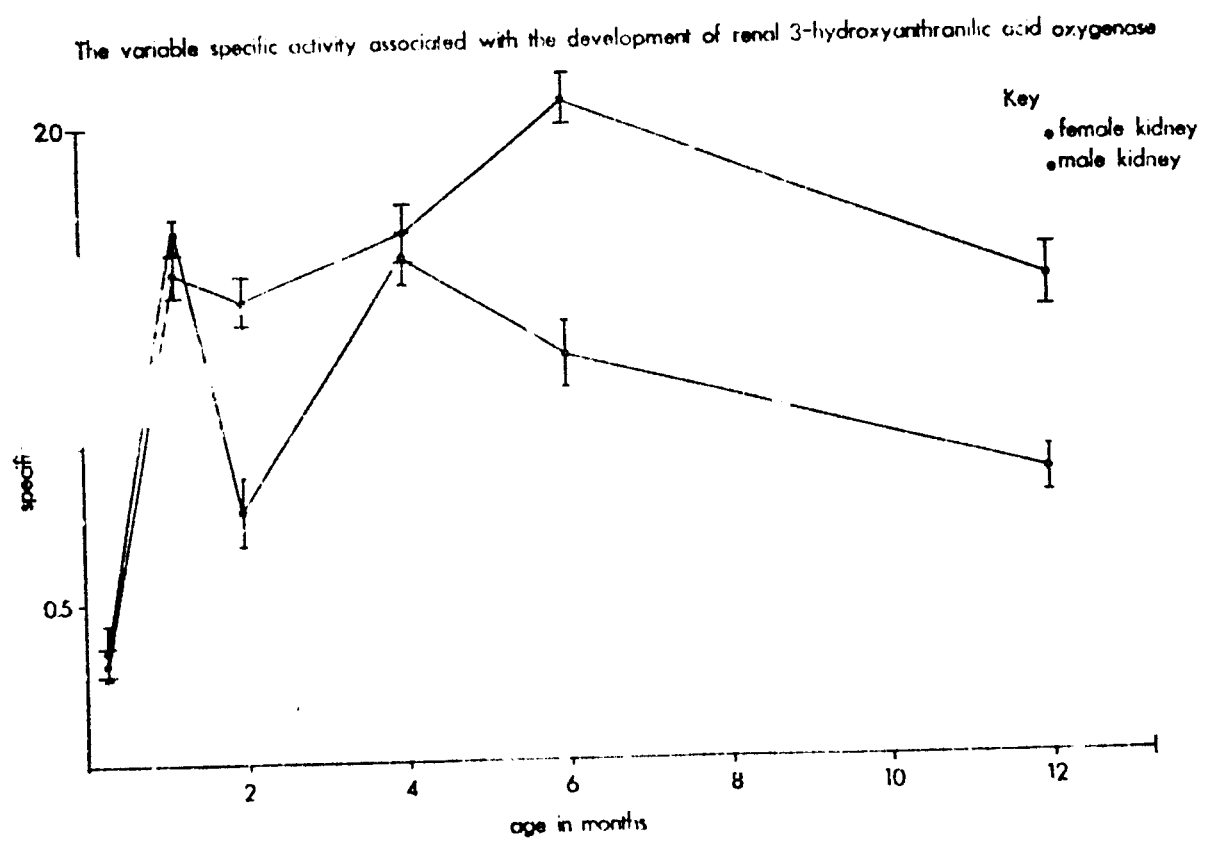
Figure 6.

Figure 6.

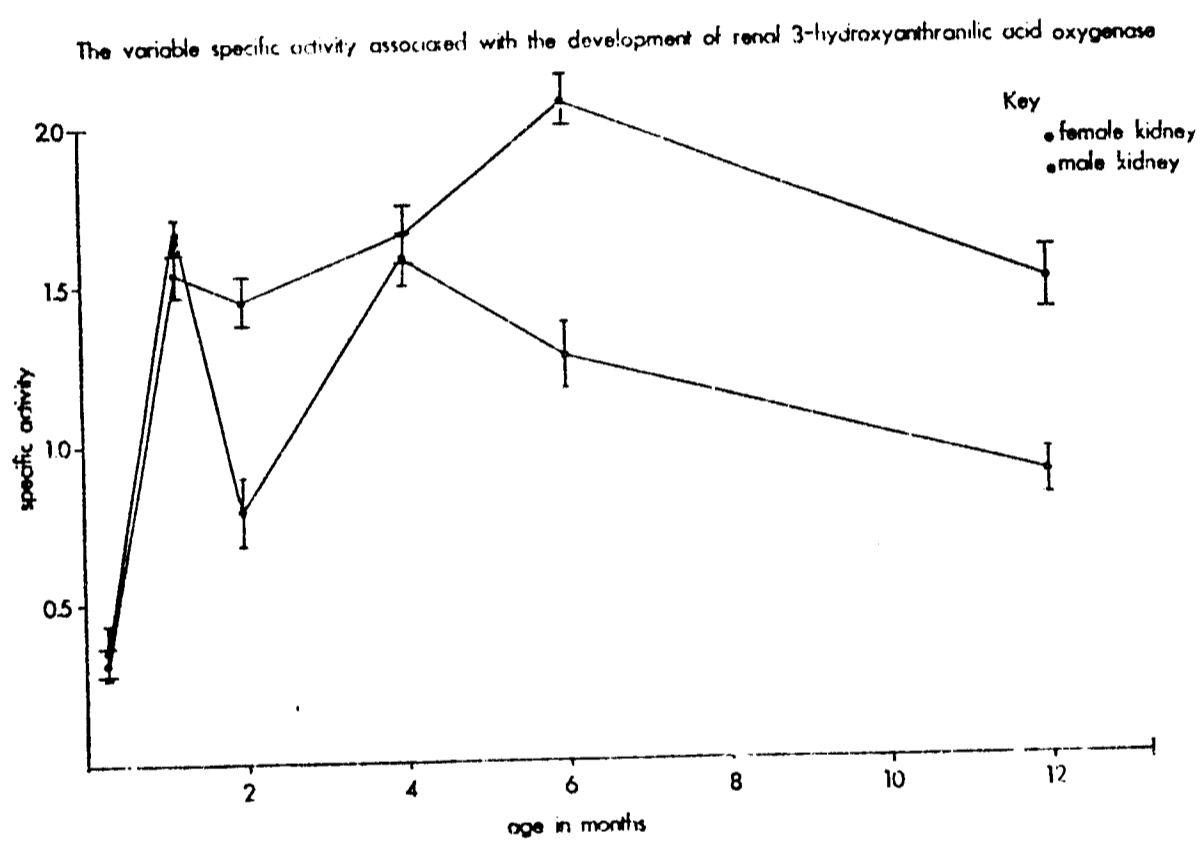
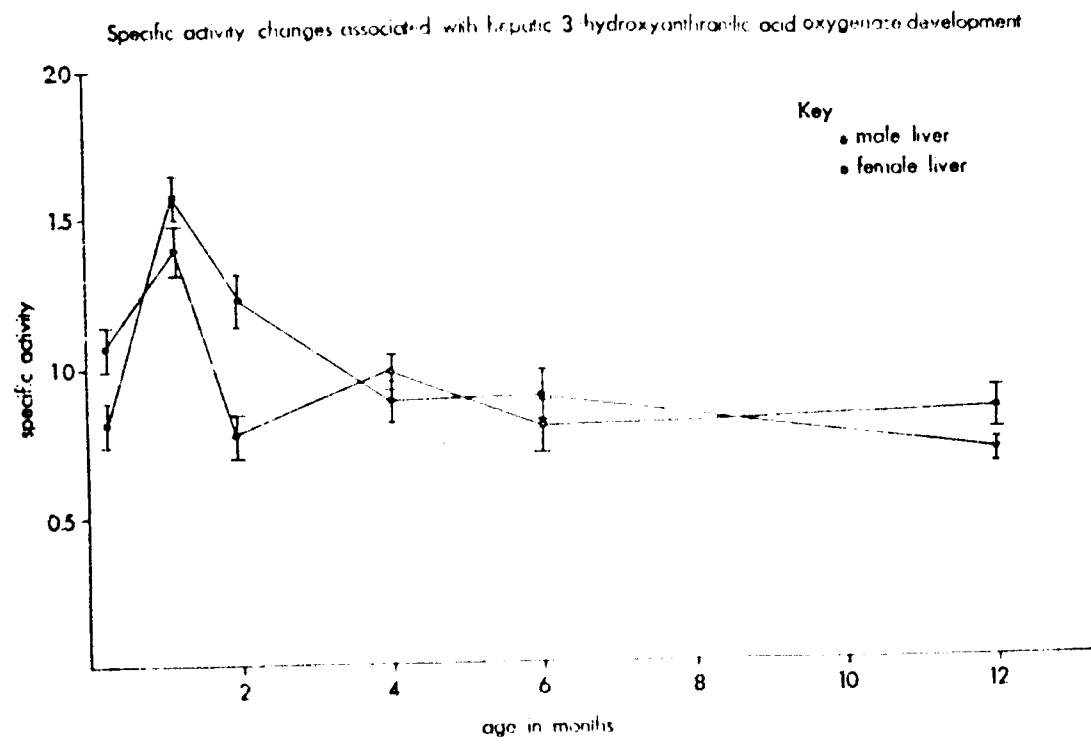


Figure 7.

2. THE INTERACTION BETWEEN CORTISOL AND 3-HAAO

Hepatic 3-HAAO responds to intraperitoneal cortisol (3 mg / 100 gm body weight) by significantly increasing its level of activity. This hepatic response and the failure of renal 3-HAAO to respond to cortisol are graphically demonstrated in Figure 8. Figure 9 shows the effects of cortisol injection on specific activity. The variability of specific activity demonstrates cortisol's generalized (simultaneous and also successive) effect on protein synthesis. At 105 minutes the specific activity of renal 3-HAAO reaches a peak. The change is attributable to a combined effect - a slightly increased enzyme activity level (Fig. 8) plus a decrease in renal protein. No satisfactory explanation is known for cortisol decreasing renal proteins at 105 minutes. Noall et al. (1957) have shown an increased level of amino acid in kidney and liver 120 minutes after hydrocortisone injection.

An attempt to obtain information about the mechanism whereby cortisol influenced hepatic 3-HAAO was made using Actinomycin D. Table 1 shows significant induction of hepatic 3-HAAO is inhibited by this protein synthesis inhibitor. Biological statistical significance is taken as a P value of 0,05 or less. This isolated result points to cortisol acting as an enzyme inducing agent and not via enzyme activation. The 70 minute time lag between cortisol injection and 3-HAAO level elevation is in keeping with this result. Actinomycin given alone increases the measurable 3-HAAO level. This increase, although not statistically significant ($P \leq 0,05$) is similar to the response of basal tyrosine aminotransferase in rat hepatoma tissue culture on exposure to actinomycin D (Peterkofsky and Tomkins, 1967). The critical factor associated with the induction of hepatic enzymes in tissue culture was found to be the concentration of actinomycin used. Garro et al. (1964) increased the level of hydrocortisone-induced tryptophan pyrrolase in rat liver by administering actinomycin D 5 hours after the hydrocortisone injection.

Figure 8.

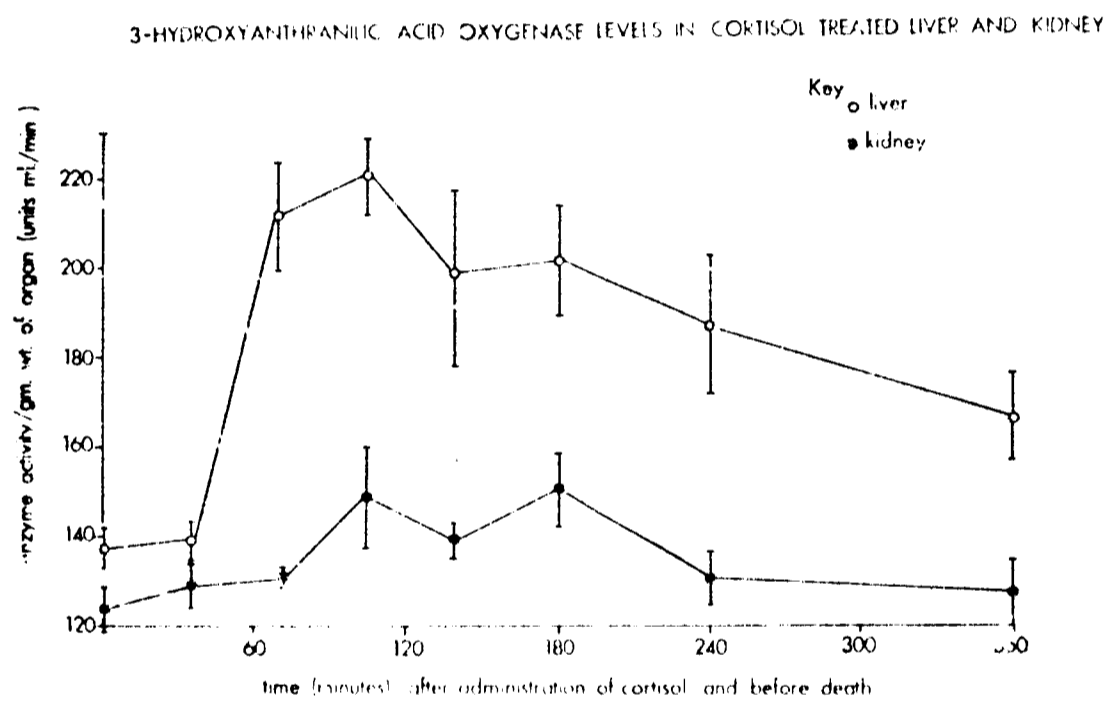


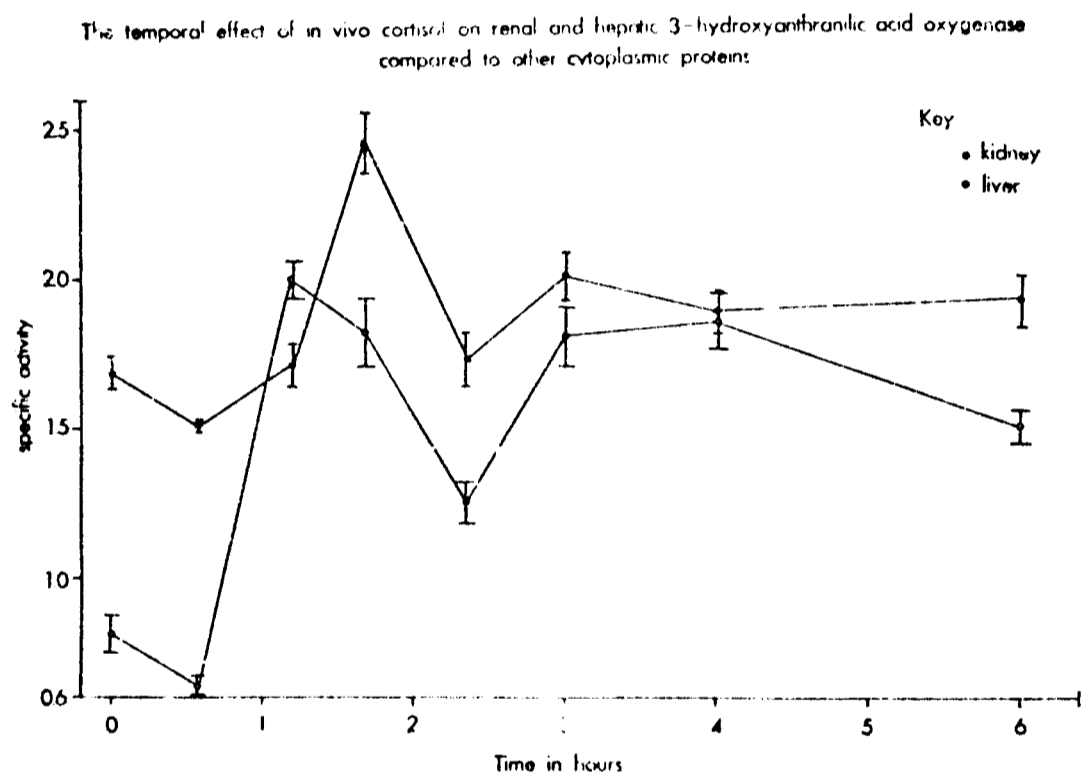
Figure 9.

Table 1.

TABLE 1: THE EFFECT OF 3-HAAD IN FIGHT WOUND OLD PAIS
OF THE SUPPLEMENTAL ACTIVITY IN VIVO OF HISA
AND ACTIN PROTEIN.

All rats killed 15 minutes post injection.

(A) The effect of In Vivo Cortisol Solution Compared to Diluent
(Mechanism of Phagocytosis) on 3-HAAD

(i) STRAIN:- SD AGE:- 8 months SEX:- male

	MEAN ENZYME ACTIVITY	N	SEM	P
SALINE	169,33	3	36,62	≤
"PARALIN"	159,73	3	24,56	0,95

Saline has been used as a control in all other experiments as cortisol's diluent has no significant effect on 3-HAAD activity.

(B) Mechanism of Cortisol Action on 3-HAAD

(ii) STRAIN:- LF AGE:- 8 months SEX:- male

	MEAN ENZYME ACTIVITY	N	SEM	P
CONTROL	133,61	5	18,28	≤
CORTISOL (3 ng / 100 gr body wt.)	195,09	5	9,12	0,05
ACTIN PROTEIN (0,125 ng / rat)	163,5	5	6,772	0,1
ACTIN PROTEIN (1 hr prior to Cortisol)	169,2	5	10,48	0,2

(iii) STRAIN:- SD

CONTROL	160,00	6	7,784	
CORTISOL (3 ng / 100 gr body wt.)	220,44	5	9,58	0,01

MEA = Mean enzyme activity per gram weight of organ.
SEM = Standard error of the mean.

Table 2.TABLE 2: THE ADAPTABILITY OF 3-HAAG IN RAT LIVER AND KIDNEY ON IN VIVO STIMULATION WITH CORTISOL

STRAIN:- Sprague-Dawley
SEX:- Male
IN VIVO STIMULUS:- (a) Intraperitoneal cortisol (3 mg / 100 gm body weight). Rats killed 3 hours post injection.
 (b) Intraperitoneal Actinocycin (0,175 mg / rat) given 4 hours prior to death.
CONTROL:- Equivalent volume of saline administered. Killed at zero time.
MEAN E.A.:- The enzyme activity per gram weight of organ.

	<u>ORGAN - LIVER</u>				N	<u>ORGAN - KIDNEY</u>		
	<u>N</u>	<u>MEAN E.A.</u>	<u>SEM</u>	<u>P_±</u>		<u>MEAN E.A.</u>	<u>SEM</u>	<u>P_±</u>
Control	5	155,2	4,757		5	100,9	2,575	
Cortisol	10	161,87	7,3715	0.4	5	105,25	3,15	0.9
Actinocycin	5	176,4	7,052	0.9	5	103,1	2,257	0.4
Actinocycin/Cortisol	5	175,6	4,635	0.5	5	99,61	4,17	0.9
	<u>AGE - 8 MONTHS</u>							
	<u>ORGAN - LIVER</u>				N	<u>ORGAN - KIDNEY</u>		
	<u>N</u>	<u>MEAN E.A.</u>	<u>SEM</u>	<u>P_±</u>		<u>MEAN E.A.</u>	<u>SEM</u>	<u>P_±</u>
Control	5	137,5	4,821		5	124,84	5,117	
Cortisol	5	201,5	11,91	0.005	5	150,72	8,337	0.1

The response of enzymes to inducing agents varies with age and species. Cortisol induces hepatic 3-HAAO in 8 month old rats of both the LE and SD strains - the response at 105 minutes after injection is however statistically more significant in rats of the SD strain (Table 1(B)). Rats of the SD strain were killed 3 hours post injection those of 8 months of age responded with a significant increase in hepatic enzyme activity, those at 15,5 weeks of age failed to respond (Table 2). Table 2 also shows that in neither case did renal 3-HAAO respond significantly to cortisol induction. It will be noted that although renal 3-HAAO did rise in 8 month old rats, the enzyme failed to reach biologically significant levels. Actinomycin D alone once again led to some increase in enzyme activity. The failure of actinomycin to influence either hepatic or renal 3-HAAO post cortisol treatment further confirms the resistance of this animal to cortisol induction.

Table 1A shows the results of an attempt to induce hepatic 3-HAAO with the diluent used in the cortisol mix-a-vial. There is no significant response of the enzyme to the substances injected in association with cortisol. The active agent in hepatic 3-HAAO induction is thus hydrocortisone sodium succinate.

3. THE INTERACTION BETWEEN L-TRYPTOPHAN AND 3-HAAO:-

In the case of liver, 3-HAAO activity was increased at 35 minutes post injection - this regardless of whether the saline / NaOH pH 10 contained L-tryptophan or not. The tryptophan treated animals have an enhanced level of hepatic enzyme activity at 180 minutes, they also have a significantly decreased level of activity at 240 minutes. Alkali treated control animals maintain an activity level not significantly different from that at zero time during this period. Whether the alkali used to solubilize tryptophan compromises the response is unknown. The responses of hepatic 3-HAAO to in vivo tryptophan and alkali treatment are tabulated in Table 3.

TABLE 3: THE RESPONSE OF HEPATIC 3-HAAO TO IN VIVO
TRYPTOPHAN TREATMENT

Hepatic 3-HAAO Response to L-Tryptophan:-

<u>TIME</u>	<u>MEAN ENZYME ACTIVITY</u>	<u>SEM</u>	<u>P_e</u>
0	150,56	5,895	
35	223,3	9,12	0,001
70	218,02	11,86	0,05
140	141,54	12,29	0,6
180	189,42	8,722	0,02
240	118,3	6,679	0,001
360	169,5	7,096	0,2

Control (Alkali pH 10 in Saline Solution):-

<u>TIME</u>	<u>MEAN ENZYME ACTIVITY</u>	<u>SEM</u>	<u>P_e</u>
0	150,56	5,895	
35	236,54	5,413	0,001
70	223,9	11,65	0,001
140	184,7	5,513	0,05
180	151,9	6,767	0,9
240	142,9	6,67	0,5
360	171,6	6,878	0,1

N = 5

The renal enzyme, unlike with cortical treatment, responds to both tryptophan and alkali. The difference between the two responses at 360 minutes is however significant - this implies a response to tryptophan per se. The marked elevation of alkali treated kidney 3-HAAO at 35 minutes is not within the statistically acceptable range of significance. The obvious technique to use in order to determine the importance of this increase would be to utilize a larger number of experimental animals. Rats of the same age, sex and strain were not available at the time. The large standard error of the mean does however point more to experimental error than a specific renal 3-HAAO response 35 minutes after alkali injection. Table 4 shows the response of the renal enzyme to in vivo tryptophan and alkali stimulation.

4. THE INTERACTION BETWEEN DIETARY DAB AND 3-HAAO:-

Figures 10(a) and (b) show the effects of dietary DAB on hepatic, renal 3-HAAO and on body weight after feeding of this carcinogen for up to 15 weeks. The animals on a DAB substituted diet maintained a lower body weight than the control. DAB influenced both liver and kidney 3-HAAO levels at 4,5 weeks; also at 7,5 and 15 weeks respectively. The increase in liver 3-HAAO at 7,5 weeks in DAB treated animals is an increase which is significant only in terms of the marked decrease in enzyme activity in the control group. These results cannot be ignored as the only obvious difference between these groups of rats is that one group was exposed to DAB. It is only in macroscopically abnormal areas of liver that the enzyme activity is decreased - 10(b) and (c). Storage of purified DAB treated enzyme (Fig. 11) does not appear to offer any great advantage over the control - in fact renal 3-HAAO after in vivo DAB treatment appears to be a little less stable than the control. The enzyme stored was from animals which had been fed on DAB for 4,5 weeks.

TABLE 4: THE RESPONSE OF RENAL 3-HAAO TO IN VIVO TRYPTOPHAN TREATMENT

Hepatic 3-HAAO Response to L-Tryptophan:-

<u>TIME (Minutes)</u>	<u>MEAN ENZYME ACTIVITY</u>	<u>SEM</u>	<u>P_c</u>
0	107,44	3,666	
35	98,42	4,522	0,3
70	115,3	3,153	0,2
140	108,07	6,16	0,9
180	115,3	7,091	0,5
240	124,6	6,087	0,1
360	139,4	3,465	0,001

Control (Alkali pH 10 in Saline Solution):-

<u>TIME (Minutes)</u>	<u>MEAN ENZYME ACTIVITY</u>	<u>SEM</u>	<u>P_c</u>
0	107,44	3,666	
35	140,3	16,69	0,2
70	116,3	4,96	0,3
140	114,8	6,296	0,3
180	103,3	4,024	0,3
240	101,3	9,55	0,6
360	122,3	3,408	0,05

N = 5

Significant difference between 360 minutes alkali and tryptophan = 0,02

Figure 10.

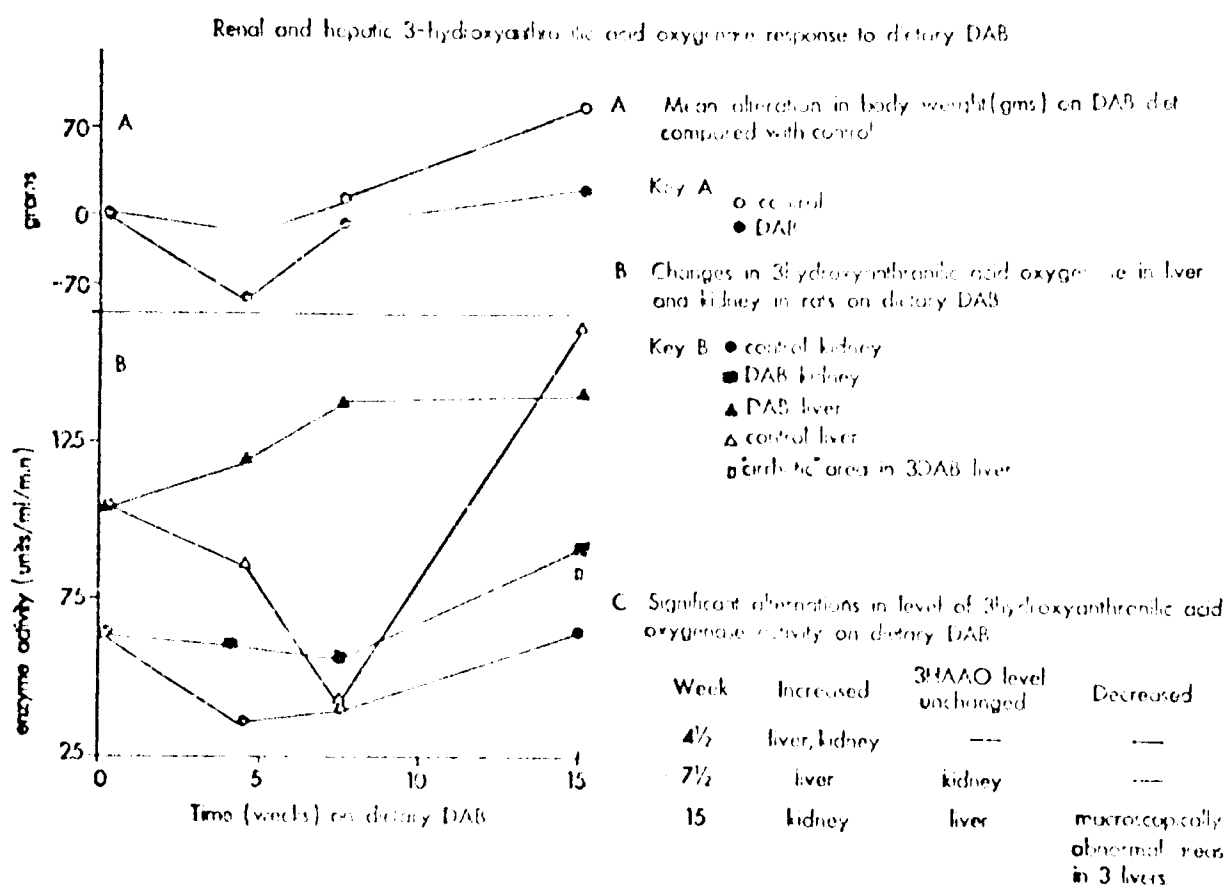
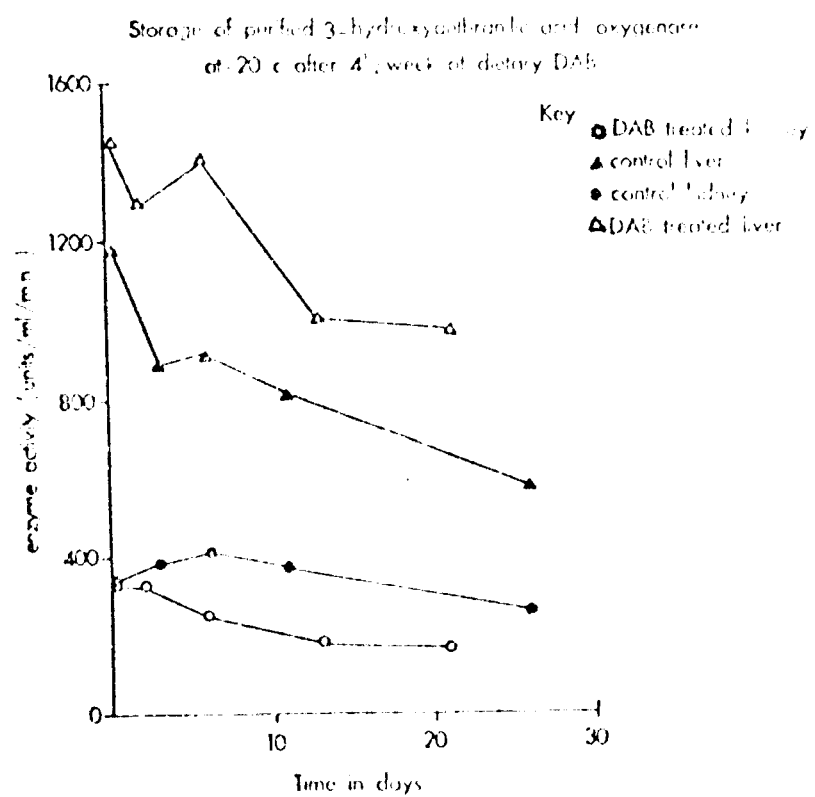


Figure 11.



THE EFFECTS OF PURIFICATION ON LIVER AND KIDNEY 3-HAAO:-

Remarks on the Technique Employed:-

Both liver and kidney show variable purification and yield. The stability on Sephadex gel filtration is especially variable, more activity tending to be lost with repeated use. Acetone proved a quicker, more difficult procedure ideal for bulk preparation where the increased loss of enzyme activity is adequately compensated for by the removal of contaminating proteins.

There is no direct correlation between per cent yield and purification - in a series of liver purifications stage 1 - 4 (p.16 Stage 2 was acetone fractionation) per cent yield varies between 11% (purification 45x) and 48% (purification 23x). In this series the maximum purification achieved by this technique is 84x (per cent yield 31). When ammonium sulphate is used in Stage 2, the results are similarly variable. In one case of kidney 3-HAAO purification (method on p.16) a per cent yield of 104% associated with a 40x purification was found. Furthermore, it is found that a good per cent yield and high fold purification achieved with the livers of a group of rats does not pre-suppose a similarly good result on purification of their kidneys.

Table 5 shows the effects of different doses of cortisol on the purification of liver and kidney 3-HAAO. In fact, pre-treatment with cortisol probably had little effect on the stability of this enzyme. It did, however, appear to sensitize hepatic 3-HAAO (3 mg cortisol / 100 gm body weight) and renal 3-HAAO (4,3 gm cortisol / 100 gm body weight) to activation by ammonium sulphate at 70% saturation. Table 5 also shows the per cent yield and purification achieved.

Table 5.

TABLE 5. PURIFICATION OF 3-OH-17β-ESTRADIOL IN VIVO COLLECTED FROM MALE RATS

STRAIN: SD Male Rats killed 140 minutes post intraperitoneal injection.

DOSE CORTISOL (mg / 100 gm body weight) 0

LIVER

Enzyme activity / gm wet weight liver				122,4				158,87				131,9			
STAGE OF PURIF.	TEA	SA	% Y.	PUR	TEA	SA	% Y.	PUR	TEA	SA	% Y.	PUR			
Initial Extr.	6 662	0,42	100	0	10 635	0,73	100	0	6 832	0,51	100	0			
Acetone fract.	2 332,5	3,64	35	5,9	5 070	4,88	47	6,7	2 470	3,79	36	7,4			
Sephadex G-100 (3,2 cc x 60 cm)	1 783,5	27,5	27	44,4	3 871,5	45,5	36	67	2 310	18,4	34	36			
70% (NH ₄) ₂ SO ₄															
Fractionation	1 176	44,27	18	71,4	2 415	191,5	23	262	1 589,5	26,3	25	51,6			
Enzyme activity / gm wet weight kidney				116,2				148,675							
STAGE OF PURIF.	TEA	SA	% Y.	PUR	TEA	SA	% Y.	PUR	TEA	SA	% Y.	PUR			
Initial Extr.	583,4	0,71	100	0	1 690,5	0,65	100	0	1 768,5	0,64	100	0			
(NH ₄) ₂ SO ₄															
Fractionation															
30%	1 197	0,9	74	1,3	1 218	1,01	71	1,5	1 268	1,18	72	1,8			
60%	1 062,5	0,97	67	1,4	1 020	1,03	66	1,6	1 275	1,39	72	2,2			
Sephadex G-100 (3,2 cc x 70 cm)	660	10,8	42	15					642	0,93	36	1,4			
70% (NH ₄) ₂ SO ₄															
Fractionation	412	10,84	27	27					452,4	19,5	26	30,6			

TEA - Total Enzyme Activity PUR - Times Purified SA - Specific Activity % Y. - Per Cent Yield

Figures 12 and 13 show activity profiles obtained from gel filtration of liver and kidney enzymes post cortisol treatment (doses 4,3 and 3 mg / 100 gm body weight) compared to their respective controls. Protein peaks are demonstrated by arrows on the corresponding activity elution profile. In the case of control kidney and 4,3 mg cortisol / 100 gm body weight liver, protein profiles failed to peak and presented instead as steadily decreasing protein concentrations. Liver (4,3 mg / 100 gm body weight) and kidney (3 mg / 100 gm body weight) differ from the other activity profiles in having double peaks.

DAB has little effect on the purification of 3-HAAO. Renal 3-HAAO does appear less stable after 7,5 weeks on a diet containing DAB - this could however be satisfactorily explained on the variability inherent in the purification procedure. Renal 3-HAAO activity and protein profiles are similar in control rats and those who have been subjected to 4,5 weeks of DAB in their diet. (Fig. 14). Figure 15 shows an activity peak in the liver of rats which received 4,5 weeks of dietary DAB. There is no corresponding protein peak.

Figure 12.

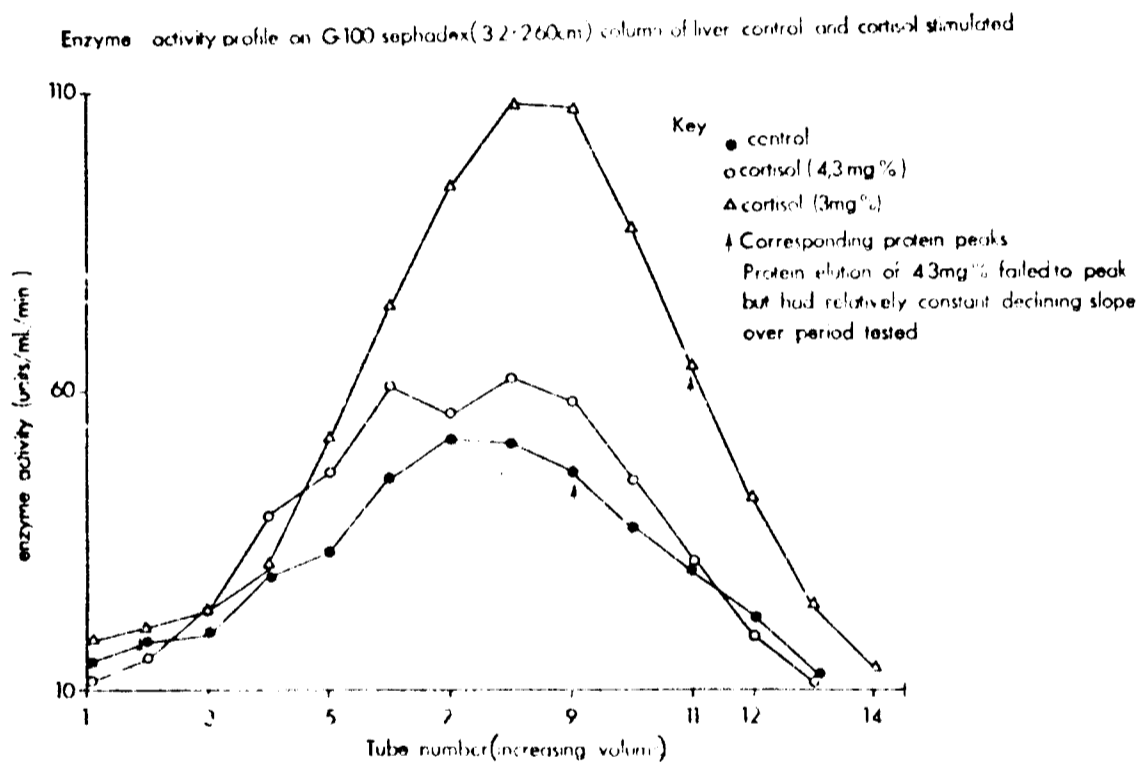


Figure 13.

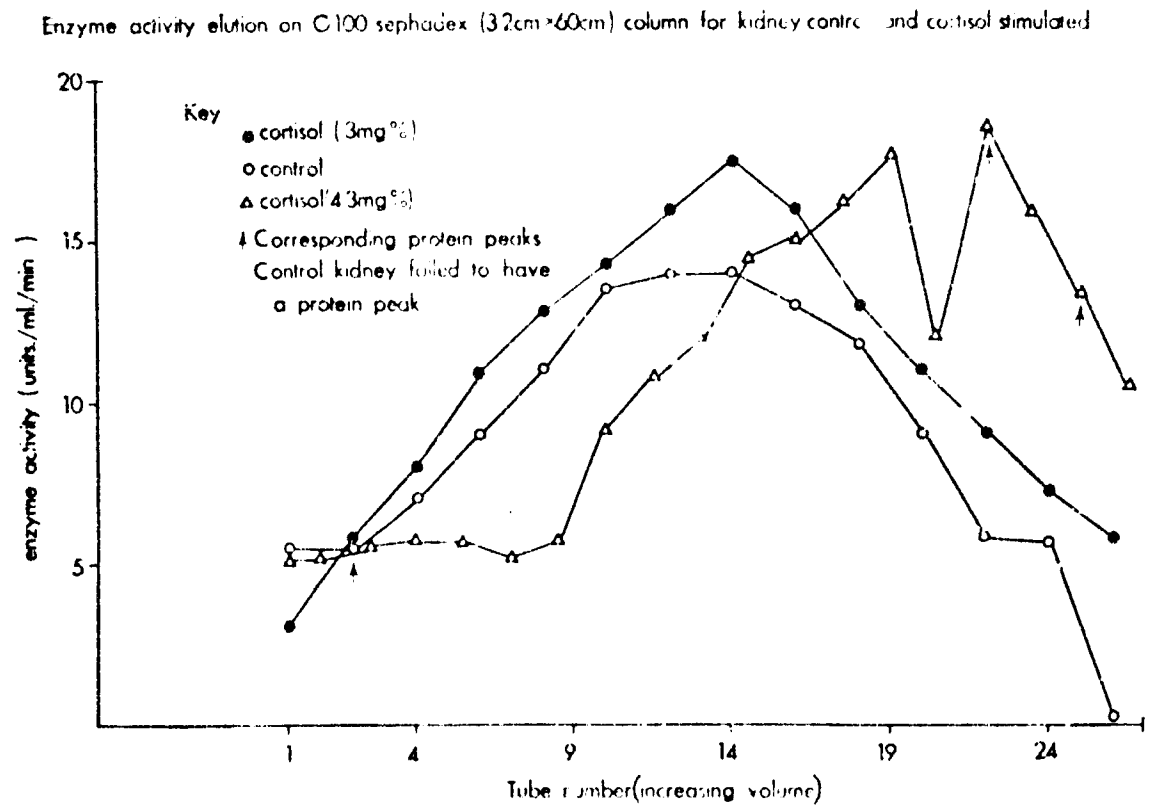
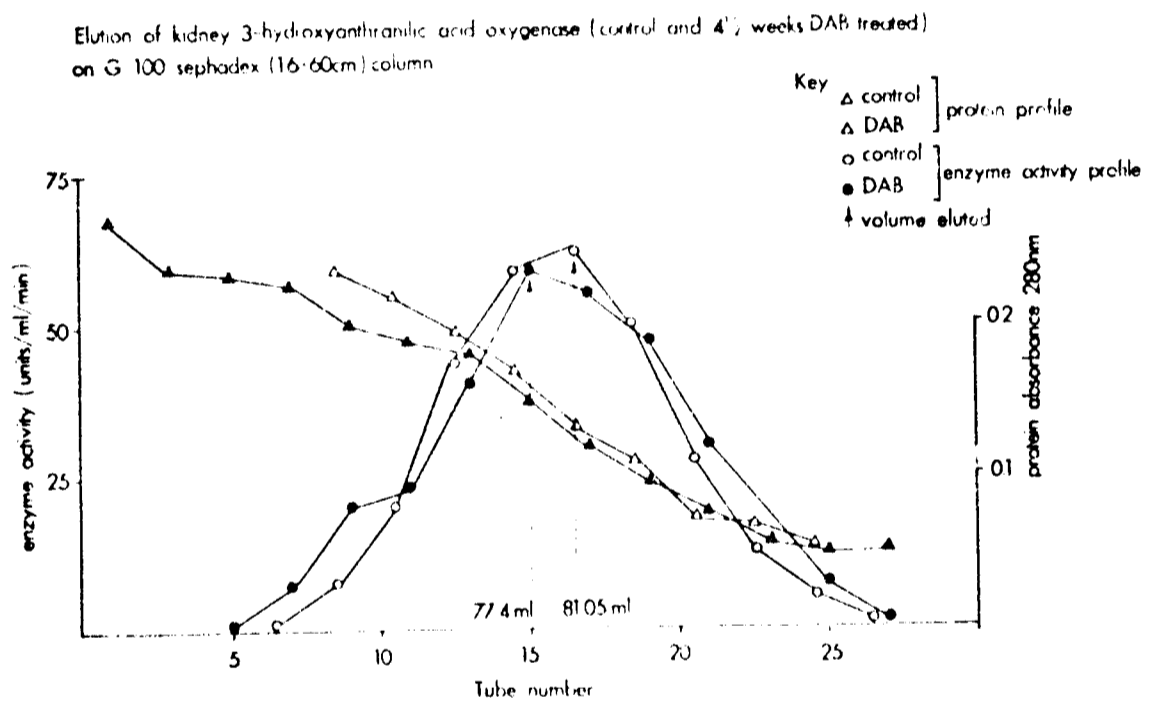


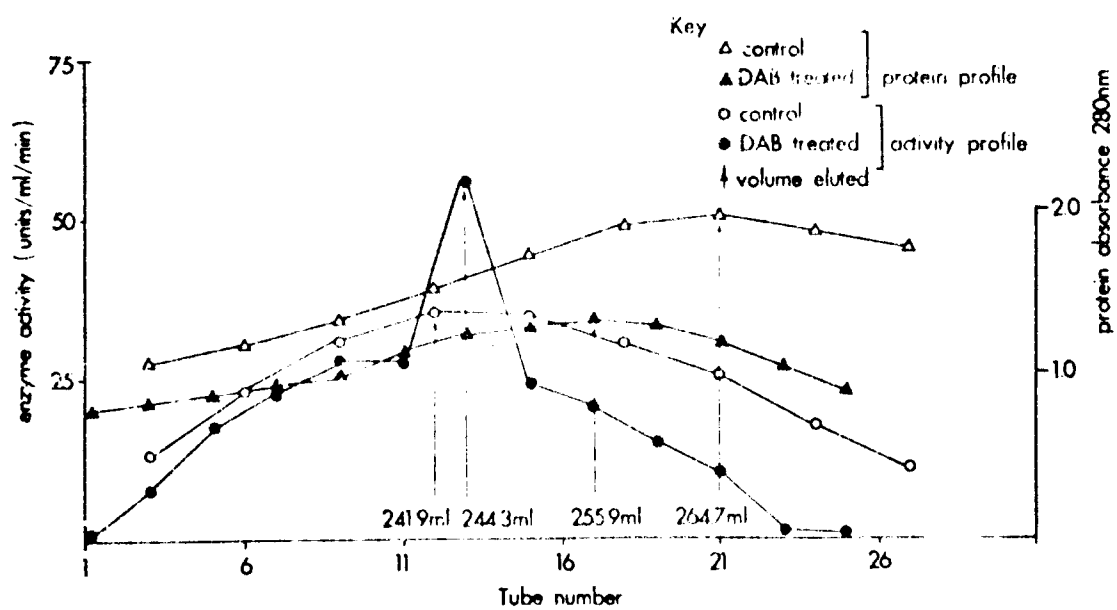
Figure 14.



Tubes represented selected on enzyme activity level, not on protein content.

Figure 15.

Elution of liver 3-hydroxyanthranilic acid oxygenase (control and 4½ weeks DAB treated) on G-100 sephadex (32·60cm) column



Tubes containing protein without enzyme activity not depicted

(C) RESULTS OF STORAGE OF 3-HAAO:-

(a) Temperature:-

Figure 16 shows the effects of 3-HAAO storage in liver and kidney homogenates at 4°C and 37°C for a period of 3 hours. Both enzymes proved more stable at 4°C than at 37°C. Theoretically enzymes are more stable at lower temperatures. When storage for a number of days was required it was decided to store the enzyme at -20°C. The effects of freezing and thawing on enzyme activity were not studied, it was, however, noted that enzyme left for over a week at 4°C lost all its activity.

(b) Choice of Storage Media:-

Figure 17 shows the stability of freeze dried purified liver enzyme on storage at -20°C. Fractions of this enzyme were dialysed prior to freeze-drying against either 0,3-M or 0,5-M ammonium acetate buffer. It became apparent that when freeze drying at 0,15-M molarity, dialysis becomes essential for enzyme stability. The dialysis step can be eliminated by using a 0,3-M solution buffer throughout. Storage of this enzyme in a soluble form in 0,15 and 0,3-M buffer, also supplementation of the 0,3-M solution by either 20% of 1 : 1 v/v or 4 : 1 v/v glycerol solutions is seen on Fig. 18. Except for storage in 1 : 1 v/v glycerol, all the storage procedures at -20°C were associated with an approximate 2/3 loss of activity over the 68 days tested.

A 1 : 1 v/v glycerol solution, although having a stabilizing effect on the enzyme, is a viscid mixture which may have undescribed kinetic effects on the enzyme.

Figure 16.

Stability of 3-hydroxyanthranilic acid oxygenase in liver and kidney homogenate at 4°C and 37°C

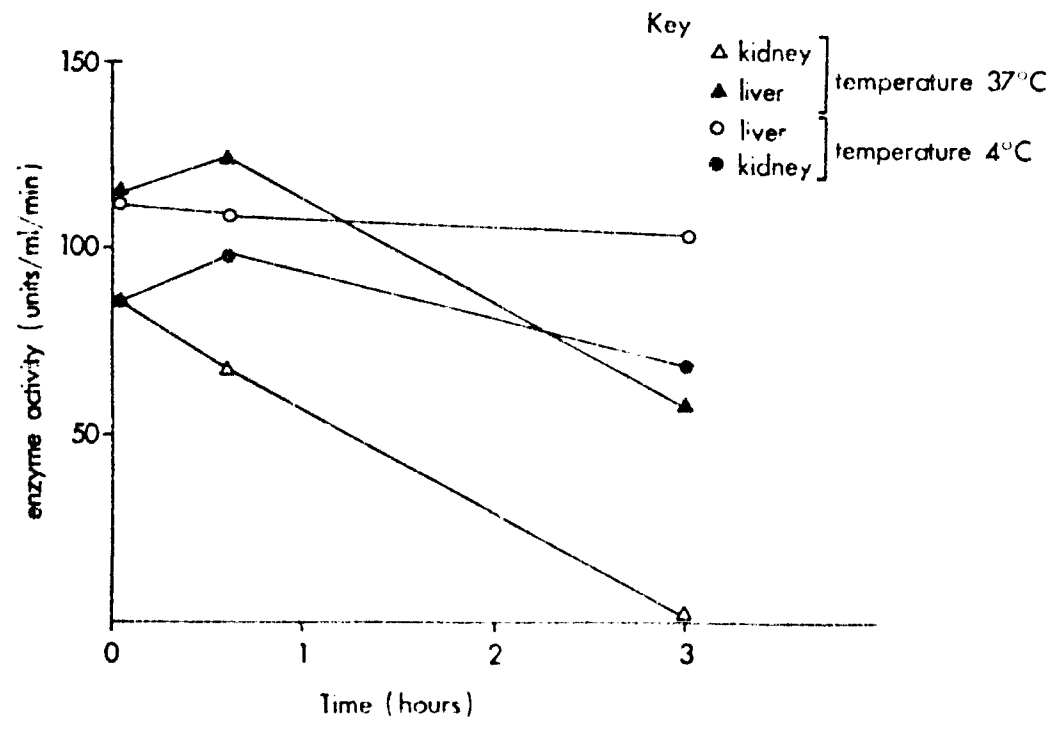


Figure 17.

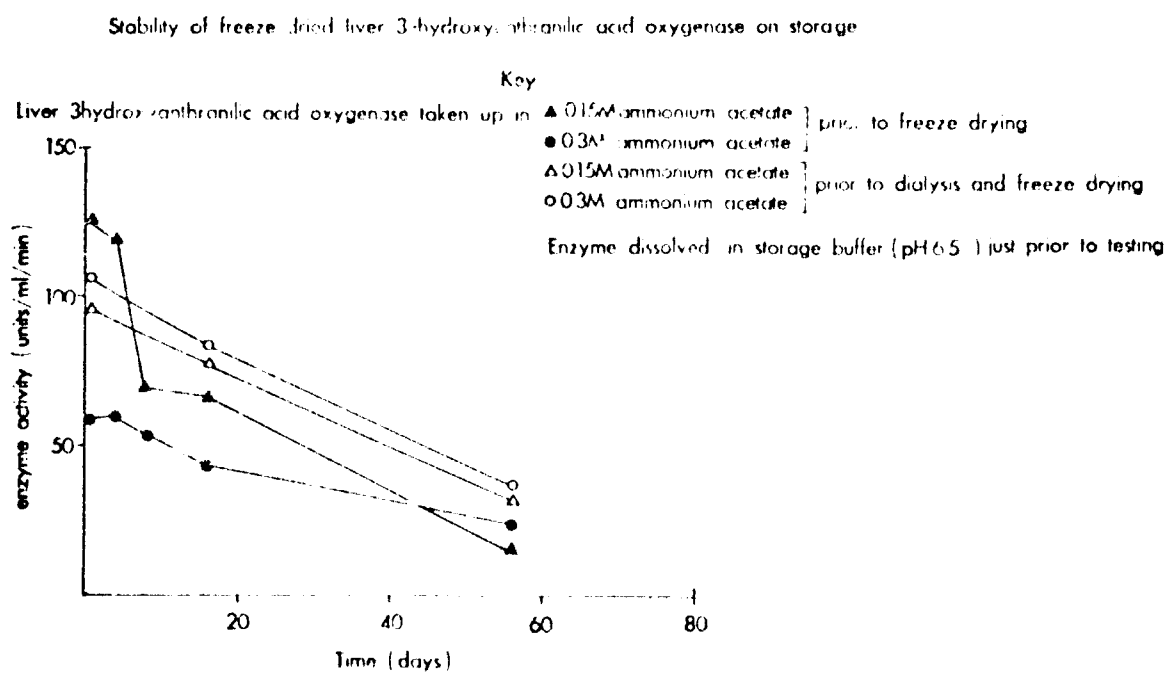
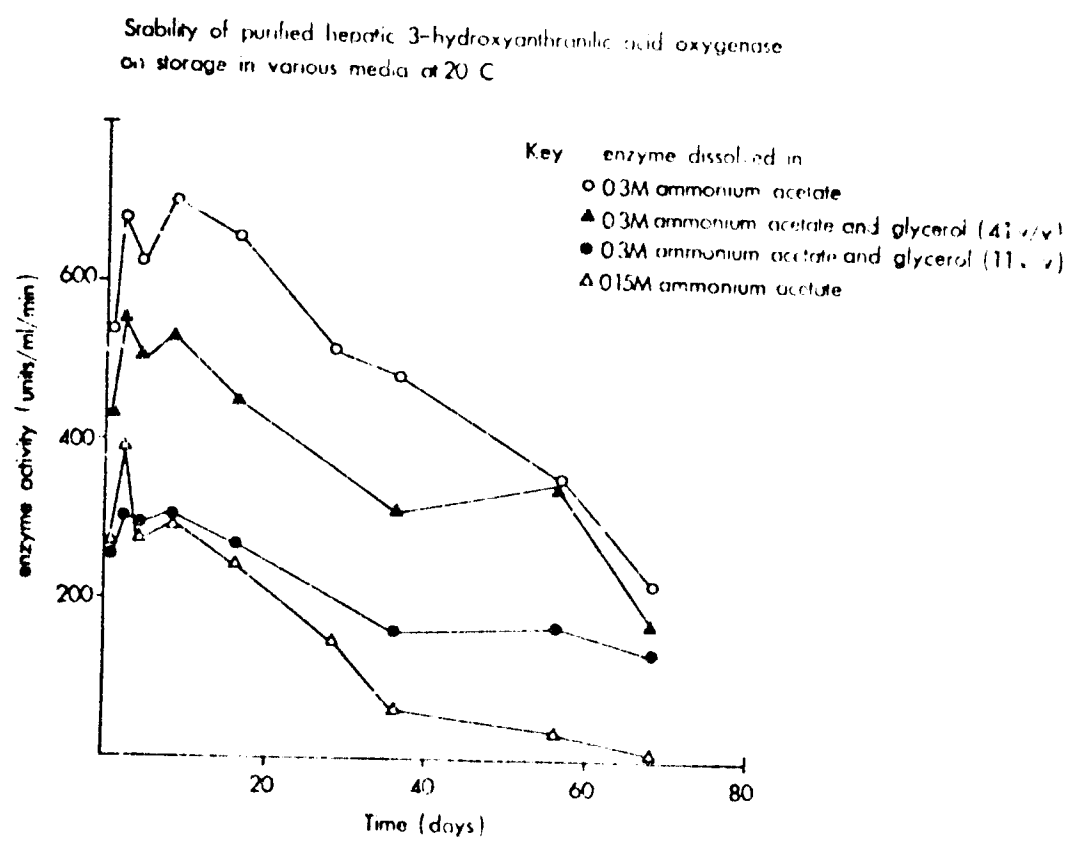


Figure 18.



(c) Stages During Purification at which the Enzyme may be Stored:-

Storage of the purified enzyme is obligatory, storage at certain intermediate stages is convenient. Figure 19 shows the stability of the purified liver and kidney enzyme at -20°C compared with these enzymes at the homogenate stage. The kidney homogenate is very unstable on storage at -20°C ; a preferable stage of storage proved to be as the 60% precipitate after ammonium sulphate fractionation. Figure 20 shows the increased stability of renal 3-HAAG at this stage compared to the homogenate (and the purified enzyme). Although it is possible to halt purification at Stage 2 (60% $(\text{NH}_4)_2\text{SO}_4$) it would still be more convenient in terms of the duration of the experiment, if the renal enzyme could be stored at the homogenate stage. An attempt was thus made to stabilize the enzyme at the homogenate stage by the addition of various additives. Additives which were added in vitro: Tryptophan and alkali incubated with the liver and kidney (homogenate) enzymes at 4°C and 37°C for 3 hours gave the following results - both enzymes were equally unstable in either additive at 37°C and neither appeared to confer any stability on the enzymes at 4°C . In vitro cortisol incubated with these enzymes at 4°C and 37°C for 6 hours suggested an increased renal stability. An attempt to confirm this by storing the enzymes with in vitro cortisol for 27 days at -20°C failed. No in vitro additive was thus found to stabilize either enzyme at the homogenate stage.

Figure 21 shows the effects of in vivo cortisol on storage of the renal enzyme at the homogenate stage. There is a dramatically enhanced stability of the enzyme as early as 35 minutes after injection. All the animals killed post cortisol (from 35 - 360 minutes) show this increased renal 3-HAAG stability at the homogenate stage. Liver 3-HAAG fails to show any change in stability over the period tested.

Figure 12.

Stability of liver and kidney 3-hydroxyanthranilic acid oxygenase on storage at 20 °C

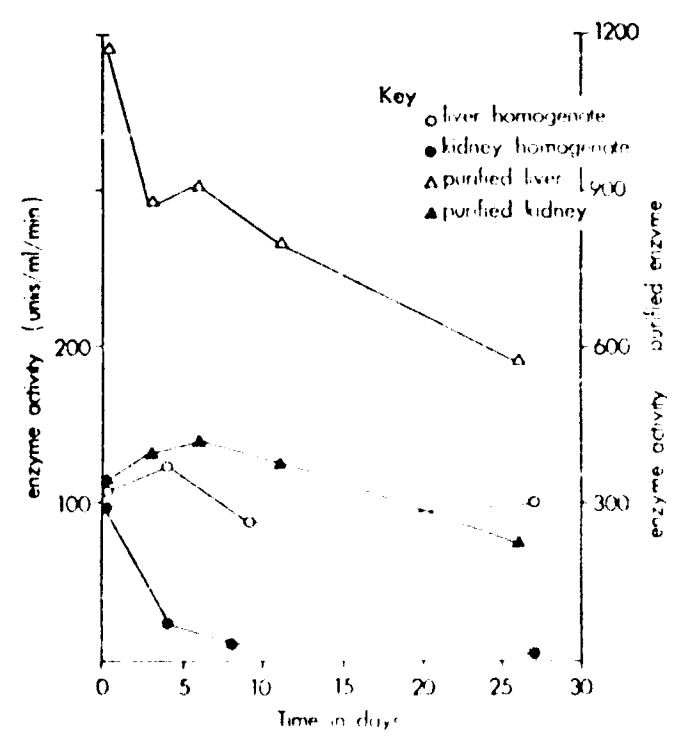


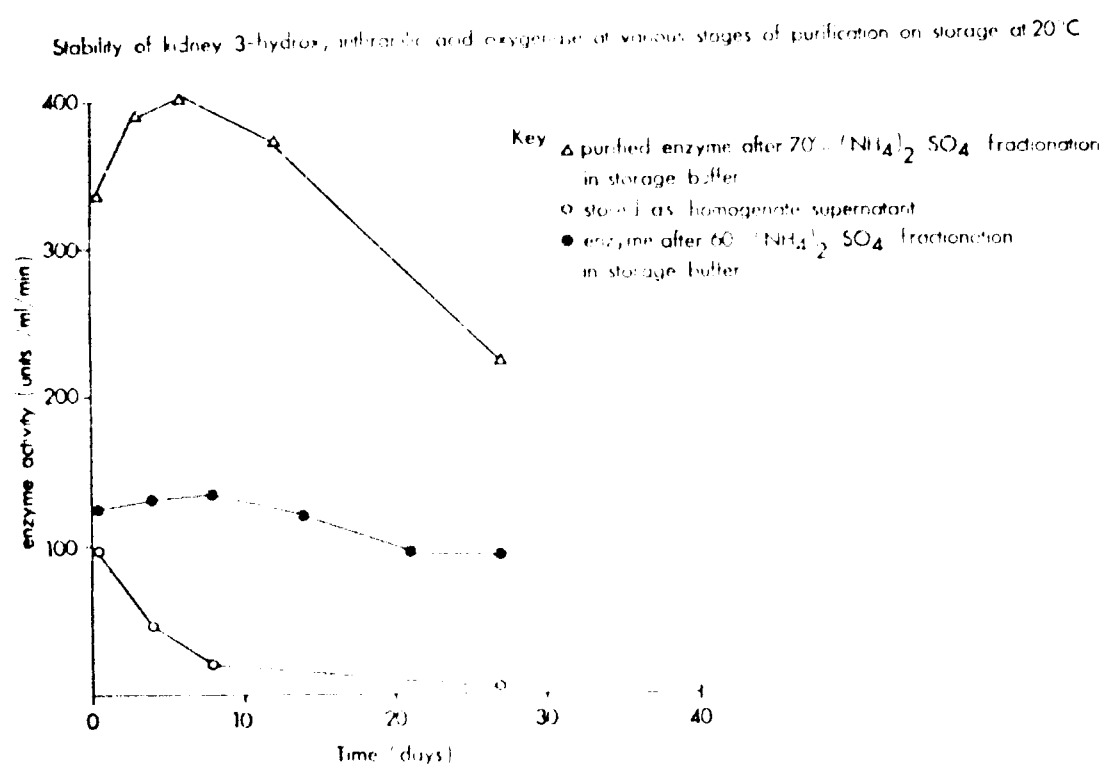
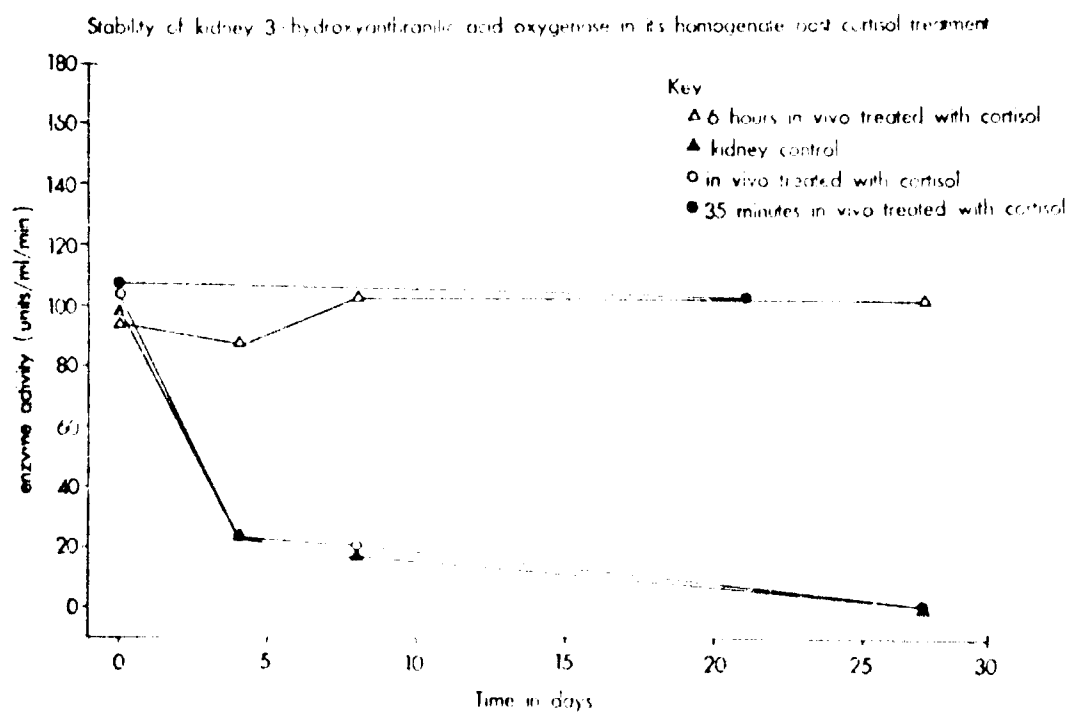
Figure 20.

Figure 21.



THE AVERAGE MOLECULE WEIGHT OF 3-HAAO FROM RAT LIVER AND KIDNEY:

(A) AS ESTIMATED ON GEL FILTRATION:-

Figure 22 shows the liver activity and Figure 23 shows the kidney activity and protein elution profiles obtained by filtration of purified GG rat enzyme through G-75 Sephadex in a 1,6 cm x 60 cm non-adjustable column. Rat liver enzyme has two protein and two enzyme peaks, only one of these protein-activity peaks coincides. The second enzyme peak is associated with a minimal rise in detectable enzyme activity and is not consistently reproducible. Owing to its doubtful significance it has not been considered in the interpretation of the results. The molecular weight of the enzyme is determined by plotting the log of the MW of the standards against the elution volume (V_e) (Fig. 24). Table 6 shows the elution volumes and the known and estimated molecular weights of proteins used in this experiment. The molecular weights of liver and kidney 3-HAAO are calculated in terms of both protein and enzyme peaks - these peaks coincide at 40 000 in the case of hepatic 3-HAAC and 33 500 in the case of renal 3-HAAO. Both enzymes have a similar protein peak, 30 500 in the case of liver and 31 500 in the case of kidney enzyme.

(B) AS ESTIMATED ON POLYACRYLAMIDE GEL ELECTROPHORESIS:-

Mobility is calculated as follows:-

$$\text{MOBILITY} = \frac{\text{distance of protein migrated}}{\text{length after destaining}} \times \frac{\text{length before staining}}{\text{distance of dye migrated}}$$

(Weber and Osborn, 1969).

Figure 22.

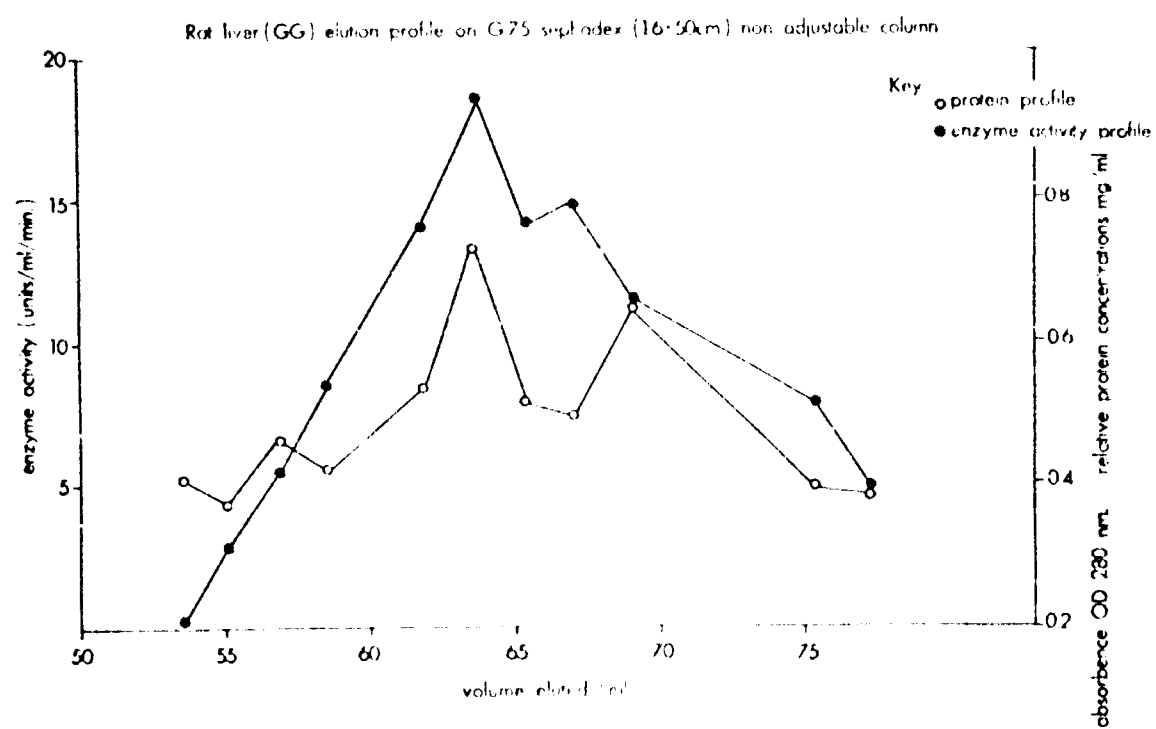


Figure reflects only those protein concentrations which are associated with enzyme activity

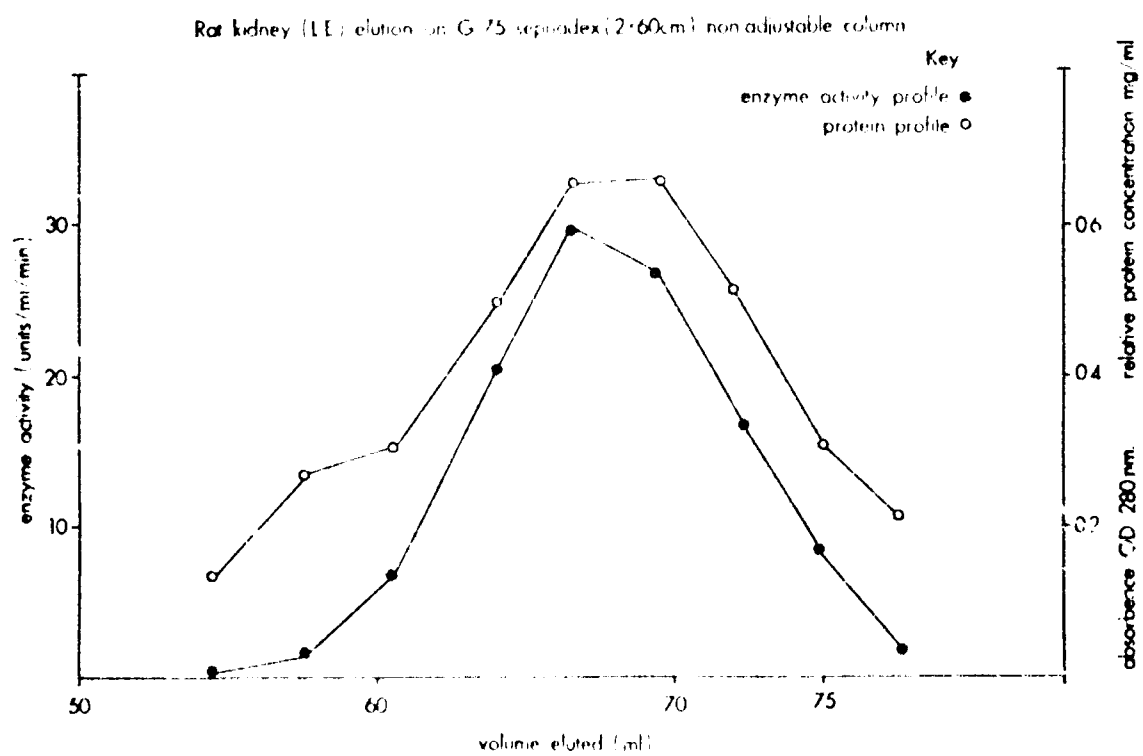
Figure 23.

Figure represents only those protein concentrations which are associated with enzyme activity

Figure 23.

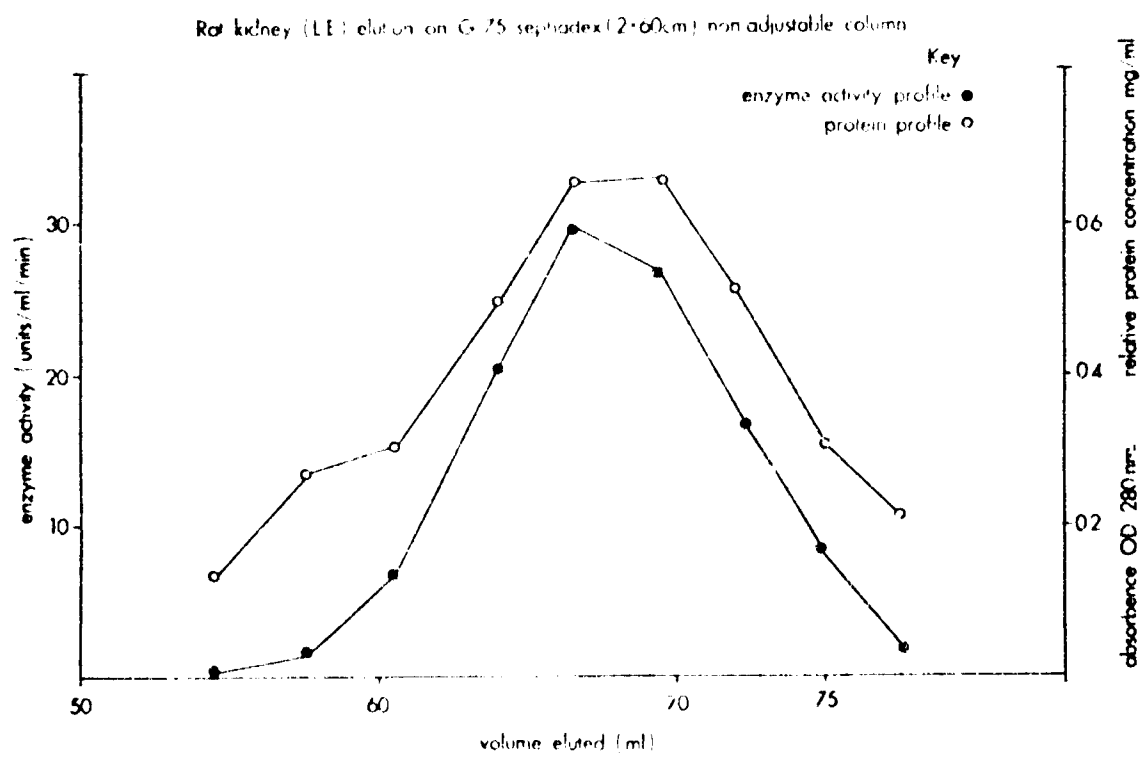


Figure represents only those protein concentrations which are associated with enzyme activity

Figure 24.

Molecular weight of 3-hydroxyanthranilic acid oxygenase
calculated on G-75 sephadex (2.60cm) non adjustable column

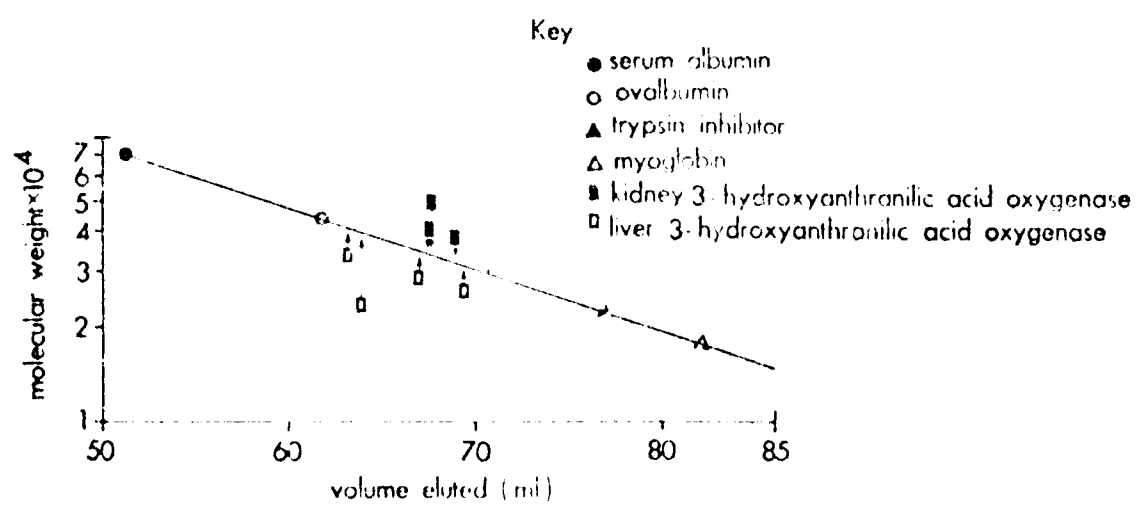


TABLE 6: MOLECULAR WEIGHT AS DETERMINED ON A NON-
ADJUSTABLE G-75 SEPHADEX COLUMN (1,6 cm x 60 cm).

	<u>M.W.</u>	<u>VOLUME FLUTED</u>	
Serum Albumin	68 000	51,25	
Ovalbumin	43 000	61,82	
Trypsin Inhibitor	21 500	76,42	
Tryptophan	17 400	82,055	
Liver (GG)	40 000	63,3	Protein + enzyme peak
	34 000	69,5	Enzyme Peak*
	30 500	69,5	Protein Peak
Kidney (LE)	33 500	66,69	Protein + Enzyme Peak
	31 500	63,4	Protein Peak

*Probably not significant

Figure 25 shows mobility plotted against the log of molecular weight. Both liver and kidney gave a number of bands on polyacrylamide gel - of these the major band of liver was at 31 000 molecular weight fragment, kidney had 4 'major' bands molecular weights ranging between 10 500 and 44 000. Table 7 shows the number of bands, the mobilities and the molecular weights (known and estimated) on polyacrylamide gel. Prolonged incubation in SDS results in an increased number of bands in both standard protein and 3-HAAO solutions. The problem with denaturing polyacrylamide gel electrophoresis is that there is no indication if the band stained has activity or not.

An attempt was made to correlate protein bands with activity by running the enzyme at 4°C as described on pages 20 and 21.

Figure 25.

Molecular weight determination of 3-hydroxyanthranilic acid oxygenase by dodecylsulphate polyacrylamide gel electrophoresis

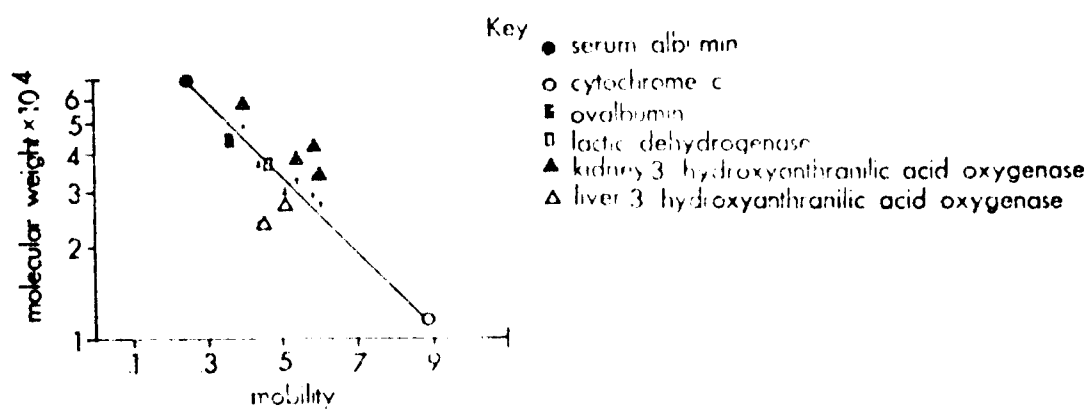


TABLE 7: MOLECULAR WEIGHT DETERMINATIONS ON DENATURING POLYACRYLAMIDE GEL ELECTROPHORESIS.

<u>SUBSTANCE</u>	<u>NUMBER OF BANDS</u>	<u>WELL DEFINED BANDS</u>	<u>MOTILITIES</u>	<u>MOLECULAR WEIGHTS</u>
Liver 3-HAAO	6	1	0,5279	31 000 (Range 11 000 - 54 000)
Kidney 3-HAAO	8	2 - 4	0,3848	44 000
			0,5412	29 000
			0,5980	25 000
			0,8829	10 500
				(Range 10 500 - 44 000)
Serum Albumin	1	1	0,247	68 000
Ovalbumin	1	1	0,3566	43 000
Lactic				
Dehydrogenase	1	1	0,4631	36 000*
Cytochrome C	1	1	0,872	11 700

*Under natural conditions this exists as an oligomer (Weber and Osborn, 1969).

In non-denaturing electrophoresis, both charge and pore size play a role (Hendrick and Smith 1969). No attempt was thus made to estimate the molecular weight. Protein and activity staining on complementary gels either overlapped or coincided. The renal activity band appears to move slightly ahead of the liver 3-HAAO band.

In summary, it appears that the molecular weight of enzyme from liver may be a little higher than that obtained from kidney. To account for the number of bands on electrophoresis and the protein and activity peaks on gel filtration, one may consider multiple forms of the enzyme (only some of which are active) or sub-units may be present. Before any definitive comparison between the molecular weights of these two enzymes can be made, one should determine that the forms of the enzyme studied are comparable.

THE 'N' TERMINAL AMINO-ACID OF THE ENZYME

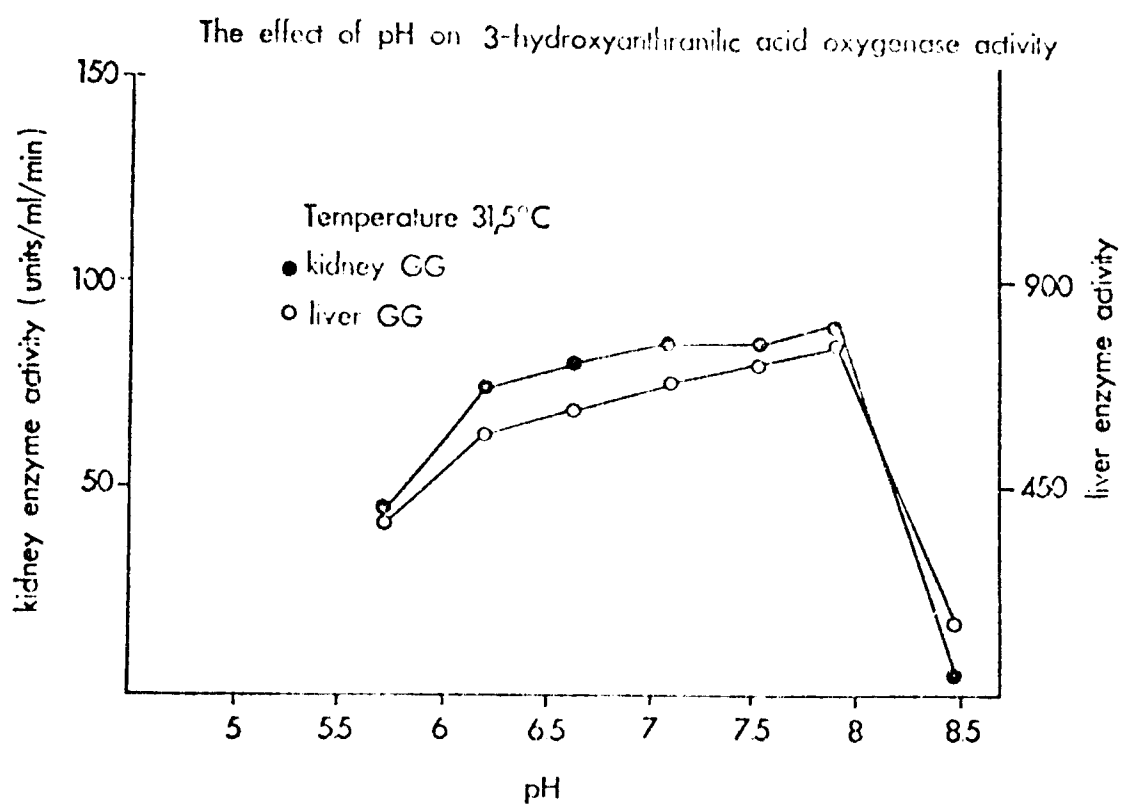
Both liver and kidney enzymes revealed 3 spots under UV light - these were identified as o-tyrosine, ϵ -lysine and an artifact dansylamine. Failure to detect an 'N' terminal amino acid could either be due to masking of that amino group or to destruction of the amino acid during dansylation and hydrolysis (Seiler 1970).

THE CATALYTIC SITE:-

(A) pH Optimum:-

Figure 26 shows the activity of liver and kidney 3-HAAO (purified GG strain) at pH's varying between 5,5 and 8,5. The overall pattern displayed by the two enzymes is similar - the peak of activity being at pH 7,9. The temperature was maintained at 31,5°C.

Figure 26.



(B) Temperature Optimum:-

Figure 27 shows the activity of purified liver and kidney 3-HAAO (GG strain) over a temperature range from 31°C to 43°C. The activity of both enzymes increases over the period tested;

(C) Michaelis Constant:-

This is determined at a pH of 7,6 and at a temperature of $29 \pm 0,5^{\circ}\text{C}$. The K_m obtained for liver 3-HAAO is $1,82 \times 10^{-5}$. In vivo cortisol or 7,5 weeks of DAB in the diet did not alter this result (Fig. 26). Figure 28 also shows the more variable results obtained for kidney on testing the K_m of 3-HAAO under similar conditions.

THE ANTIGENIC SITE:-

The controls - i.e. serum taken from both rabbits prior to injection and serum taken from the control rabbit at intervals equivalent to the antibody producing animal - all failed to react on exposure to the antigen. This is true for both the gel diffusion and titration techniques.

(A) Gel Diffusion:-

The antibody reacted with liver and kidney antigen of both species as shown diagrammatically in figure 29. The enzyme isolated from DAB and cortisol treated was still capable of this interaction. In all cases a single precipitation line with no spur formation was produced - figure 30 demonstrates the precipitation line between the GG liver antibody and SD kidney antigen.

Figure 27.

Effect of temperature on the catalytic activity of 3-hydroxyanthranilic acid oxygenase

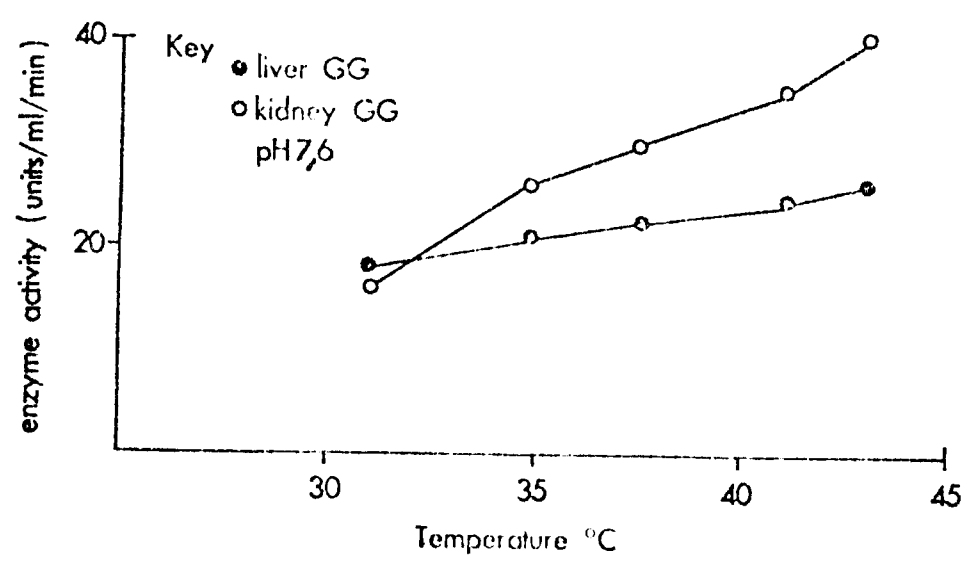


Figure 28.

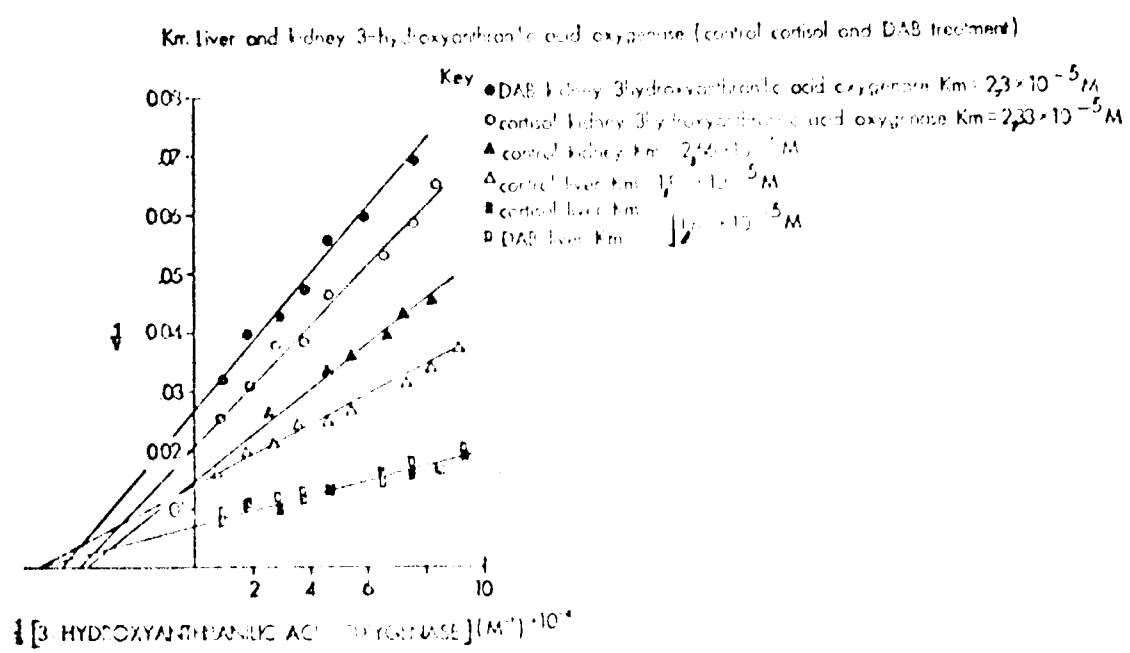
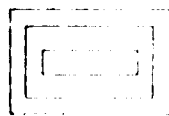
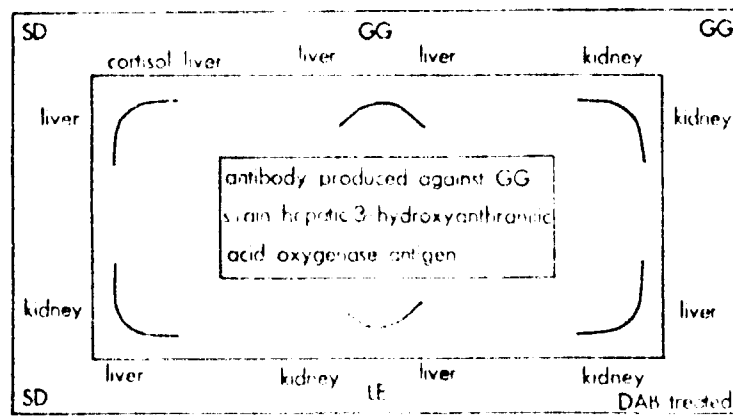


Figure 29.

DIAGRAMMATIC REPRESENTATION OF ANTIBODY-ANTIGEN PRECIPITATION ON GEL DIFFUSION



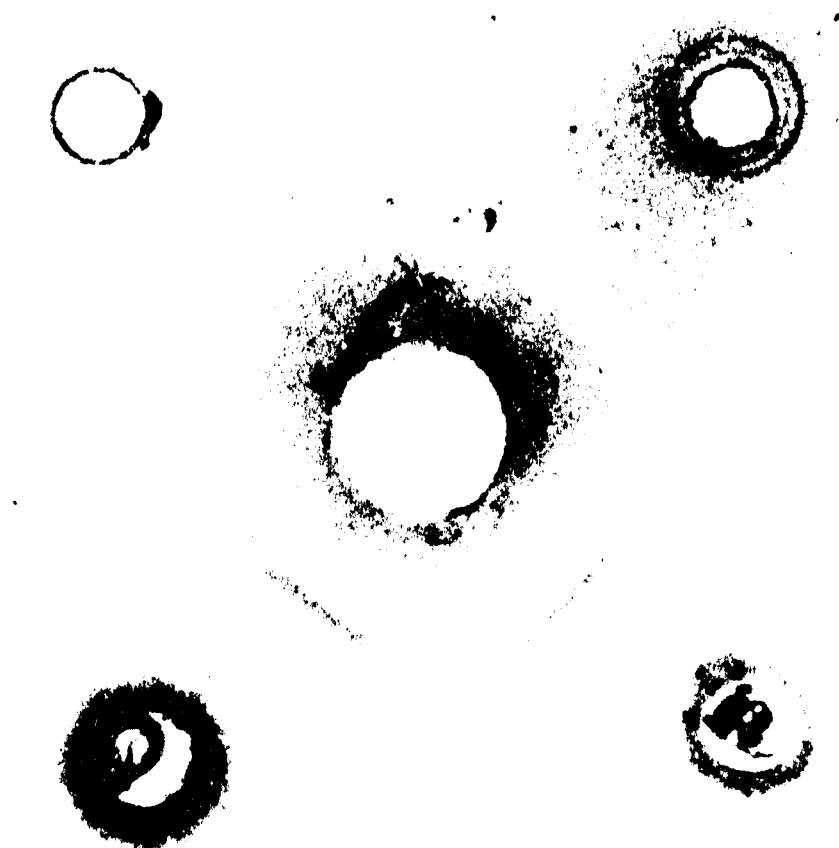
The variety of antigens reacted against the antibody

Antibody-antigen precipitation line

- single line in all cases
- no spur formation between adjacent pairs

Figure 30.

Precipitation of the antigen-antibody complex between the antibody central wells and the antigen (in the lower two wells) can be seen.



The absence of precipitation lines does not exclude the presence of a complementary antigenic binding site, the presence of a precipitation line is synonymous with the presence of complementary antigen and antibody binding sites on the proteins tested.

(B) Antibody - Antigen Titration:-

The results obtained in attempting to elucidate the mode of action whereby cortisol elevates the level of 3-HAAO were disappointing. No information was obtained which pointed to induction of the enzyme and scant information for activation was present.

The only positive results obtained in this experiment were to confirm the results obtained on gel diffusion - i.e. that the antibody - antigen reaction between liver stimulated antibody and liver and kidney antigen does occur.

A DAB - 3-HAAO COMPLEX:-

A sulphur containing amino acid is implicated in the binding of the azo dye - both methionine and cysteine have been implicated (Ketterer and Christodoulides 1968). Savage (1973) showed SH groups present in 3-HAAO of baboon liver. An attempt was made to demonstrate the presence of a DAB - 3-HAAO complex in the purified enzyme preparations (p.26).

Addition of formic acid directly to the purified enzyme of both liver and kidney of DAB fed (4,5 and 7,5 weeks) animals failed to produce any pink coloration of the solution. Trichloroacetic acid when added to DAB treated enzyme in polyacrylamide gel also produced no evidence of DAB being present in the purified enzyme solution.

D I S C U S S I O N

(A) THE POST-NATAL DEVELOPMENT OF 3-HAAO IN RAT LIVER AND KIDNEY

The development of rat 3-HAAO is influenced by both nuclear and cytoplasm determinants. Certain of these influences are clearly delineated, others are open to surmise.

(a) The Genetic Aspects:-

1. The structural Gene:-

The presence of an active structural gene is synonymous with the presence of its product. 3-HAAO is known to have structural genes in liver and kidney. These are active by 7 days after birth and remain so into adult life.

2. Architectural Genes:-

The enzyme is found in the cytoplasm of both organs. The techniques employed cannot exclude its presence from other sites. Dollneret *et al.* (1966) found an association between the site of an enzyme within a cell and the "over shoot" phenomenon unlikely. The architectural gene is probably not responsible for the "over shoot" demonstrated in 3-HAAO.

3. Regulatory Genes:-

Any single assay of enzyme activity reflects not only the rate of enzyme synthesis, but also the rate of enzyme catalysis (Correll *et al.* (1965). The most probably explanation for the over shoot phenomenon observed in male liver and female kidney is an imbalance within the regulatory gene. Both the catalytic (Ganschow and Schinke (1969) and the synthetic genome (Doyle and Schinke (1969) have been shown to be individually responsible for enzyme levels within the liver. The actual locus controlling 3-HAAO level within the liver and kidney remains obscure. The over shoot phenomenon may either

be due to a delayed development of this locus, or the locus may change with increasing maturity.

There is no apparent acute requirement for nicotinic acid at 59 days in the male liver or at 4 months in the female kidney. Besides, Young (1967) has found that the excessive increase in the activity of one enzyme is not necessarily co-ordinated with an increase in functionally related enzymes. The excessive increase of 3-HAAO probably manifests a genetic rather than a homeostatic response. Moog (1971) remarked that enzymes which undergo a large increase in activity frequently temporarily rise above their adult level before stabilizing. The relatively high specific activity found in male liver at 38 days, followed by the excessive level of measurable 3-HAAO at 59 days, could be interpreted as a large increase in the productivity of this enzyme's synthetic genes during this period.

4. Temporal Genes:-

The levels of enzyme activity are predicted by the temporal gene. Short term changes are superimposed on these predictable levels via factors which affect the ratios between the loci of the regulatory gene.

The temporal development of the enzyme within male and female kidney is synchronous. Liver is out of step. Liver which is exposed to a higher concentration of molecules is more prone to short term adaptation than kidney.

(b) A Cytoplasmic Aspect:-

Cytoplasmic factors may influence enzyme expression at either the level of transcription or translation (Hanninen (1971)). Cortisol is known to combine with hepatic cytoplasmic receptors - certain of these cortisol receptor complexes have later been located in the nucleus (Feilgelson and Feilgelson (1972)).

The role of cortisol in the development of 3-HAAO is discussed

The Pituitry-Adrenal-Liver Axis:-

The pituitry in the young rat does not respond to stress before at least 8 days after birth; the adrenal responds to ACTH after about 4 - 6 days (Jailer 1950). Adrenal corticosteroid concentration increases until 185 days and thereafter stored steroid levels rapidly decrease. Joost (1966) concluded that foetal liver has an early ability to respond to steroid hormones. In the neonate it takes only 24 hours before the hepatocyte is capable of taking up and binding cortisol with almost adult efficiency. By 11 days cortisol is concentrated in rat liver with adult dexterity. There is, however, a difference within the cytosol. Between 16 and 39 days cortisol binders II and III defined by Morey and Litwack (1969), Singer and Litwack (1971) reverse their ratios. By 39 days the hepatocyte binders are identical to those found in the adult (Singer and Litwack 1971). Inducibility of hepatic tyrosine amino transferase parallels the shift in cortisol binding pattern.

It would thus appear that the pituitry-adrenal liver axis is intact as early as 18,5 days, and that by 39 days the ability of the liver to bind cortisol reaches maturity. The pattern of development imposed upon 3-HAAO in both liver and kidney prior to 18,5 days continues uninterrupted until well past 39 days of age. It seems unlikely that the development of this enzyme awaits the maturity of the pituitary-adrenal-liver axis.

Failure of Cortisol to Induce 3-HAAO:-

At 15,5 weeks 3-HAAO in male rat liver resists induction by cortisol. Possibly the cortisol is being bound by transcortin which has a higher affinity for cortisol than the hepatic dehydroxy system (Sandberg and Slaunwhite 1963). The hepatic dehydroxy steroid dehydrogenase system (Burton and Jeyes 1968) may also play a role in this resistance to cortisol. Haining and Correll (1969) found that tryptophan pyrrolase responded better to induction between 6 and 12 months, than at either 1 or 24 months. Possibly a similar mechanism underlies the absence of response at 15,5 weeks and the significant response at 8 months of 3-HAAO. This mechanism may well involve gene potential.

Cortisol as an Explanation for the Sexual Differences in 3-HAAO Development in Liver:-

For the first 20 days of post-natal life, plasma steroid levels increase simultaneously in both sexes (Decker et al. 1965). Transcortin is of similar concentration until 30 days, thereafter the female levels increase (Gala and Westphal 1965). Steroid bound to transcortin is biologically inactive (Slaunwhite et al. 1962). Prior to 26 days corticosteroids are metabolized in a similar manner by both sexes - after 32 days males produce metabolites B and D (Singer and Litwack 1971). In summary, the sexual discrepancies of steroid plasma binding of steroid concentration and of hepatic steroid metabolism occur after 20 days and are established by 32 days of age. The sexual difference in hepatic 3-HAAO development precedes the sexual discrimination of cortisol metabolism.

Cortisol completely fails to account for the sexual difference in 3-HAAO development. These differences ma,

possibly, partially be attributed to the hepatic microsomal enzyme system.

At 4 weeks male and female hepatocytes are equally capable of metabolizing hormones, drugs and other molecules. By 6 weeks the male hepatocyte is more efficient (Conney 1967). Possibly the less efficient detoxification by the female hepatic microsomal enzyme system results in a prolonged stimulation by effective molecules. This may explain the relatively high levels of this enzyme in the female liver after 2 months of age.

The sexual difference in renal 3-HAAO is one of quantity. Both the aetiology and the function of the raised level of 3-HAAO in the male kidney remain obscure.

The development of the genetic apparatus of 3-HAAO has been monitored via measurement of the cytoplasmic level of this enzyme. Prior to maturity, cortisol has no detectable influence on the genetic expression of 3-HAAO.

(B) LIVER 3-HAAO VERSUS KIDNEY 3-HAAO:-

Enzymes performing similar biochemical functions may occur in different forms. They may have different physicochemical or adaptive properties. The former may be conferred on the enzyme at the level of the structural gene, the latter may result from the physicochemical properties or be due to different internal environments.

(a) A consideration of the Physico Chemical Properties of 3-HAAO:-

The structural gene determines the amino acid sequence of a protein. Alteration of a single base within the

structural gene may result in the production of isoenzymes. Certain specific areas of 3-HAAO have been considered -

(i) The Catalytic site and the influence of pH, temperature and substrate:-

No obvious differences were found.

It must be recalled that the active site probably consists of groups separated by peptide bonds or even situated on different chains (Boyer 1960). The similarity between liver and kidney's response therefore reflects a similarity in structure which is not confined to a single amino acid. Liver and kidney 3-HAAO has been found to be isodynamic in this study.

(ii) The Antigenic Site:-

The rabbit produced a single detectable antibody to liver 3-HAAO. The probability that the purification techniques are adequate is increased and kinetic studies become meaningful. Liver and kidney have a similar antigen site

(iii) The 'N' Terminal:-

Neither enzyme has an 'N' terminal detectable by means of dansylation.

(iv) Molecular Weight Determination:-

Multiple bands were observed on polyacrylamide gel separation of both liver and kidney. More than one peak of protein and activity were detected on Sephadex filtration. These factors suggest the presence of possible isoenzymes of sub units or of variably active forms of the same enzyme.

(v) The Stability of 3-HAAO:-

On purification and storage 3-HAAO, proved unstable. Except for renal 3-HAAO on storage at the homogenate stage, both liver and kidney enzymes display a similar stability

on storage and also on purification. Renal 3-HAAO's stability as a homogenate is markedly improved after in vivo cortisol treatment. Hepatic 3-HAAO at a similar stage of purification is inherently more stable - in vivo cortisol does not further enhance this stability. The question as to whether this is an organ specific difference or an enzymatic dissimilarity arises.

(b) The Internal Environment of Liver and Kidney 3-HAAO:-

The liver is recognized as a more rapid protein synthesizing organ than other tissues (Bellman 1963), tryptophan is however probably not concentrated by liver as Civen and Knox (1959) found, the concentration of free tryptophan is only slightly higher in liver than in plasma. Steroids non-specifically increase the level of free amino acids in both liver and kidney (Noall et al. 1957). Specific interaction between substrate and cozymes is influenced by other intracellular factors (Yielding and Tonkins 1962). The dissimilar functions of liver and kidney suggest different internal cellular environments in the two organs.

The ability of steroids to influence the protein synthesis of enzymes is probably confined to interaction within target organs. Endocrine products accumulate in target organs and are effective in triggering concentrations (Szego 1971). Target organs selectively bind steroids to cytosol receptors, these mobile receptors transfer the steroid to the nucleus (Hechter and Soifer 1971). Not only does the target cell concentrate the steroid, e.g. cortisol is concentrated by the hepatocyte, (Litwack and Baserga 1967), it transports the hormone to the nucleus, e.g. aldosterone to the kidney cell nucleus, (Jensen and Jacobson 1962), cortisol to the hepatocyte nucleus (Beato and Feilgelson 1972). Kidney and Liver both have the ability to act as target organs - they both are capable of protein synthesis in appropriate circumstances.

Non-target organs do have their cytoplasm exposed to hormones but they differ from target organs in that they lack the ability to transport the hormone to the nucleus (Boulieu 1971). Non-target organs do not develop a nuclear concentration of the hormone (Stumpf et al. 1971), and protein synthesis becomes unlikely.

In vitro cortisol fails to produce any effects on 3-HAAO - in vivo cortisol does effect this enzyme. This suggests that cortisol either requires a viable genetic apparatus or that one of cortisol's metabolites is the active component. The latter is more likely to apply as kidney is not recognized as a target organ of cortisol. Renal 3-HAAO is stabilized by in vivo cortisol. The exogenous dose of cortisol that stabilized renal 3-HAAO had no effect on the stability of liver 3-HAAO. The inherently enhanced stability of liver 3-HAAO may be achieved by endogenous cortisol - it is difficult to assess the true concentration of a hormone at its site of action (Viltee 1961). It is suggested that the apparently different response of liver and kidney 3-HAAO is a dose-dependent phenomenon explained on a target organ response.

At the present level of investigation, all that can be stated is that liver and kidney 3-HAAO are heterozygous i.e. they are isodynamic but are derived from different organs.

(C) 3-HAAO - AN ADAPTABLE ENZYME

(a) Cortisol and 3-HAAO:-

There are basically three levels at which enzyme regulation may occur (Yielding 1971):-

1. Modification of the activity / efficiency of the enzyme. This occurs when tryptophan pyrrolase is

exposed to tryptophan (Greengard and Feilgelson 1961). This enhanced catalytic power is achieved at a cytoplasmic level.

2. The availability of enzyme substrate and the removal of products.

This is the basis of the 'mass action' theory of enzyme adaptation. (Mandelstam 1952).

3. Changes in the concentration of the enzyme as determined by synthesis and degradation.

Cortisol increases the synthetic rate of tryptophan pyrrolase, tryptophan decreases its rate of degradation (Schimke et al. 1965). Both an adaptive increase in enzyme synthesis or a decrease rate of degradation are defined as enzyme induction.

Hormones are known to modify genetic expression at the level of transcription (nucleus) and translation (cytoplasm) (McKerns 1969). Hormones thought to influence NAD metabolism include adrenal, thyroid, female gonadal and growth hormone (Greengard 1965). In this study, the effects of the adrenal glucocorticoids on one of these enzymes is considered. Glucocorticoids increase the level of tryptophan pyrrolase via increased protein and m-RNA synthesis (Masuda and Duncan 1971). The effects of glucocorticoids on 3-HAAO have not previously been described.

(b) Cortisol Acting at the Cytoplasmic Level:-

Cortisol accumulates mainly in the cytoplasmic compartment of the hepatocyte (Litwack et al. 1963 and 1965, Litwack and Baserga 1967, Morey and Litwack 1969).

Acting at a cytoplasmic level, steroids are known to alter enzymatic structure by interfering with covalent bonds among sub-units, i.e. steroids are known to achieve a change in

tertiary structure (Yielding and Lockins 1962). Cortisol, acting as a ligand, is known to retard hydrolysis of certain proteins (Ryan 1973). Cortisol, although it fails to increase the level of activity in the kidney, stabilizes renal 3-HAAO on storage in the homogenate. Only in vivo cortisol elicits this response. This does not exclude the possibility that cortisol acts at a cytoplasmic level; it may imply that a metabolite is the active component. Certainly only a small per cent of metabolically unaltered cortisol is found in the hepatocyte (Fiola and Litwack 1966).

Hormones are transported to the nuclei of their target organs (Beato and Feigelson 1972; Jensen and Jacobson 1962). The cytoplasm of both target and non-target organs are exposed to hormones. It is thus likely that the kidney cell experiences cytoplasmic but not nuclear exposure to cortisol.

In this study an interaction between a cortisol metabolite and 3-HAAO at the cytoplasmic level may account for the enhanced renal 3-HAAO stability on storage at the homogenate stage post in vivo cortisol therapy.

(c) Cortisol, 3-HAAO and the 'Mass Action' Theory:-

Mandelstam (1952) described the mass action theory on the basis of an equilibrium phenomenon. Combination of an enzyme with substrate upsets this equilibrium with the precursor substance. The higher the substrate concentration, the greater the demand for enzyme and the higher the rate of enzyme synthesis.

Cortisol is known to induce tryptophan pyrrolase - the first and most likely rate-limiting enzyme of the tryptophan-nicotinic acid pathway (Rose and Braidman 1971). The possibility that the elevated levels of 3-HAAO detected in liver post cortisol

therapy are due to increased movement down this pathway exists. It is unlikely that the primary action of cortisol on 3-HAAO is at the level of tryptophan pyrrolase for the following reasons:-

1. Cortisol mediates an enhanced level of tryptophan pyrrolase via increased protein synthesis - this is not proceeded by an enhanced conversion of inactive to active enzyme (Greengard 1963). In 1966 Greengard went a step further and showed that after cortisol treatment the major component of tryptophan pyrrolase which is increased is an inactive form. Under physiological conditions most of the tryptophan pyrrolase in the cytosol is in an inactive form (Knox et al. 1966). All the tryptophan pyrrolase induced by cortisol is thus not immediately available to the tryptophan-nicotinic acid pathway and hence not to the 'mass action' hypothesis. In fact Civen and Knox (1969) showed that cortisol did not increase the rate of tryptophan metabolism as measured by the excretion of xanthenic and kynurenic acids in pyridoxal deficient rate. Although steroids may influence the entry of amino acids into cells induction with cortisol, unlike with tryptophan, is not associated with an increased level of free tryptophan within the liver (Tomkins and Maxwell 1966).

2. Knox and Auerbach (1965) increased the level of tryptophan pyrrolase activity using cortisol and found urinary excretion of kynurine doubled. Both tryptophan and cortisol have been shown to increase the urinary excretion of tryptophan metabolites (Brown and Price 1956, Aitman and Green 1966) - these metabolites are found prior to 3-HAAO in the tryptophan-nicotinic acid pathway. This points not only to the production of a certain concentration of active tryptophan pyrrolase, but also illuminates the limited extent moved down the pathway by at least a proportion of the product. Rose and Mc Ginty (1968) found some 3-HAAO excreted after cortisol

induction. This implies inadequate 3-HAAO available for the proportion of substrate potentially able to pass down this pathway to nicotinic acid.

3. Tryptophan pyrrolase can be induced by cortisol in newborn rats after 12 days of age (von Bekkum), 3-HAAO was not induced by cortisol in 15,5 week old rats (p. 39). Mass movement down this pathway in the 15,5 week old rat either fails to reach / induce 3-HAAO or induces 3-HAAO in statistically insignificant quantities.

4. In 1957 no adaptation had been found subsequent to the first reaction in tryptophan metabolism (Lin and Knox). Excess dietary leucine significantly increases tryptophan pyrrolase but has no effect on either 3-HAAO or nicotinote-phosphoribosyl transferase (Afoorunissa and Narasingarao 1973).

5. The effect of steroids on liver enzymes is sequential rather than simultaneous of the maximum response tryptophan pyrrolase is between 4 - 6 hours (Feigelson et al. 1962, Rosen and Milholland 1971). The maximum response of 3-HAAO precedes this. Although the maximum response depends on the half life of the enzyme, this fact does not favour the mass action hypothesis.

The activity of cortisol induced tryptophan pyrrolase, the temporal response of tryptophan pyrrolase and 3-HAAO, the enhanced excretion of metabolites prior to 3-HAAO in the tryptophan-nicotinic acid pathway and the lack of response in other enzymes on this pathway all oppose the concept of the 'mass action' hypothesis being the sole mode of induction of 3-HAAO by cortisol.

(d) Cortisol Acting at the Level of the Genetic Apparatus of 3-HAAO:-

Tomkins et al. (1969) suggest that cortisol may mediate induction via regulation of the conformation of the allosteric molecule. The steroid may influence the synthesis, degradation or transport of this repressor substance from the cytoplasm to the nucleus. Karlson and Sekeris (1966) feel cortisol acts at the level of the nucleus via binding to histones. A negatively charged derivative of cortisol may play a role in the relatively non specific depression of genetic information associated with cortisol induction (Filla and Litwack 1966). Cortisol is thought to act at the level of gene transcription in the case of tryptophan pyrrolase (Yu Fu Li and Feigelson 1969) and other hepatic enzymes tested in rat hepatoma (Yu and Feigelson 1971). Ashmore and Morgan (1967) feel that cortisol's initial action on the nucleus activates synthesis of specific RNA resulting ultimately in certain enzymes being produced. Purine nucleotide synthesis is one of the consequences of glucocorticoid activity on the liver (Feigelson et al. 1962, Feigelson and Feigelson 1963 and 1965).

This enhanced RNA synthesis associated with hormonal enzyme induction is inhibited by actinomycin D (Garren et al. 1964). Actinomycin is thought to inhibit RNA polymerase via binding with DNA primers (Goldberg et al. 1962). This complex between actinomycin is selective and reversible (Barnabei et al. 1965). Actinomycin D given alone is known to increase enzyme activity - this increase is attributed to an enhanced enzyme synthesis in the presence of inhibition of synthesis of inhibitors of translation (Tomkins et al. 1966). Actinomycin does not interfere with the pre-existing m-RNA acting as a template for protein synthesis - it does not preclude enhancement of enzyme activity via allosteric effects, conversion of inactive molecules or even increased synthetic

ability of existing m - RNA (Roy et al. 1964). Actinomycin inhibited cortisol induction of both tryptophan pyrrolase (Michkin and Shore 1967) and 3-HAAO (see P. 37). Cortisol thus induces via protein synthesis. As previously discussed, it is unlikely that the movement of metabolites down this pathway could completely account for the increased level of 3-HAAO; it thus seems likely that the primary action of cortisol is at the level of 3-HAAO's genetic apparatus.

The rapidity of this enzyme's response to cortisol, the knowledge that RNA polymerase activity peaks at 3 hours post intraperitoneal cortisol, the effect of cortisol on the renal enzyme and the nebulous results obtained on antibody antigen titrations, all suggest a multifocal mode of action.

THE ADAPTABILITY OF 3-HAAO ON TRYPTOPHAN STIMULATION:-

The ability to survive within a changing environment is intimately linked with the adaptability of the metabolic process. In animals, adaptation can be achieved via either hormonal regulation or alteration of a dietary substrate (Feigelson et al. 1962). Tryptophan induction of tryptophan pyrrolase is associated with two phenomena - conversion of inactive to active forms of the enzyme plus an increase in the total protein moiety of this enzyme (Greengard and Acs 1962). Actinomycin has no influence on this response (Greengard et al. 1963). Tryptophan administration should thus increase the movement down the tryptophan-nicotinic acid pathway and elevate the level of 3-HAAO activity - this was found true of both liver and kidney. The drop in hepatic 3-HAAO activity which followed tryptophan induction could possibly be explained as enzyme depletion due to excessive movement down the tryptophan-nicotinic acid pathway - the acute demand for 3-HAAO exceeding the synthetic potential of

the genetic apparatus at that time. The alkali used may have further compromised the response.

Tryptophan administration leads not only to the elevation of tryptophan pyrrolase, it also elevates at least four other hepatic enzymes (Foster 1966; Perino et al. 1965). In the case of at least one of these enzymes an intact adrenal is required (Kenney and Fera 1961).

The peak of tryptophan induced hepatic 3-HAAO occurs towards the end of maximum induction by cortisol and proceeds cortisol's induction of tryptophan pyrrolase. Induction of 3-HAAO by cortisol is not primarily via mass movement down this pathway; tryptophan induction of this enzyme may however be due to this mechanism. Certain modes of action have not been excluded.

THE ADAPTABILITY OF 3-HAAO ON DAB INGESTION:-

In theory it seemed possible that DAB could attach to 3-HAAO. DAB is known to bind to certain cytoplasmic proteins which are themselves almost entirely absent from the resultant tumour (Sorof et al. 1958). In DAB induced hepatomas the level of 3-HAAO activity is notably decreased (Shimoyama et al. 1965). The cytoplasmic protein to which the dye binds has certain properties similar to those of 3-HAAO - its isoelectric point is 8,4 (3-HAAO failed to migrate in polyacrylamide gel at 8,4) no 'N' terminal has been detected and this protein shows microheterogeneity (Ketterer et al. 1967). The molecular weight of this protein is 45 000, that of 3-HAAO is lower. The lower molecular weight form could be justified if the enzyme had separated into sub-units during purification.

As the dye is only hydrolysed off from this protein by strong alkali (Miller and Miller 1952), purification of 3-HAAO will not disturb the dye - protein complex. Testing with formic and trichloroacetic acid showed no evidence for 3-HAAO being cytoplasmic protein to which DAB attached. Michaelis constant and antibody experiments confirmed that DAB does not influence the catalytic or antigen site on 3-HAAO. It thus seems unlikely that DAB directly influences 3-HAAO at a cytoplasmic protein level. Dietary DAB did however enhance this enzyme's activity in both liver and kidney.

In terms of the activity of renal 3-HAAO it is of interest to note that bound dye is virtually absent from kidney (Miller and Miller 1947), while one of the hepatic DAB binders is a cortisol binder (Litwack and Morey 1970). The carcinogenicity of DAB is associated with its ability to bind DNA (Dingman and Sporn 1967). No striking relationship exists between renal tumours and DAB and nothing to the contrary was found in this study. This study did however show that DAB, or one of its metabolites, enhances the activity of 3-HAAO - the mechanism involved remains obscure.

Less difficult to explain is the decreased level of 3-HAAO in macroscopically abnormal areas of liver after 15 weeks on the DAB diet. These areas had suffered hepatocyte loss and connective tissue gain thus enzyme activity is not comparable on a weight to weight basis. Why certain liver cells should be more susceptible to noxious agents than others is beyond the scope of this dissertation.

3-HAAO is an adaptable enzyme with an adaptable pathway. Stimuli may act at the level of the nucleus or the cytoplasm

but in either case the effectiveness of this stimulus is influenced by the physiological state pre-imposed by other regulatory factors.

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Author Jamison J R

Name of thesis Studies on 3-Hydroxyanthranilate Oxygenase in Rat liver and kidney 1974

PUBLISHER:

University of the Witwatersrand, Johannesburg

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