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THE EFFECT OF NORETHANDROLONE ON THE
LIVER MICROSOMAL ENZYME WHICH
DEMETHYLATES AMINOPYRINE

BY

GARRETT JOHN GROSS

A thesis submitted
in partial fulfillment of the requirements for the
degree Master of Science, Major in
Pharmacology, South Dakota
State University

1967

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LIVER MICROSOMAL ENZYME WHICH
DEMETHYLATES AMINOPYRINE

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable as meeting the thesis requirements for this degree, but without implying that the conclusions reached by the candidate are necessarily the conclusions of the major department.

✓ Thesis Adviser

Head, Pharmacology
Department

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INTRODUCTION

With the introduction of numerous and more complex drugs in medicine annually, the metabolism of drugs by liver microsomal enzymes and factors influencing their metabolism has become a subject of increasing interest. A variety of drugs are metabolized by enzyme systems located in liver microsomes (1). Reactions catalyzed by these enzymes include N-dealkylation, deamination, aromatic hydroxylation, ether cleavage, alkyl chain oxidation, azo link cleavage, sulfoxide formation, and glucuronide formation.

The report that 3'-methyl -4- dimethylaminoazobenzene, a potent liver carcinogen, did not cause hepatomas in rats when administered with 3-methylcholanthrene (2) stimulated early investigations to study the effects of various chemical compounds on drug metabolizing enzymes found in liver microsomes. Since this first investigation numerous studies have been done with a variety of drugs in an effort to obtain information concerning the mechanism involved in drug potentiation and synergism.

Studies by Conney et al. (3) showed that pretreatment of rats with phenobarbital induces the synthesis of enzymes that metabolize aminopyrine and stimulates liver protein synthesis. Similarly, phenobarbital and chlorcyclizine markedly stimulate the activity of enzyme systems in liver

microsomes that hydroxylate testosterone and Δ^4 -androstene-3,17 dione (4). Burns (5) has shown that the anti-inflammatory drug, phenylbutazone, increases the metabolism of aminopyrine.

Administration of certain drugs enhances the ability of liver microsomes to metabolize the same or closely related compounds (4). Conney et al. (3) has shown that the administration of phenylbutazone, aminopyrine, 3,4 benzpyrene, or phenobarbital will increase the ability of rat liver microsomes to metabolize phenylbutazone, aminopyrine, 3,4 benzpyrene or hexobarbital. The ability of drugs to stimulate drug metabolizing enzymes is paralleled in vivo by an accelerated rate of drug metabolism and by a shortened duration of drug action.

Dixon et al. (6) showed that diabetes induced by alloxan affects certain hepatic microsomal drug metabolizing enzymes. A depressed ability to metabolize drugs is evident in vitro and sleeping times after hexobarbital are prolonged. Insulin can reverse these effects of alloxan on sleeping time. It is possible that factors leading to sévere depletion of hepatic glycogen will affect the rate at which drugs are metabolized by the microsomal enzymes.

The ability of some of the chlorinated hydrocarbon insecticides to induce the synthesis of hepatic microsomal enzymes has been observed. Chlordane increases the activity

of the enzymes that metabolize hexobarbital, aminopyrine, and chlorpromazine (7). More recently, Kinoshita et al. (8) has shown that DDT and toxaphene increase the activity of enzymes that metabolize p-nitroanisole and aminopyrine.

Booth and Gillette (9) undertook a study to determine the effect of a number of androgenic steroids on various microsomal enzyme systems. Their data showed that anabolic steroids as well as androgenic hormones markedly enhance the activity of the microsomal enzyme systems which metabolize drugs.

The ability of steroids to enhance the activity of the liver microsomes is apparently not related to their androgenic potency (10). For example, testosterone propionate is a more potent androgen than 19-nortestosterone, but these steroids are about equally effective in stimulating the activity of liver microsomal enzymes. This finding suggests that the increase in enzyme activity might be related to the anabolic activity of the steroids.

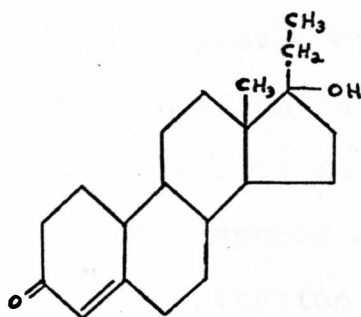
The anabolic steroids testosterone propionate, 19 nortestosterone and methyltestosterone stimulated the activity of some of the microsomal enzyme systems which metabolize drugs (10). The present investigation was undertaken to obtain information on the effect of the anabolic steroid, norethandrolone, on the liver microsomal enzyme systems in weanling and adult female and male rats. The influence

of norethandrolone on the activity of liver microsomal enzyme systems has not been previously studied. The experiment was designed to determine the effect of norethandrolone on the activity of the enzyme (N-demethylase) responsible for the demethylation of aminopyrine. The results of this study may be useful in the final evaluation of the mechanism of action of norethandrolone and similar compounds.

MATERIALS AND METHODS

Female and male weanling (23 days old, 40-50 Gms) and adult (250-450 Gms) Sprague-Dawley rats were used for this study. The animals were housed in an air-conditioned room (65-75°F) and were provided with feed (Purina Laboratory Chow) and water ad libitum.

The compound under investigation was norethandrolone (17 α -ethyl-19 nortestosterone; Nilevar[®], Searle), an anabolic steroid. It was obtained from G. D. Searle and Company of Chicago, Illinois and has the following chemical structure:



Norethandrolone

The drug was dissolved in corn oil and administered in a concentration of 1 mg/ml to the weanling rats and 10 mg/ml to the adult rats. All drug injected animals received a dose of 1 mg/kg of norethandrolone daily by subcutaneous injection.

The effect of norethandrolone on the microsomal enzyme system in the rat liver which demethylates

aminopyrine was tested in four different groups of rats: group one, male weanling rats; group two, female weanling rats; group three, adult male rats, and group four, adult female rats. A fifth group of weanling male rats was injected with 1000 mg/kg of corn oil. In all tests non-injected animals from the same shipment served as controls. The rats were sacrificed at various intervals during the three- to four-week experimental period. A portion of the liver was removed and the activity of the enzyme responsible for the demethylation of aminopyrine was determined.

Aminopyrine is N-demethylated by the microsomal enzymes of the liver to formaldehyde and 4-aminoantipyrine. Formaldehyde, the product used to measure enzyme activity, was isolated by the modified method of La Du (11). The rats were sacrificed by decapitation and a portion of the liver was immediately removed, weighed and homogenized with a Potter Elvehjem glass homogenizer in sufficient 0.1 M phosphate buffer, pH 7.4 to provide a 10% liver homogenate. Duplicate 1.0 ml (100 milligrams of tissue) and 1.5 ml (150 milligrams of tissue) aliquots from each rat liver homogenate were placed in separate Warburg reaction vessels. The flasks also contained 100 micromoles of nicotinamide, 75 micromoles of $MgCl_2$, 0.2 micromoles of

NADP, 5 micromoles of aminopyrine, 0.5 ml of 0.1 M semicarbazide, 2 micromoles of ATP, 3.8 micromoles of glucose-6-phosphate, and sufficient 0.1 M PO_4 buffer, pH 7.4 to give a final volume of 5.0 ml.

The vessels were incubated in a constant temperature bath in an atmosphere of air at 37°C for one hour. At the end of the incubation period the vessels were placed in an ice water bath and the reaction was stopped by the addition of 5 ml of 10% trichloroacetic acid. The precipitated protein was removed by centrifugation at 3700 X g for 10 minutes. The supernatant was decanted and further acidified by the addition of 5 ml of 50% trichloroacetic acid to make a final concentration of 20% trichloroacetic acid and a total volume of 15 ml. The final acidified supernatant was placed in a distillation apparatus and the first 5 ml of distillate were collected. A 1 ml aliquot of this distillate was assayed for formaldehyde content.

The enzyme activity is directly related to the amount of formaldehyde formed in the demethylation of aminopyrine. Formaldehyde was determined by the chromotropic acid method of Mac Fayden (12) as modified by Mueller, Miller (13) and La Du et al. (11). In this procedure 1 ml of the final distillate containing formaldehyde was mixed with 4 ml of chromotropic acid solution (12) and heated in a boiling water bath for 30 minutes. The tubes were then

removed and allowed to cool to room temperature. The solutions containing formaldehyde developed a purple color and were read colorimetrically on a Spectronic 20 at 570 mμ.

In order to determine the adaptability of La Du's (11) method to this study the following tests were performed.

An experiment was undertaken to determine demethylase activity according to the method of La Du (11). Five Warburg reaction vessels were prepared. A 10% adult male rat liver homogenate was used and tissue levels of 50 mg, 100 mg, 150 mg, and 200 mg were placed in four flasks with the fifth one serving as the blank. The ingredients in each flask were in the concentrations mentioned above except that ATP and glucose-6-phosphate were not included. Enzyme activity was observed at 100 mg, 150 mg, and 200 mg tissue levels shown in Fig. 1. No enzyme activity was found in the 50 mg tissue level.

Other investigators have shown that the addition of glucose-6-phosphate and ATP enhance the activity of certain enzymes found in the microsomal fraction of the liver (15). Six flasks were prepared with two having 150 mg of tissue, one with 200 mg, one with 250 mg, one with 300 mg, and one blank. One of the flasks containing 150 mg of tissue contained 1 mg of ATP and 1 mg of glucose-6-phosphate.

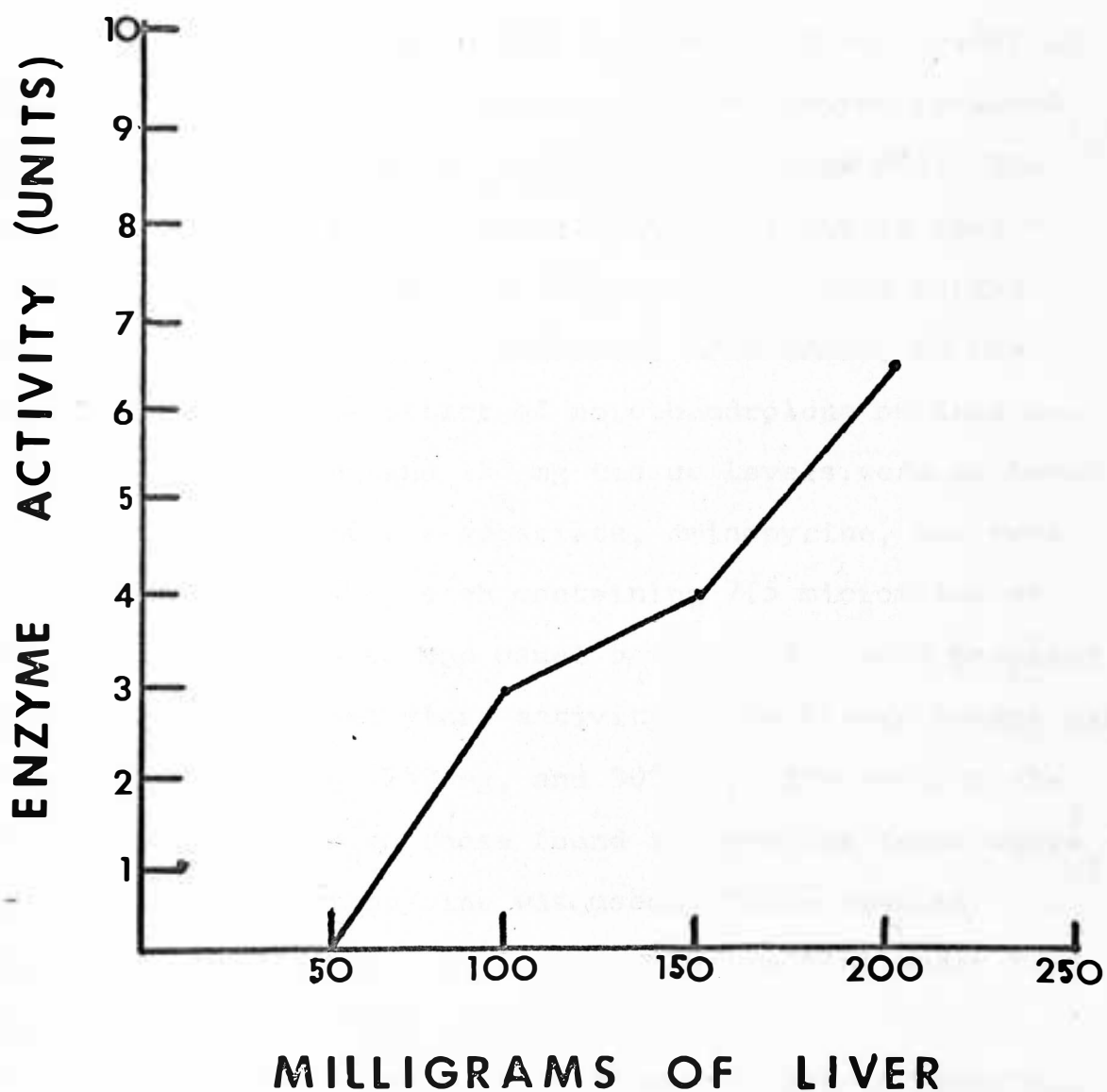


Fig. 1. The activity of the enzyme which demethylates aminopyrine in adult male rats. Each unit represents .01 micrograms of formaldehyde liberated by 150 mg of tissue in 60 minutes.

The remaining flasks were set up according to the method of La Du (11). Enzyme determinations indicated that the addition of 1 mg of glucose-6-phosphate and 1 mg of ATP increased the activity two- to three-fold.

An experiment was conducted to determine if there was a relationship between tissue levels and the amount of formaldehyde formed. The amount of formaldehyde produced by 150 mg, 200 mg, 250 mg, and 300 mg was measured. The results of these determinations (Fig. 2) indicate that tissue levels in excess of 250 mg do not produce further increases in formaldehyde formation. Therefore, in the determination of the effect of norethandrolone on demethylase activity 100 mg and 150 mg tissue levels were employed.

To determine if the substrate, aminopyrine, was rate limiting, four flasks, each containing 7.5 micromoles of aminopyrine instead of the usual 5 micromoles were prepared and assayed for demethylase activity. The tissue levels used were 150 mg, 200 mg, 250 mg, and 300 mg. The results obtained were similar to those found in previous tests where 5 micromoles of aminopyrine was used. These results indicated that aminopyrine is not rate limiting under the conditions used in this study.

Two tests were run to determine the rate of formaldehyde distillation. In the first test, five flasks containing from 0.125 micromoles to 1 micromole of standard U.S.P. formaldehyde were prepared. The other ingredients

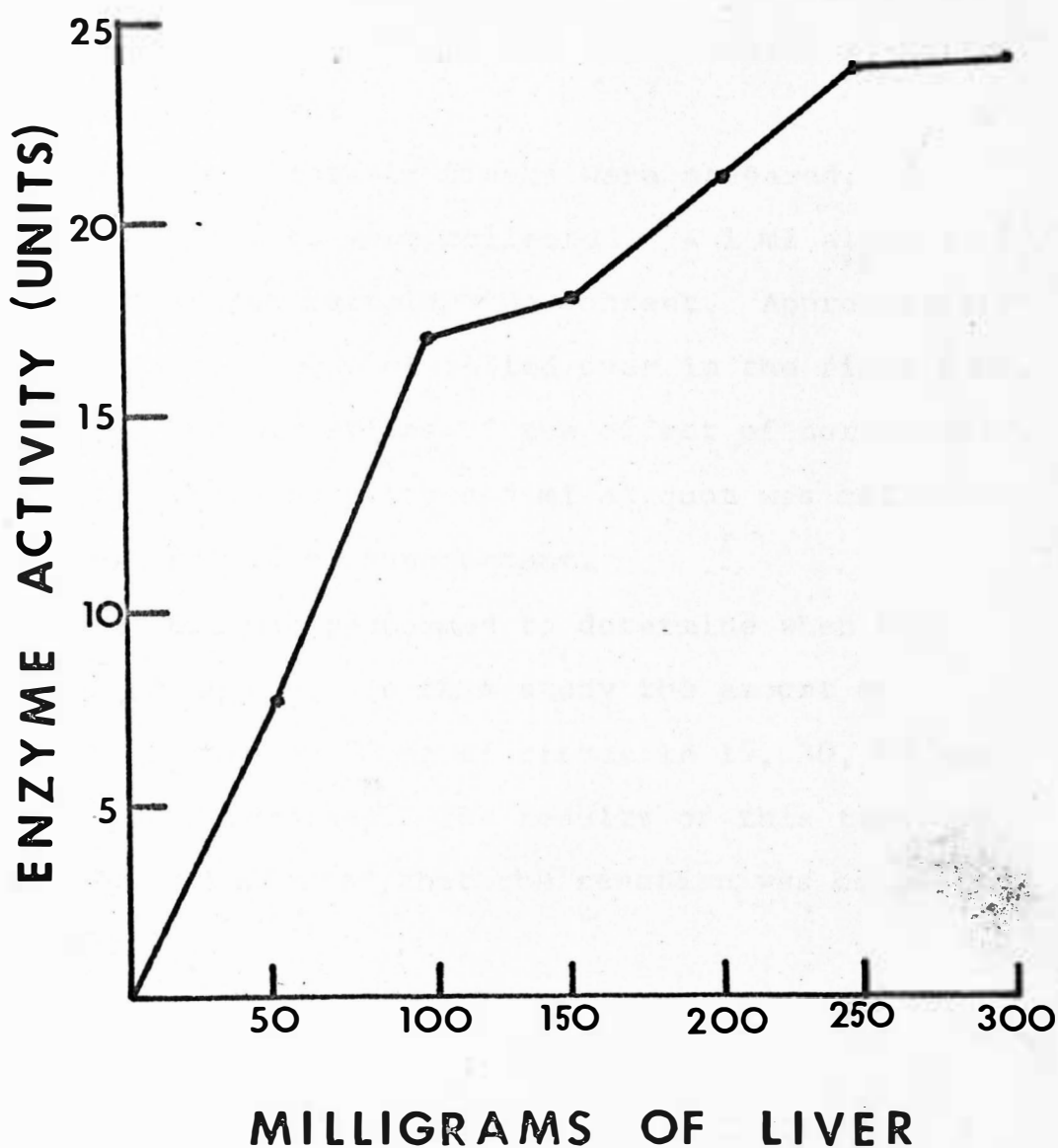


Fig. 2. The activity of the enzyme which demethylates aminopyrine in adult male rats. Each unit represents .01 micrograms of formaldehyde liberated by 150 mg of tissue in 60 minutes.

were the same as the above tests. From each sample a 2 ml and an 8 ml aliquot was collected. A 1 ml aliquot from each of these samples was assayed for formaldehyde content. Approximately 90% of the formaldehyde distilled over in the first 2 ml.

In the second test six flasks were prepared, and 5 ml and 2 ml aliquots were collected. A 1 ml aliquot of each was assayed for formaldehyde content. Approximately 99%+ of the formaldehyde distilled over in the first 5 ml. In all future determinations of the effect of norethandrolone on demethylase activity a 5 ml aliquot was collected from the original 15 ml supernatant.

A final test was performed to determine when the reaction was complete. In this study the amount of formaldehyde formed by 50 mg of tissue in 15, 30, 60 and 90 minutes was determined. The results of this test as shown in Fig. 3 indicated that the reaction was completed in sixty minutes.

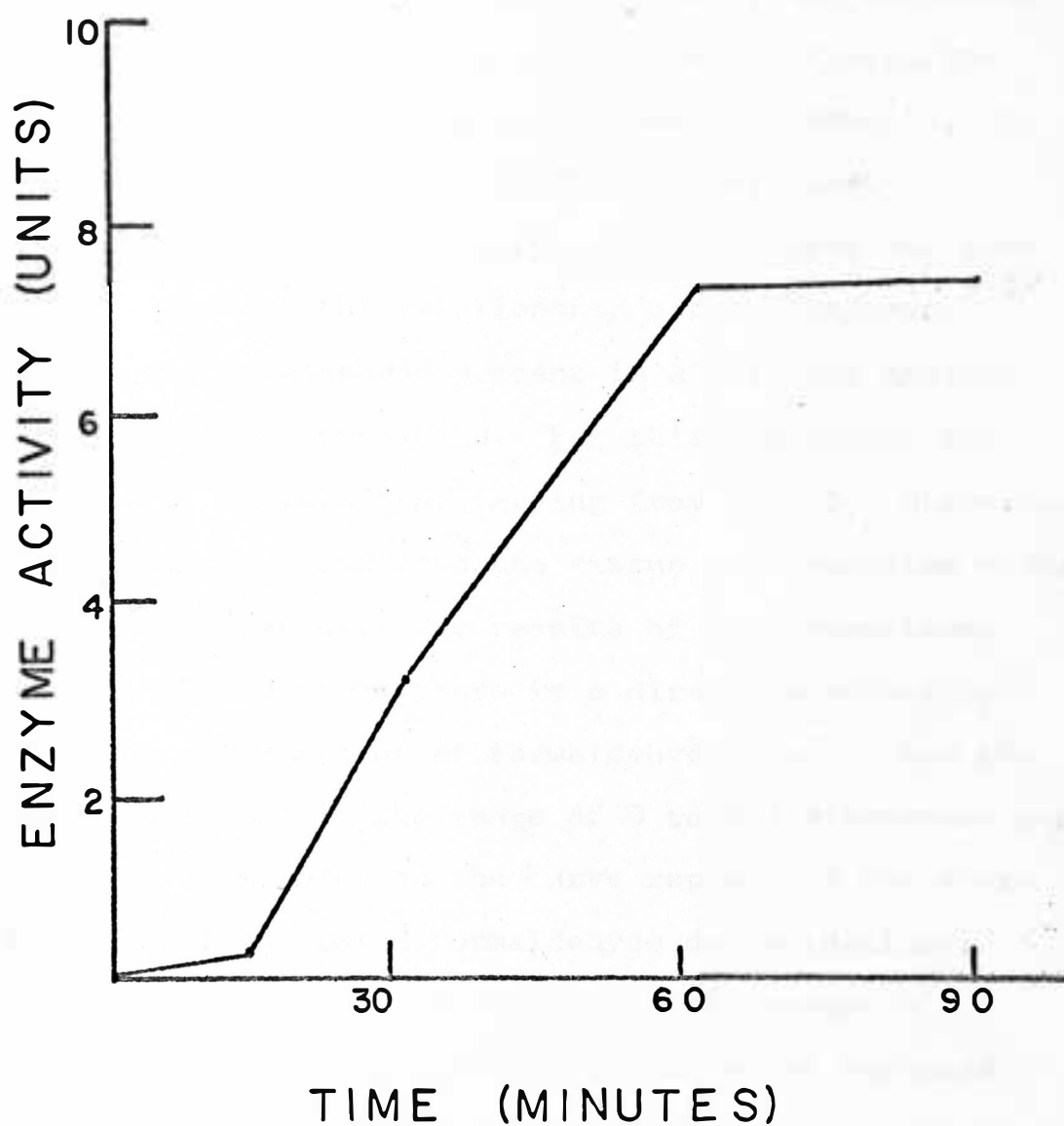


Fig. 3. The activity of the enzyme which demethylates aminopyrine in adult male rats. Each unit represents .01 micrograms of formaldehyde liberated by 150 mg of tissue in 60 minutes.

RESULTS

The observation that certain anabolic steroids enhance the activity of liver microsomal enzymes responsible for the metabolism of drugs and toxic compounds (9) stimulated the present investigation concerning the effect of norethandrolone, a potent anabolic steroid, on the enzyme system which demethylates aminopyrine.

Standard formaldehyde curve.--An experiment was performed to determine the relationship between the concentration of formaldehyde present in a solution and the per cent absorbance at 570 mu. For this experiment concentrations of formaldehyde varying from 0 to 0.5 micrograms per milliliter were added to the tissue free reaction media employed in this study. The results of this experiment shown in Fig. 4 indicate there is a direct relationship between the concentration of formaldehyde present and the per cent absorbance in the range of 0 to 0.5 micrograms per milliliter. Each point on the curve represents the average value obtained from 3 formaldehyde determinations. Enzyme activity is directly related to the amount of formaldehyde formed and, therefore, this curve was used to interpret the results of this study.

The influence of norethandrolone on N-demethylase activity in weanling male rats.--To determine the effect of norethandrolone on N-demethylase activity groups of

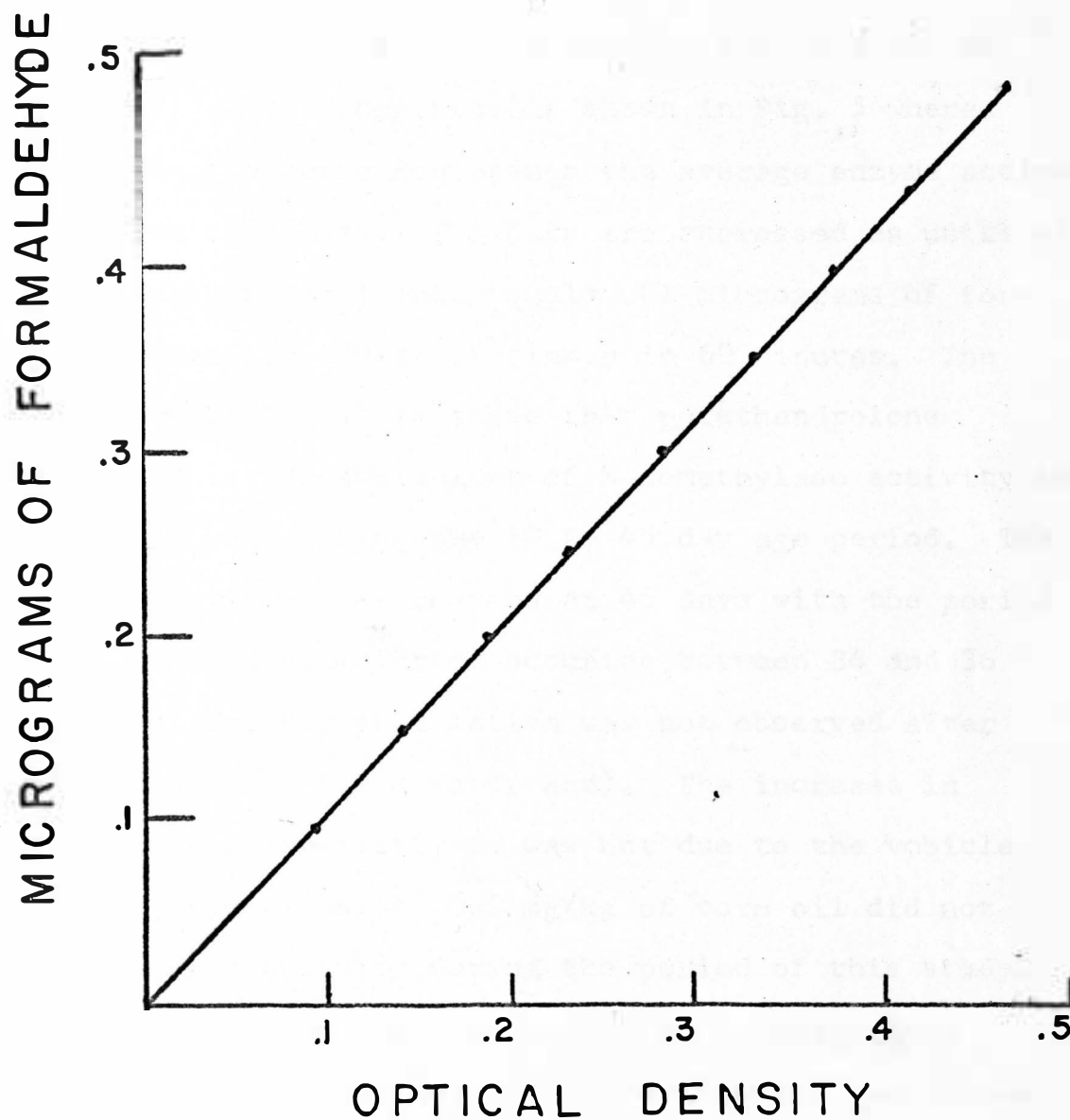


Fig. 4. Standard formaldehyde curve. Optical density versus micrograms of formaldehyde present.

3 weanling male rats were given daily subcutaneous injections 1 mg/kg of norethandrolone. The animals were sacrificed at various intervals during the four week investigational period. A portion of the liver was removed from each animal and assayed for N-demethylase activity. The effect of norethandrolone on the liver microsomal enzyme which demethylates aminopyrine is shown in Fig. 5 where each point on the curve represents the average enzyme activity of groups of 3 rats. The data are expressed as units of enzyme activity where 1 unit equals .01 micrograms of formaldehyde formed by 150 mg of tissue in 60 minutes. The results shown in Fig. 5 indicate that norethandrolone markedly stimulated development of N-demethylase activity in weanling male rats during the 30 to 45 day age period. The maximum enzyme level was reached at 45 days with the period of the most rapid stimulation occurring between 34 and 36 days of age. Further stimulation was not observed after 22 daily injections (45 days of age). The increase in development of enzyme activity was not due to the vehicle since daily injections of 1000 mg/kg of corn oil did not stimulate enzyme activity during the period of this study.

The influence of norethandrolone on N-demethylase activity in weanling female rats.--The effect of norethandrolone on the liver microsomal enzyme which demethylates aminopyrine in weanling female rats is shown in Fig. 6.

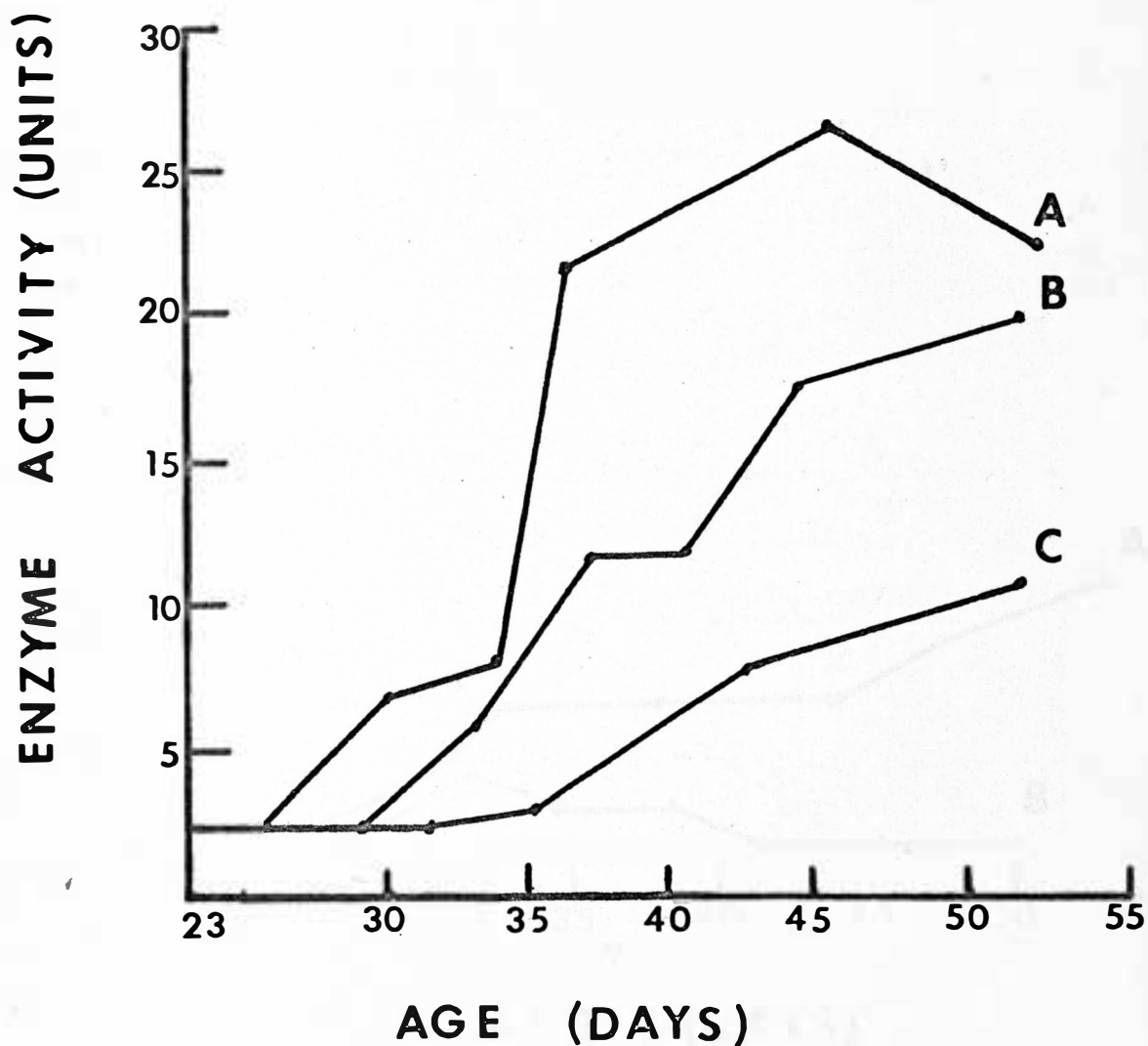


Fig. 5. The effect of norethandrolone on the activity of the enzyme which demethylates aminopyrine in weanling male rats where A represents the drug-injected animals, B the control animals, and C the corn oil injected animals. Each unit represents .01 micrograms of formaldehyde liberated by 150 mg of tissue in 60 minutes.

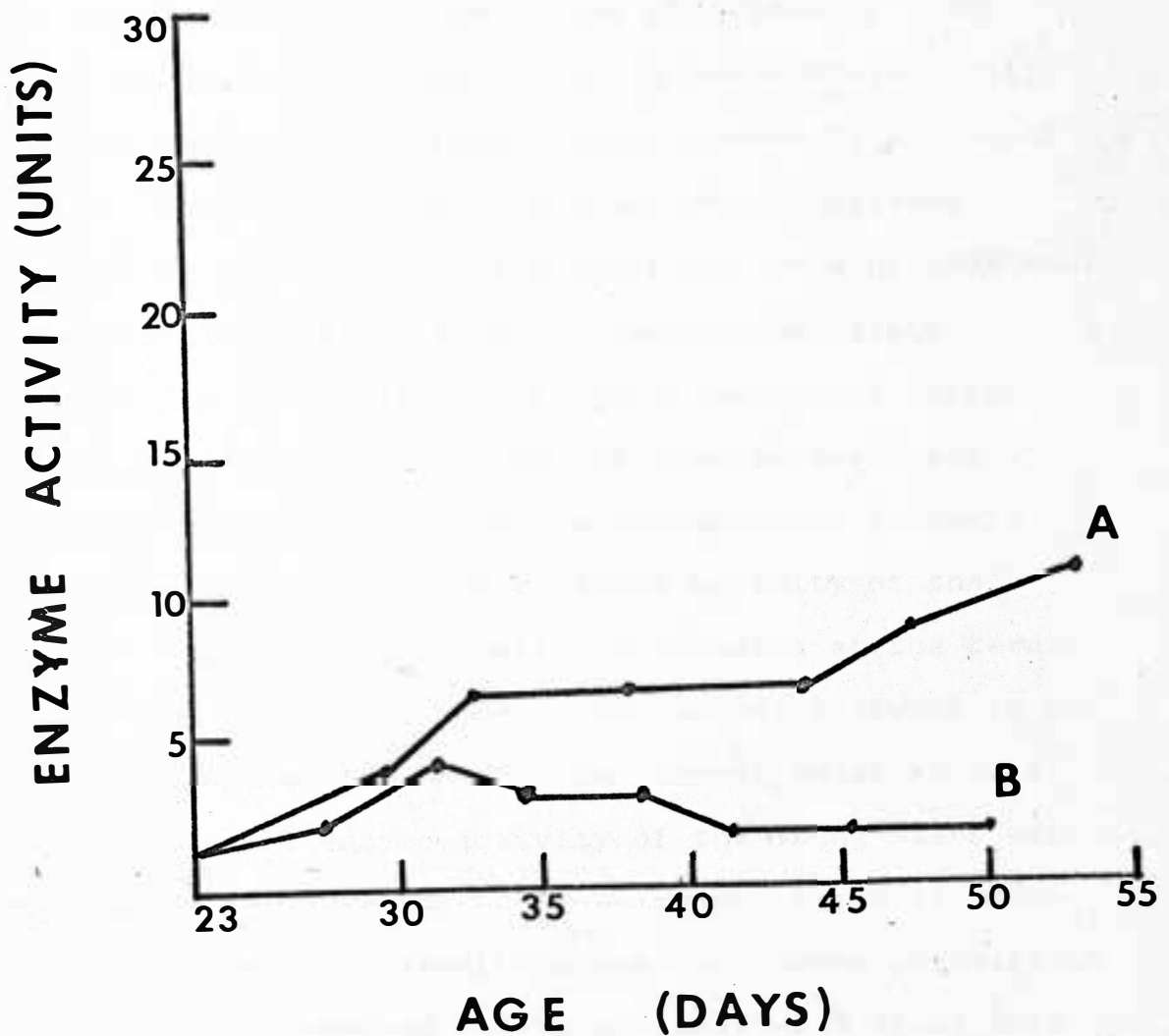


Fig. 6. The effect of norethandrolone on the activity of the enzyme which demethylates aminopyrine in weanling female rats where A represents the drug-injected animals and B the control animals. Each unit represents .01 micrograms of formaldehyde liberated by 150 mg of tissue in 60 minutes.

Groups of 3 rats were given daily subcutaneous injections of 1 mg/kg of norethandrolone. The animals were sacrificed at various intervals during the 3 - 4 week experimental period. The points on the curve represent an average of enzyme determinations on the livers of groups of 3 animals and the activity is expressed in units where 1 unit equals .01 micrograms of formaldehyde formed by 150 mg of tissue in 60 minutes. The enzyme activity increased approximately three-fold during the first week of treatment and then leveled off until day 43 when it was again stimulated for the remainder of the experimental period. These results differ from those obtained in the experiment on the young male rats where the activity increased mainly from day 30 to 45. The enzyme activity of the livers of young females was still increasing at the termination of the experiment whereas the enzyme activity in the young males tapered off toward the control value at this time. The overall enzyme activity of the young males was significantly higher than the females, but was never more than twice the control level whereas the enzyme activity of the young females reached levels as great as 5 times that of the controls.

The influence of norethandrolone on N-demethylase activity in adult male rats.--Many experiments dealing with drug-induced enzyme induction have been performed on

weanling rats. A study was undertaken to determine if norethandrolone affects the enzyme activity in adult male rats. Groups containing 2 animals were given daily subcutaneous injections of 1 mg/kg of norethandrolone. The animals were sacrificed at various intervals throughout the 4 to 5 week experimental period. The results of this study are shown in Table 1 where the control value represents the average of enzyme assays performed on livers of 2 animals assayed at various time intervals. The data are expressed as micrograms of formaldehyde formed by 150 mg of tissue in 60 minutes. The results indicate that daily injections of 1 mg/kg of norethandrolone does not significantly affect N-demethylase activity in the adult male rat.

The influence of norethandrolone on N-demethylase activity in adult female rats.--The effect of norethandrolone on the liver microsomal enzyme which demethylates aminopyrine in adult female rats is shown in Table 2. The results are expressed as micrograms of formaldehyde formed by 150 mg of tissue in 60 minutes. The drug values represent the average of groups of 2 animals at various time intervals and the control values are the average of enzyme measurements of 6 animals from the same shipment. The results indicate that daily injections of 1 mg/kg of norethandrolone cause a three- to four-fold increase in N-demethylase activity at 3 to 4 weeks after the initiation of this study.

TABLE I

EFFECT OF NORETHANDROLONE ON THE ACTIVITY OF THE ENZYME WHICH
DEMETHYLATES AMINOPYRINE IN ADULT MALE RATS

Days of Injection	Enzyme Activity (micrograms CH_2O /150 mg) tissue/60 minutes	Per Cent Of Control	Per Cent Change In Activity
Control	.12 (.08-.15)	--	--
23	.11 (.11-.13)	92	-8
27	.13 (.12-.14)	108	8
32	.16 (.14-.17)	133	33
39	.13 (.12-.14)	108	8

TABLE II

EFFECT OF NORETHANDROLONE ON THE ACTIVITY OF THE ENZYME WHICH
DEMETHYLATES AMINOPYRINE IN ADULT FEMALE RATS

Days of Injection	Enzyme Activity (micrograms CH_2O /150 mg) tissue/60 minutes	Per Cent Of Control	Per Cent Change In Activity
Control	.03 (.02-.06)	--	--
21	.08 (.06-.10)	267	167
24	.07 (.06-.08)	233	133
28	.11 (.10-.12)	367	267

DISCUSSION

The administration of various drugs and foreign compounds has been shown to stimulate certain drug metabolizing enzyme systems found in liver microsomes. Numerous studies have been undertaken to determine the mechanism of action of these drugs on the liver microsomal enzymes. Remmer (14) has shown that the repeated administration of phenobarbital and several other drugs results in a quantitative increase of the smooth-surfaced endoplasmic reticulum of the liver cell. The marked increase in drug metabolizing enzymes was found to occur in the enlarged smooth membrane fraction of the endoplasmic reticulum. These results and evidence offered by Conney et al. (3) seem to indicate that phenobarbital and other foreign compounds stimulate liver protein synthesis.

The effects of the anabolic steroids differ in many ways from those of other foreign compounds (9). For example, hydrocarbons and barbiturates increase the liver to body weight ratio about 20-30% after a few days administration while prolonged treatment with the anabolic steroids does not significantly change this ratio. Testosterone propionate and 19-nortestosterone, two anabolic steroids, have been shown to increase the weight of the levator ani muscle and the seminal vesicles while benzpyrene and phenobarbital do not alter these weights.

Kato (16) found in studies using hexobarbital, pentobarbital, meprobamate, carisoprodal, and strychnine that the maximum activity in the liver microsomal drug-metabolizing enzymes is found in rats 30 days old and after this age the activity is progressively decreased. In the present investigation on the effect of norethandrolone, an anabolic steroid, on the microsomal enzyme that demethylates aminopyrine, the maximal enzyme activity in young males occurs at 45 days of age and the activity in the young females was still increasing at 52 days of age. The results of our study and findings of other investigators (9,16) suggest that the mechanism by which anabolic steroids stimulate the activity of the microsomal enzymes of the liver which metabolize certain drugs differ from those of the non-steroidal enzyme inducers.

Fouts (15) has studied the microstructure of the smooth-surfaced endoplasmic reticulum in an attempt to obtain information on the mechanism by which drugs act on liver microsomal enzyme systems. The reticulum is difficult to study because it is sensitive to the physiological state of the cell and seems to change its arrangement as the activity of the cell changes. The reticulum can exist randomly as a series of canals, or highly ordered like a pipeline. Fouts (15) has advanced the theory that these reticulum connections or tubings could be altered by

drugs. The changing of these canals to a highly ordered system could be a possible explanation for the mechanism by which norethandrolone and other anabolic steroids act on liver microsomal drug metabolizing enzyme systems. At the present time, however, the mechanism by which the anabolic steroids exert their effects is not known.

In another study, Fouts (17) found that the compound need not be metabolized by the body to cause stimulation. A potent, non-specific drug stimulator is barbital, which is secreted from the body unchanged.

There have been numerous reports describing sex differences in the metabolism of drugs by liver microsomal enzyme systems. Quinn et al. (18) has found that liver microsomes of male rats oxidize hexobarbital and aminopyrine at a more rapid rate than do those of female rats. The production of androgens is apparently the dominant factor in determining the sex variation in drug-metabolizing enzymes. Booth & Gillette (9) have shown that 19-nortestosterone, methyltestosterone, 4-chloro-19-nortestosterone acetate, and 4-androstene-3,17 dione, given to intact female rats every other day for 4 weeks, stimulates the microsomal enzymes which metabolize hexobarbital, monomethyl-4-aminoantipyrine and naphthalene. In accordance with these findings, the present investigation on the effect of norethandrolone, on the liver microsomal enzyme

that demethylates aminopyrine showed that the activity of the enzyme reached a significantly greater level in the young male rats than in the young female rats. Our study also demonstrated that norethandrolone did not stimulate enzyme activity in adult male rats while stimulation was observed in the adult female rats. Booth & Gillette (9) have shown that steroid-induced stimulation of enzyme activity is primarily due to the anabolic properties of the drugs. It is known that the anabolic potency of norethandrolone is approximately that of testosterone (19) and, therefore, since the normal levels of circulating testosterone in the adult female are extremely low compared to that of the adult male the injection of 1 mg/kg of norethandrolone would be expected to have a more pronounced effect on the enzyme activity in the adult female than in the adult male.

SUMMARY

1. To determine the effect of norethandrolone on the activity of the liver microsomal enzyme which metabolizes aminopyrine the system of La Du (11) was modified by the addition of 1 mg of ATP and 1 mg of glucose-6-phosphate.

2. Daily injections of norethandrolone (1 mg/kg) to weanling male rats over a period of 3 to 4 weeks caused a marked increase in the activity of the liver microsomal enzyme (N-demethylase) which metabolizes aminopyrine.

3. Daily injections of norethandrolone (1 mg/kg) to weanling female rats over a period of 3 to 4 weeks caused a marked increase in the activity of the liver microsomal enzyme (N-demethylase) which metabolizes aminopyrine.

4. Daily injections of norethandrolone (1 mg/kg) to adult male rats over a period of 4 to 5 weeks caused a slight increase in the activity of the liver microsomal enzyme (N-demethylase) which metabolizes aminopyrine.

5. Daily injections of norethandrolone (1 mg/kg) to adult female rats over a period of 4 to 5 weeks caused a marked increase in the activity of the liver microsomal enzyme (N-demethylase) which metabolizes aminopyrine.

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