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EFFECT OF DIETARY OROTIC ACID AND ADENINE SULFATE ON HEPATIC MICROSOMAL ENZYMES IN MALE AND FEMALE RATS*

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Abstract—The addition for 6 days of 1% orotic acid to the diet of rats reduced the activity of aniline hydroxylase in the 9000 *g* supernatant from 1683 ± 111 (S. E.) to 640 ± 96 n-moles/liver/10 min in the male, but had no effect in the female. Further, there was no significant change in microsomal protein. The addition of 0·25% adenine sulfate along with the orotic acid gave the intermediate value of 1244 ± 116 nmoles/liver/10 min. On the other hand, the small decrease after orotic acid in ethylmorphine *N*-demethylase activity of microsomes was not statistically significant. Orotic acid decreased the level of cytochrome P-450 from 0·093 ± 0·004 to 0·058 ± 0·005 $E_{(450-490)}$/mg of microsomal protein in male rats without affecting the concentration of this cytochrome in females. Further, in male and female rats, the activity per unit of cytochrome P-450 decreased from 76·6 ± 5·6 and 67·5 ± 2·4 nmoles *p*-aminophenol formed/10 min/unit of cytochrome P-450, respectively, to 60·3 ± 5·3 and 52·2 ± 1·5. The soluble fraction from orotic acid-fed animals increased aniline hydroxylase by 30–50 per cent in microsomes from control and treated animals.

RATS fed a diet containing orotic acid for 7–10 days develop a fatty liver characterized by the deposition of large droplets of triglycerides in the tubules of the endoplasmic reticulum. The liver otherwise appears to be functionally normal, unlike that of animals receiving many other lipotrophic agents. This effect of orotic acid seems to be caused by an impairment in the synthesis of the β-lipoproteins which transport fats from the liver.[1] This effect may be related to the observed decrease in the hepatic concentration of adenine nucleotides, since the concomitant addition of adenine sulfate to the diet prevents the fatty infiltration.

Rubin[2] has reported that the addition of orotic acid to the diets of male rats had no effect on the hexobarbital sleeping time or *in vitro* on the hepatic hexobarbital hydroxylase activity. Further, it did not affect the induction of this enzyme by phenobarbital. The metabolism of hexobarbital is far more rapid in male than in female rats. This difference tends to disappear under such stresses as starvation and other dietary manipulations.[3] Yet the stress of orotic acid feeding did not seem to have this effect.

It has become increasingly apparent that most substrates of the mixed-function

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oxidases in liver microsomes may be classified into two general groups manifested by changes in the absorbance spectrum: the difference spectrum caused by type I substrates is characterized by a trough at about 420 nm and a peak at about 385 nm, whereas that caused by type II substrates is characterized by a peak at about 430 nm and a trough at about 394 nm.[4]

Since hexobarbital is a type I substrate, we decided to compare the effect of orotic acid on the metabolism of another type I substrate, ethylmorphine, and on a type II substrate, aniline. We have found that the addition of orotic acid to the diet had little effect on ethylmorphine *N*-demethylase, but significantly reduced aniline hydroxylase activity in male, but not in female rats.

MATERIALS AND METHODS

All animals used in these experiments were Osborne–Mendel rats (female 160–180 g; male 200–220 g) obtained from the National Institutes of Health colony and individually housed in hanging wire cages. They were fed a diet containing 16% casein, 70% sucrose, 5% corn oil, 5% vitamin mixture (Nutritional Biochemical Corp.) and 4% salt mixture, USP XIV. Orotic acid and adenine sulfate were added without substitution for other constituents to give levels of 1 and 0·25% respectively. After 6 days on the diet, the animals were killed by decapitation; the livers removed, weighed, chilled on ice and homogenized with 3 vol. of KCl (150 mM)-Tris (20 mM) buffer, pH 7·4, in a motor-driven, Teflon glass homogenizer with 0·1 to 0·15 mm clearance. The homogenates were centrifuged at 9000 *g* for 15 min in a Sorvall RC-2 centrifuge with an SS-34 rotor. The supernatants were aspirated and either used directly or centrifuged at 105,000 *g* for 60 min in a Spinco model L ultracentrifuge with a No. 40 rotor. The resulting microsomal pellets were homogenized in KCl-Tris buffer and the suspensions recentrifuged at 105,000 *g* for 60 min. The microsomal pellets were suspended in KCl–Tris to give the equivalent of 1 g liver per ml of buffer. Protein was determined by the method of Sutherland *et al.*[5] and the protein concentration was adjusted to 6 mg per ml.

Mixed-function oxidase activity was determined for the hydroxylation of aniline and the *N*-demethylation of ethylmorphine. Duplicate incubations for both reactions were performed in the following fashion: To a 20-ml serum bottle was added 1 ml of a solution containing Tris-HCl (150 μmoles, pH 7·4 at 25°), $MgCl_2$ (15 μmoles), glucose 6-phosphate (30 μmoles), NADP (1·5 μmoles), and glucose 6-phosphate dehydrogenase (2 units*). Either 1 ml of 3 mM aniline hydrochloride or 3 mM ethylmorphine hydrochloride in water and 1 ml of the enzyme preparation were added to give a total incubation volume of 3 ml. The samples were incubated in air at 37° for 10 min. Under these conditions both reactions are linear with respect to time and protein concentration. The hydroxylation of aniline was stopped by placing the vessels in an ice bath. The amount of *p*-aminophenol formed was determined by the addition of a 2·6-ml portion of the incubate to a 45-ml stoppered centrifuge tube containing 25 ml ether and about 1 g of solid NaCl. The tubes were shaken vigorously by hand. A 20-ml portion of the ether phase was transferred to a second 45-ml stoppered centrifuge tube to which was added 1% aqueous phenol (1·6 ml) followed by 0·5 M K_3PO_4 (0·4 ml). The tubes were shaken, the color was allowed to develop for 20–30 min, and the absorbancy of the aqueous phase was

*One unit reduces 1 μmole NADP/min at 37° and pH 7·4.

determined at 650 nm. The *N*-demethylation of ethylmorphine was stopped by the addition of 5% $ZnSO_4$ (1 ml) to each vessel, followed by a 3:1 mixture of saturated solutions of $Ba(OH)_2$:$(Na_2)B_4O_7$ (2 ml). After centrifugation, the formaldehyde in the clear supernatant was determined according to the method of Nash.[6]

The level of cytochrome P-450 was determined by passing carbon monoxide through a suspension of microsomes (2 or 3 mg protein/ml) for 5 min and measuring the absorbancy at 450 and 490 nm in a Gilford 2000 spectrophotometer before and after the addition of a few milligrams of sodium hydrosulfite (dithionite). The concentration of cytochrome P-450 was taken as the change in the difference spectrum on the addition of dithionite.

All data were analyzed by either two-way analysis of variance or the Student *t*-test, as indicated by the experimental design. $P < 0.05$ was taken as significant.

RESULTS

The inclusion of orotic acid in the diet significantly decreased aniline hydroxylase activity in the liver 9000 *g* supernatant of males, but did not significantly affect the activity in that of females (Table 1). Adenine sulfate, which by itself did not affect aniline hydroxylase, partially prevented the impairing effects of orotic acid in the liver preparation from male rats.

TABLE 1. ANILINE HYDROXYLASE IN THE 9000 *g* SUPERNATANT FROM THE LIVERS OF MALE AND FEMALE RATS FED A DIET CONTAINING OROTIC ACID OR ADENINE SULFATE FOR 6 DAYS*

Rats	Treatment	*p*-Aminophenol formed			
		(nmoles/g liver/10 min)		(nmoles formed/liver/10 min)	
		Control	1% Orotic acid	Control	1% Orotic acid
Male	Control	123 ± 10	43 ± 6	1683 ± 111	640 ± 96
	0·25% Adenine sulfate	112 ± 11	81 ±11	1613 ± 183	1244 ± 116
Female	Control	60 ± 7	47 ± 8	538 ± 32	678 ± 119
	0·25% Adenine sulfate	77 ± 14	68 ± 3	504 ± 62	635 ± 29

* Preparations and assays are given in the text. There were four animals per treatment. Values are average ± standard error. The effect of orotic acid in male rats was significant ($P < 0.001$) both on a per gram and per liver basis. The interaction of orotic acid and adenine sulfate was also significant ($P < 0.05$ on a per gram basis; $P < 0.025$ on a per liver basis).

Since we supplemented the diet with orotic acid for 6 days, the livers of the treated animals were only 5–10 per cent heavier than controls. The decrease in enzymatic activity per gram of liver in males is not due to the dilution of the same amount of enzyme by triglycerides, since the activity per liver is also significantly depressed (Table 1). Nor is the decrease due to a loss of hepatic microsomal protein, since the amount of microsomal protein per liver is not significantly affected by the orotic acid diet (Table 2).*

* It should be noted that it is sometimes difficult to measure the level of proteins in treated animal due to the increase in the turbidity of the solutions. In this experiment, the turbidity was determined in samples to which 0·5 ml water was added instead of the Folin reagent.

On the other hand, the orotic acid diet and the adenine sulfate–orotic acid diets did not alter the rate of ethylmorphine demethylation by the liver 9000 *g* supernatant of either male or female rats (Table 3). Since both ethylmorphine and hexobarbital cause type I spectral changes, these results are in agreement with the observations of Rubin.[2] Although the adenine sulfate diet appeared to decrease the activity of the ethylmorphine *N*-demethylase in the experiment reported in Table 3, the effect did not always occur in other experiments.

TABLE 2. TOTAL HEPATIC MICROSOMAL PROTEIN OF MALE RATS FED A DIET CONTAINING OROTIC ACID OR ADENINE SULFATE FOR 6 DAYS*

Treatment	Microsomal protein (mg/liver)	
	Control	1% Orotic acid
Control	317 ± 28†	291 ± 24
0·25% Adenine sulfate	312 ± 6·7	319 ± 29

* Preparations and assays are described in the text.
† There were five animals per treatment. Values are average ± standard error.

TABLE 3. ETHYLMORPHINE *N*-DEMETHYLASE PER LIVER BY MICROSOMES FROM THE LIVERS OF MALE AND FEMALE RATS FED A DIET CONTAINING OROTIC ACID OR ADENINE SULFATE FOR 6 DAYS*

Rats	Treatment	HCHO formed (μmoles/liver/10 min)	
		Control	1% Orotic acid
Male	Control	55·3 ± 3·2†	43·7 ± 5·8
	0·25% Adenine sulfate	40·9 ± 4·7	49·9 ± 5·9
Female	Control	11·1 ± 1·7†	11·5 ± 0·5
	0·25% Adenine sulfate	9·6 ± 0·7	12·2 ± 1·8

*Preparation and assays are described in the text.
† There were five animals per treatment. Values are average ± standard error.

It seemed possible that the impairing effects of the orotic acid diet on the aniline hydroxylase might be due to the accumulation of an inhibitor in the soluble fraction. When the 9000 *g* supernatant was reconstituted by combining the microsomal and soluble fractions from animals fed orotic acid and from control animals (Table 4), it became evident that the decrease in aniline hydroxylase activity caused by the orotic acid diet was due to an impairment in the liver microsomes and not to an inhibitor in the soluble fraction. The activity of ethylmorphine *N*-demethylase also tended to be lower in the microsomes from the animals fed orotic acid than in those from controls, but the decrease was not statistically significant (Table 3). The soluble fraction from the rats fed the orotic acid diet actually enhanced aniline hydroxylation by microsomes

TABLE 4. EFFECT OF SOLUBLE FRACTION ON THE ANILINE HYDROXYLASE AND ETHYLMORPHINE *N*-DEMETHYLASE BY HEPATIC MICROSOMES FROM MALE RATS FED OROTIC ACID FOR 6 DAYS*

Aniline hydroxylase	*p*-Aminophenol formed (nmoles/10 min/mg protein) soluble fraction	
Microsomes	Control	1% Orotic acid
Control	1·29	1·76
1% Orotic acid	0·64	0·90

	HCHO formed (nmoles/10 min/mg protein)	
Ethylmorphine *N*-demethylase	Soluble fraction	
Microsomes	Control	1% Orotic acid
Control	27·5	28·3
1% Orotic acid	24·4	21·1

* Preparations and assays are given in the text.

TABLE 5. EFFECT OF URIDINE 5′-MONOPHOSPHATE (UMP) ON THE ANILINE HYDROXYLASE OF MICROSOMES FROM A MALE RAT*

Concn of UMP (M)	*p*-Aminophenol formed (nmoles/10 min/mg protein)	% Control
0	3·81	—
1×10^{-3}	4·43	116
5×10^{-3}	4·67	122

* Preparations and assays are given in the text.

from both control and orotic acid-treated animals, but did not appreciably affect ethylmorphine *N*-demethylation. Since uridine 5′-phosphate (UMP) accumulates in the liver soluble fraction of rats receiving orotic acid, it was noteworthy that UMP added to the incubation mixtures stimulated aniline hydroxylation (Table 5). The magnitude of the increase caused by UMP, however, was insufficient to account for all the stimulatory effect of the liver soluble fraction from orotic acid-fed rats.

Studies with liver microsomes revealed that the orotic acid diet decreases the amount of cytochrome P-450 in males, but not in females (Table 6). This decrease in the cytochrome P-450, however, can account for only about $\frac{2}{3}$ of the decrease in aniline hydroxylase activity. Thus, the activity per unit of cytochrome P-450 is significantly decreased in animals fed orotic acid. In this experiment there was also a slight decrease in the activity of aniline hydroxylase in female rats, while in the experiments with the 9000 *g* supernatant there was no decrease (Table 1). The slight stimulatory effect of the soluble fraction from the treated animals (Table 4) with the slight depression of the microsomal enzymatic activity observed here balances out in the 9000 *g* supernatant to give the same levels as in the controls.

TABLE 6. ANILINE HYDROXYLASE ACTIVITY AND CONCENTRATION OF CYTOCHROME P-450 PER MG OF HEPATIC MICROSOMAL PROTEIN AND ANILINE HYDROXYLASE ACTIVITY PER UNIT OF CYTOCHROME P-450 IN MALE AND FEMALE RATS RECEIVING A CONTROL DIET OR A DIET SUPPLEMENT WITH OROTIC ACID FOR 6 DAYS*

Rats	Treatment	Aniline hydroxylase (nmoles *p*-aminophenol formed/10 min/mg protein)	Cytochrome-P-450 ($\Delta E_{450-490}$/mg protein)	Aniline hydroxylase (nmole *p*-aminophenol formed/10 min/unit cytochrome P-450)
Male	Control	7·07 ± 0·50†	0·093 ± 0·004	76·6 ± 5·6
	1% Orotic acid	3·50 ± 0·40	0·058 ± 0·005	60·3 ± 5·3
Female	Control	4·27 ± 0·12	0·063 ± 0·002	67·5 ± 2·4
	1% Orotic acid	3·17 ± 0·16	0·061 ± 0·003	52·2 ± 1·5
Statistical significance of:				
	Sex	$P < 0·001$	$P < 0·005$	NS‡
	Orotic acid	$P < 0·001$	$P < 0·001$	$P < 0·005$
	Interaction of sex and orotic acid	$P < 0·005$	$P < 0·001$	NS‡

* Preparations and assays are given in the text.
† Values are averages of six animals ± standard error.
‡ Not significant.

TABLE 7. ANILINE HYDROXYLASE PER GRAM OF LIVER BY 9000 *g* SUPERNATANT FROM THE LIVERS OF MALE AND FEMALE RATS FED A PURIFIED DIET OR STANDARD LABORATORY CHOW FOR 6 DAYS*

Rats	*p*-Aminophenol formed (nmoles/g liver/10 min)	
	Chow	Purified diet
Male	109 ± 17†	163 ± 25
Female	113 ± 19	99 ± 8

* Preparations and assays are given in the text.
† The values are averages of four animals ± standard error.

The marked sex difference in cytochrome P-450 content and aniline hydroxylase activity in liver preparations from control rats (Tables 1 and 6) was unexpected, because other investigators have found less than a 10 per cent sex variation in these parameters.[3] In the other studies, however, the rats were fed a chow diet rather than a purified diet. As shown in Table 7, this difference in diet accounts for the failure of others to find a sex difference in aniline hydroxylation.

DISCUSSION

Orotic acid is an intermediate in the biological synthesis of pyrimidine nucleotides. When the diet of the rat is supplemented with this compound, the hepatic uridine nucleotide concentration increases while the adenine nucleotide concentration decreases. It is not entirely clear as to how or, even whether, these changes in nucleotide concentrations cause the massive accumulation of hepatic triglycerides. There is evidence that orotic acid causes a selective inhibition of the synthesis of the

β-lipoproteins which transport the triglycerides from the liver cells. This inhibition of protein synthesis and the resulting triglyceride accumulation are reversed by the addition of adenine sulfate along with the orotic acid. These data would suggest that the synthesis of at least some proteins may be regulated by the balance of nucleotide concentrations in the liver cell.

We have noted a similar decrease in the concentration of a single enzyme system, the aniline hydroxylase in the male rat. This reduction in activity is not related to a loss of microsomal protein and is out of proportion to the loss of cytochrome P-450. It is unlikely from the evidence we have presented that this effect is due to some inhibitory factor in the soluble fraction of the cell. But rather there appears to be a change not only in the cytochrome P-450 content but also in the properties of the enzyme involved in the hydroxylation.

It is possible that the changes in liver nucleotides may act to repress the synthesis of one of the "isozymes" of aniline hydroxylase. Since these enzymes have not been solubilized and purified, the kinetic evidence for the existence of isozymes is, at this time, highly circumstantial. Without some method for demonstrating their existence, it is not possible to examine this hypothesis.

Equally possible, and equally difficult to test, is the hypothesis that the abnormal nucleotide concentration ratios may cause changes in the sequence of amino acids by causing misreadings of the code, either in translation or transcription.

Alternatively the effects may be more direct. Recent work has indicated that orotic acid feeding increases the concentration of lipid peroxides.[7] It is possible that aniline hydroxylase of male rats may be more sensitive to lipid peroxidation than that of female rats.

It should be noted that the treated animals did not eat as much as controls and thus failed to gain as much weight. Starvation would not seem to be a likely cause for the observed changes in enzyme concentrations, since in starvation aniline hydroxylase in females increases while in males it does not change.[3, 8] Further, the rate of metabolism of substrates like ethylmorphine is significantly lower in the starved male rat than in the fed, but is relatively unaffected in the female. These changes in the male are the opposite of those we observed in the present studies.

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