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DETERMINANTS OF HEPATIC AMINOPYRINE DEMETHYLASE ACTIVITY*

LESTER F. SOYKA

Department of Pediatrics and Human Development,
Stanford University School of Medicine,
Stanford, Calif. 94305, U.S.A.

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Abstract—A study has been made of age and maturational determinants as well as of the incubation conditions which effect aminopyrine demethylase activity of rat liver. The specific activity of *N*-demethylation *in vitro* by the 9000 *g* supernatant fraction increased about 3-fold in the first 30 days after birth. The relative deficiency of the newborn was not affected by increasing the supply of NADPH. No evidence for an inhibitor or for the presence of an inactive form of the enzyme during the newborn period was found. After puberty, the male rat, under the influence of androgen, showed a further increase per unit of microsomal protein. Nonetheless, the marked increase in overall capability to demethylate resulted from the increase in liver size. Undernourished rats had a defect in metabolism greater than that which could be accounted for on the basis of decreased body and liver weight. The finding of different effects of pH on the enzymatic activity of newborns and adults suggests the presence of age-dependent differences in the enzyme(s) or inhibitor(s) involved in the demethylation of aminopyrine.

OXIDATIVE *N*-dealkylation, perhaps truly a hydroxylation reaction,[1] removes alkyl groups from secondary or tertiary amines to form aldehydes and primary amines. It is an important step in the metabolism of a number of clinically useful drugs, e.g. *N*-methyl barbiturates[2] and narcotic alkaloids such as codeine and morphine,[3] ephedrine[4] and amphetamine. Aminopyrine is an active substrate for this reaction and has been frequently employed in studies of hepatic microsomal drug metabolism.[5–10]

Reports have indicated that demethylation of aminopyrine is deficient or absent in the newborn mouse and guinea pig,[11] rabbit[12] and rat.[13] However, the extensive studies of Kato *et al.*[14] showed that rates of drug metabolism are not necessarily stable after the neonatal period. The metabolic capacity of rats for pentobarbital and zoxazolamine reached a maximum at 30 days and declined thereafter. Values at 15 and 250 days of age were equivalent. Similarly, Catz and Yaffe[15] found a striking decrease in effect and increase in metabolism of hexobarbital in the mouse at 3 weeks of age.

We were prompted to examine the influence of age as a determinant of demethylation capacity in the rat in order to define more quantitatively its development during the newborn period and to study variations during adulthood.

MATERIALS AND METHODS

Pregnant rats of the Sprague–Dawley strain were obtained from the Hormone Assay Laboratory, Chicago, Ill. They were kept at a constant temperature (23°) and

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continual illumination. Rodent pellets* and tap water were allowed *ad libitum*. The cages were covered with towels prior to delivery to limit maternal cannibalism. The day of birth was considered the first day of life. Animals were not fasted prior to sacrifice, for the sake of uniformity, since it was not feasible to fast newborn rats. It is recognized that loss of endoplasmic reticulum occurs during the centrifugal isolation procedure when glycogen deposits are plentiful.[16]

The following procedures were carried out in a cold room. The livers were quickly and totally removed, washed in 1·15% KCl, pH 7·4, until clear of blood, blotted dry and weighed. A 1-g aliquot, or 2–3 livers pooled in the case of newborns, was homogenized with 3–5 vol. of 1·15% KCl in a modified Potter–Elvehjem homogenizer.† The homogenate was centrifuged at 9000 *g* (average) for 25 min. Supernatant equivalent to 0·3 g of whole liver was used as an enzyme source for the incubations which were carried out in duplicate. The supernatant was used immediately because storage caused a decrease in activity within a few hours. This property of rat liver homogenates has been discussed by Gillette.[1] Conditions were usually as described by Mazel *et al.*:[17] each incubation flask contained (in micromoles) aminopyrine, 10; NADP, 0·65, glucose 6-phosphate (G-6-P), 1·0; nicotinamide, 50; and $MgCl_2$, 25, in 0·5 M phosphate buffer, pH 7·4, in a final volume of 6·0 ml. Semicarbazide hydrochloride, 45 μmoles, was added to trap formaldehyde (HCHO) released during incubation. After 15 min of incubation in a Dubnoff metabolic shaker at 37°, the reaction was terminated by addition of 2 ml of 20% $ZnSO_4$ and 2 ml of a saturated solution of $Ba(OH)_2$. After centrifugation, 5 ml of the supernatant was taken for estimation of HCHO by the Nash reaction. Correction was made for HCHO production other than that from substrate by incubation of flasks containing tissue and the NADPH generating system, but omitting substrate and an unincubated "zero-time" flask containing all components. These "blank" values differed for each age group and for the amount of G-6-P added so that individual blanks were necessary in each study. Concentrations of enzyme, substrate and cofactors, as well as the duration of incubation and pH, were altered in various studies; exact details are given in the table captions.

RESULTS

Incubation conditions

Time course. The length of time during which the reaction proceeded by zero kinetics depended upon the relationship between the activity of the NADPH generating system and that of the enzyme present. Therefore, incubation conditions were arranged to provide linearity for at least 15 min. With larger amounts of enzyme and/or more active enzyme (male vs. female) 1 μmole G-6-P was inadequate (Fig. 1A). Even if linearity for 15 min was accomplished (Fig. 1B), the use of too small a quantity of G-6-P limited formaldehyde production so that the activity of adult liver appeared to be less than that from 20-day-old animals if studied after 30 min of incubation (Fig. 1, C and D). The decrease in rate after 15 or more min was restored by a second addition of G-6-P, but not of substrate or NADP (Table 1).

Cofactors. Additional studies were necessary to establish optimum conditions, since it appeared that in a number of reports in the literature a linear reaction had not been followed.

* Ralston-Purina Co., St. Louis, Mo.
† Kontes Glass Company, Ridgeland, N.J.

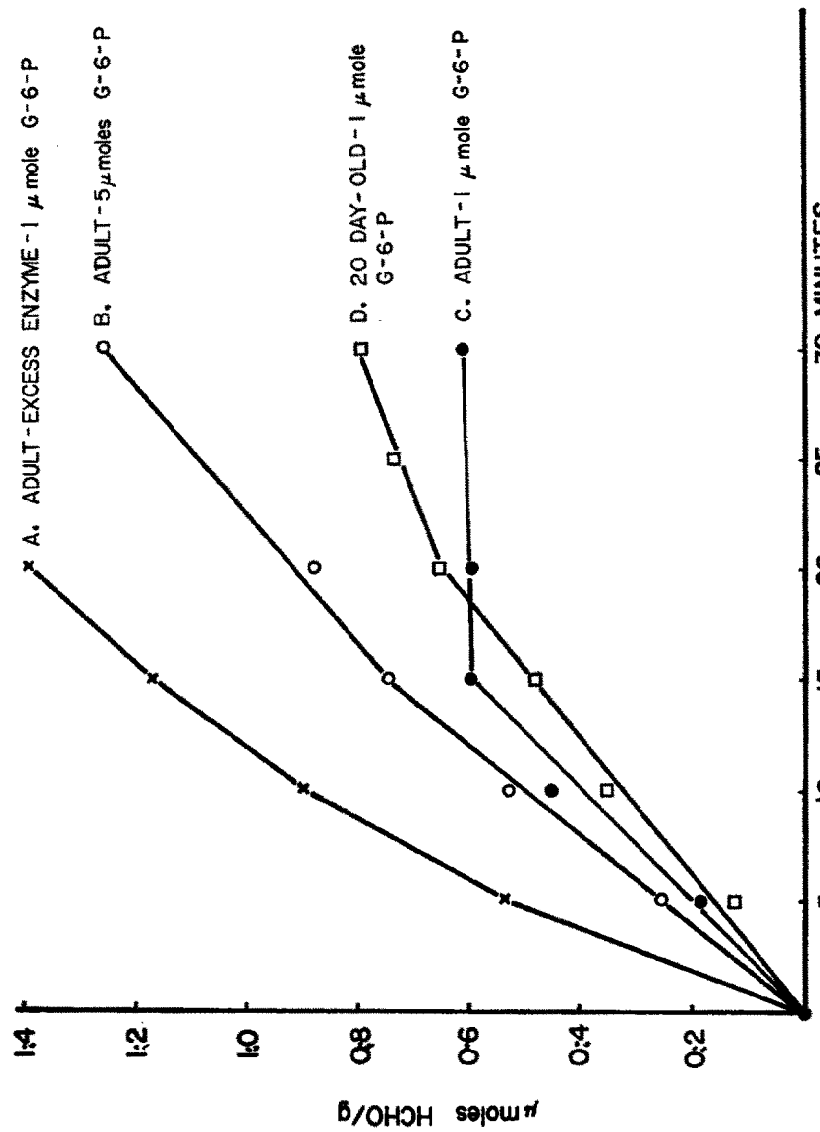

FIG. 1. Time course of formaldehyde production from aminopyrine. The quantities of enzyme (9000 *g* supernatant) and G-6-P were altered as detailed below. Each flask contained equal amounts of substrate and the other cofactors. Incubation was at 37° in air. Values are the means of duplicate determinations. A, supernatant equivalent to 0·5 g liver from an adult male rat with 1 μmole G-6-P; B, supernatant equivalent to 0·3 g liver from an adult female rat with 5 μmoles or C, 1 μmole G-6-P; D, supernatant equivalent to 0·3 g liver from a 20-day-old rat with 1 μmole G-6-P.

Increasing the amount of G-6-P from 1 to 15 μmoles caused an approx. parallel increase in formaldehyde production by supernatant from both newborn (8-day-old) and adult liver, thus preserving the ratio of activities. This point was further established by an experiment in which both NADP (0·32–2·6μ moles) and G-6-P (1 or 5 μmoles) concentrations were varied (Fig. 2). Activity increased with both concentrations of G-6-P when NADP was increased from 0·32 to 0·65 μmole, but not

TABLE 1. EFFECT OF A SECOND ADDITION OF SUBSTRATE OR COFACTORS AFTER 15 min OF INCUBATION ON FORMALDEHYDE (HCHO) PRODUCTION FROM AMINOPYRINE

Additions	HCHO (mμmoles/g wet wt. of liver)
None	160
10 μmoles Aminopyrine	160
0·65 μmole NADP	220
1·0 μmole Glucose 6-phosphate	420
NADP + G-6-P	420

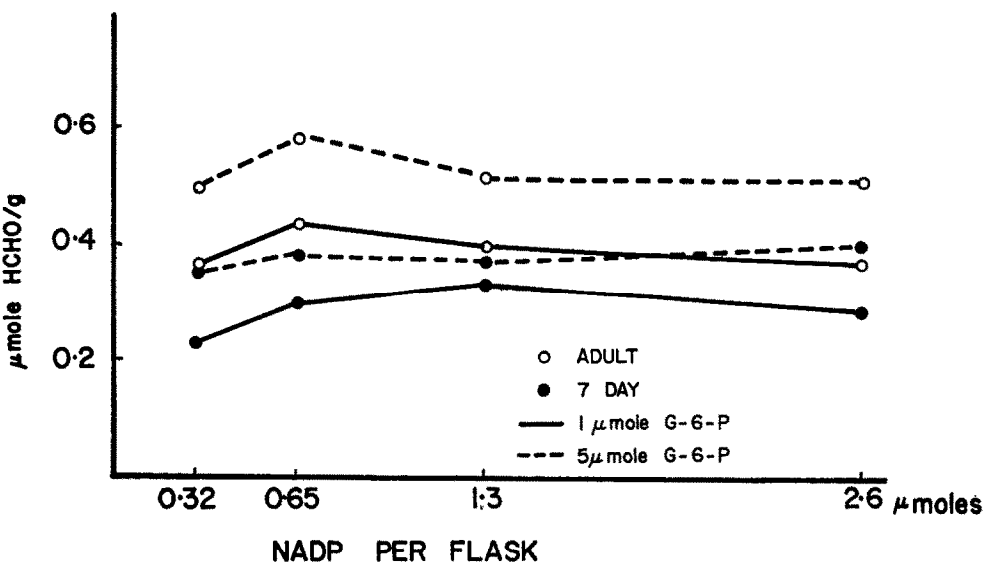

FIG. 2. Formaldehyde production from aminopyrine as related to the quantities of NADP and G-6-P. Each flask contained 10 μmoles aminopyrine and 9000 *g* supernatant equivalent to 0·3 g liver. With the largest amount of NADP employed, the final concentration was $4{\cdot}32 \times 10^{-4}$ M; with 5 μmoles G-6-P, it was $8{\cdot}33 \times 10^{-4}$ M. Flasks were incubated for 15 min at 37°.

with larger amounts. Formaldehyde production was higher with 5 μ moles G-6-P than with 1 μmole at each concentration of NADP. Nonetheless, the ratio of adult to newborn was approx. 1:4 with each of the eight combinations of NADP and G-6-P concentrations studied. Addition of 5–10 units of glucose 6-phosphate dehydrogenase (G-6-PD)* had no effect (not illustrated).

Enzyme. The 9000 *g* supernatant from adult rats is known to contain the bulk of drug-metabolizing liver enzymes when prepared by the usual procedures. This was not

* Nutritional Biochemical Co., Cleveland, Ohio; 8000 units/mg.

TABLE 2. DISTRIBUTION OF AMINOPYRINE DEMETHYLASE ACTIVITY IN FRACTIONS OF RAT LIVER HOMOGENATES OBTAINED BY CENTRIFUGATION*

Age	Enzyme source			
	Whole homogenate	200 *g* Pellet	9000 *g* Pellet	9000 *g* Supernatant
2 hr	167	50	83	83
2 days	317	183	183	317
10 days	407	133	60	483
20 days	367	50	100	450
60 days	327	173	267	1040
5 months†	400	250	183	490

* Livers were pooled or a 1·5-g aliquot taken, homogenized with 5 vol. of 1·15% KCl and brought to a final volume of 10·5 ml. A 4·5-ml aliquot was taken as the "whole homogenate." The remainder was centrifuged at 200 *g* for 10 min at 4°. The supernatant was then centrifuged at 9000 *g* for 25 min. The fractions were diluted so that each flask contained tissue derived from 0·3 g liver. A separate zero-time flask for each fraction at each age was used for the blank value. Values are in HCHO produced (mμmoles/g wet wt. of liver) and are the means of two determinations.

† Pregnant.

found to be true in the case of fetal and newborn animals. In a series of experiments, the activity of the whole homogenate was compared with that of the fraction sedimenting at 200 *g*, at 9000 *g* and the 9000 *g* supernatant. Substantial activity was found in the 200 *g* and 9000 *g* pellets; in fact, the latter fraction was as active as the 9000 *g* supernatant in animals 2 hr old (Table 2). Even in older rats, the 9000 *g* pellet contained about ¼ the activity of the supernatant.

A striking and unexplained finding, possibly due to the presence of phosphatases and oxgenases, was the uniformly low activity of the whole homogenate compared to that of the 9000 *g* supernatant, which exhibited a steady increase in activity with age. In addition, the total activity of the 9000 *g* pellet and the supernatant equalled that of the homogenate only at 1 day of age. Afterwards, the total activity of the fractions increasingly exceeded that of the homogenate, so that by 60 days of age, it was four times greater.

In part, the lesser activity of the homogenate could be overcome by increasing the concentration of the components of the NADPH generating system. By doubling the amount of NADP (1·3 μmoles), a 5-fold increase in G-6-P (50 μmoles) and the addition

TABLE 3. EFFECT OF THE pH OF INCUBATION MEDIA ON FORMALDEHYDE PRODUCTION FROM AMINOPYRINE BY 9000 *g* SUPERNATANTS OF LIVER HOMOGENATES OF IMMATURE AND ADULT RATS*

Age	HCHO (pH 7·4) (mμmoles/g wet wt.)	Per cent of value at pH 7·4				
		pH of Media				
		6·9	7·4	7·6	7·9	8·5
6–21 days	212 ± 35	56 ± 16(6)	100(8)	155 ± 18(3)	145 ± 29(7)	50 ± 13(4)
4–16 months	431 ± 134	80 ± 4(6)	100(7)	110(2)	76 ± 12(6)	6 ± 1(3)
P	0·001	>0·05	—	—	<0·05	0·02

* Values are mean ± S.E.M. Numbers in parentheses indicate number of separate experiments, each performed in duplicate.

of G-6-PD (10 units), a 7-fold increase was produced in whole homogenate activity compared to a 2·5-fold increase with the 9000 *g* supernatant. Nonetheless, under these conditions the total activity of the fractions was still 2·5 times greater than that of the homogenate.

pH of media. To determine if qualitative differences existed in the demethylase enzyme(s), studies were made of the pH optima. Activity relative to that at pH 7·4 was lower at pH 6·9, but higher at pH levels of 7·6, 7·9 and 8·5 for supernatant from newborn (6–21 days) than from adult rats (Table 3). Activity per gram wet weight of adult liver at pH 7·4 was twice that of the newborns in these experiments.

Influence of age

Activity (formaldehyde production) per unit of tissue (here expressed per gram of tissue, but similar per milligram of protein) increased 3- to 4-fold from birth to about 30 days of age and was rather stable thereafter (Table 4).

TABLE 4. FORMALDEHYDE PRODUCTION FROM AMINOPYRINE AS RELATED TO AGE AND SEX

Age (days)	No.	Sex	Body wt. (g)	Liver wt. (g)	HCHO production (mμmoles) per g wet wt.*	HCHO production (mμmoles) per total liver*	per g wet wt. relative to day 1
1	12	Both	5·7	0·25	222 ± 22	55 ± 6	
2	5	Both	7·0	0·26	204 ± 49	51 ± 11	1·0
3	7	Both	9·3	0·32	273 ± 55	89 ± 19	1·2
5	8	Both	14·7	0·56	274 ± 67	154 ± 38	1·2
8	6	Both	14·0	0·54	349 ± 27	188 ± 14	1·6
12	6	Both	22·2	0·71	438 ± 21	310 ± 13	2·0
32	6	M	70	3·16	923 ± 66	2936 ± 361	4·2
35	10	F	96	3·71	646 ± 43	2457 ± 249	2·9
35	4	M	125	4·89	820 ± 88	4151 ± 430	3·7
45	4	F	145	6·38	454 ± 49	2848 ± 211	2·0
45	2	M	189	8·23	924	7705	4·2
50†	3	F	120	5·54	308	1689	1·4
50†	3	M	159	6·86	648	4439	2·9
72	5	F	167	6·22	368 ± 46	2257 ± 243	1·7
72	3	M	231	8·00	949	7648	4·3
80	6	M	282	11·31	684 ± 38	7695 ± 404	3·1
150	4	F	281	9·51	773 ± 118	7875 ± 1568	3·5

* Mean ± S.E.M.
† Undernourished (see text for discussion).

As noted above, apparent enzyme activity depended upon duration of incubation, concentration of cofactors, etc. Nevertheless, since these alterations affected each age group to the same extent, a valid interpretation of the data was made possible by relating them to an arbitrary standard, e.g. activity on day 1 (last column, Table 4).

Although liver size bore a rather constant relationship to body weight (about 4 per cent), when aminopyrine demethylase activity per animal was considered, the capacity for demethylation increased 150 times from birth to adulthood in the female and as much as 300 times in the male.

Basis of deficiency of newborn. Studies were performed which failed to support three possible explanations for the deficient activity of the newborn: (1) the presence of an inhibitor in newborn liver, as suggested by Fouts and Adamson; (2) a masked form of the enzyme being present and capable of activation, as has been shown with mammalian

hepatic *p*-hydroxyphenyl-pyruvate oxidase;[18] (3) that the enzyme(s) is relatively inefficient and requires a larger supply of NADPH to carry out the demethylation reaction.

An inhibitor was sought by combining the 9000 *g* supernatant from newborn and adult rats in various proportions as the enzyme source. In each case, formaldehyde production was within 10 per cent of the predicted value (Table 5).

TABLE 5. A STUDY OF THE POSSIBLE INHIBITORY EFFECT OF NEWBORN RAT LIVER ON FORMALDEHYDE PRODUCTION BY ADULT LIVER*

9000 *gr* (% adult)	Supernatant (% newborn)	HCHO produced (μmoles/g)	Predicted (μmoles/g)	Found/Predicted × 100 (%)
100	0	1·35		
75	25	1·13	1·09	104
50	50	0·93	0·83	111
25	75	0·63	0·57	110
0	100	0·31		

* Values are the means of triplicate determinations. Each incubation flask contained 10 μmoles aminopyrine, 0·65 μmole NADP, 10 μmoles G-6-P with 0·5 M phosphate buffer containing $MgCl_2$, nicotinamide and semicarbazide at pH 7·4 and 9000 *g* supernatant equivalent to 0·3 g wet weight of liver. The experiment was repeated with essentially similar results.

Activation of the enzyme(s) was attempted by the method of Goswami and Knox.[18] The 9000 *g* supernatant from 3 g of liver from 10-day old animals was divided into two 10-ml portions. To one portion was added glutathione (GSH, 0·5 m-mole) and 2,6-dichlorophenolindophenol (DCPIP, 15 μmoles) in 0·5 ml of 0·1 M phosphate buffer, pH 7·4; to the other portion of supernatant, buffer only was added. After incubation for 15 min at 37°, the supernatant was used as enzyme source in the standard assay procedure. Formaldehyde production was significantly lower with the treated supernatant. Addition of GSH and DCPIP directly into flasks during incubation also resulted in decreased formaldehyde production.

Because increase in the quantity of the components of the NADPH generating system, especially G-6-P, led to increased yields of formaldehyde, the effect of stepwise increases of NADP, G-6-P and G-6-PD was studied. The largest quantities used were 5 μmoles, 50 μmoles and 50 units respectively. Although production increased, the ratio of adult to newborn (10 days old) remained approx. 2, the same ratio as that seen in Table 4 when 0·65 μmole NADP and 1 μmole G-6-P were used.

Influence of sex

After sexual maturation, activity per unit of liver became about twice as high for males as for females (Table 4). In this study, a sex-related difference was detected after 24 days of age. This corresponds to the time of testicular descent, which was found to occur in 85 per cent of Wistar rats between 18 and 31 days.[19] In a single experiment, a sex difference (males higher than females) was also noted with S-methylisothiourea, but not with 6-methylaminopyrine as substrate. The difference per unit of liver was, of course, magnified when total metabolic capacity was considered, because of the greater liver weight of the male.

To ascertain if this difference was hormonally determined, a group of young adult female rats was treated with testosterone. Only 7 daily injections of 1 mg were administered to avoid changes in body or liver weights.

Specific and total activity increased 23 and 38 per cent respectively (Table 6). Because of marked group variability, these increases were not statistically significant. Nonetheless, these results are compatible with those of Quinn *et al.*,[6] who reported a decrease in hexobarbital sleeping time and an increased capacity to oxidize the drug

TABLE 6. INFLUENCE OF ANDROGEN ON AMINOPYRINE DEMETHYLASE ACTIVITY OF THE FEMALE RAT*

Group	Body wt.		Liver wt.		HCHO production (mμmoles/g)		HCHO production (mμmoles/liver)	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
Testosterone	273	18	9·6	0·9	649	137	6106	803
Control	284	31	10·4	0·5	528	149	5446	1506
P	N.S.		N.S.		N.S.		N.S.	

* Five adult female Sprague–Dawley rats were injected with 1·0 mg testosterone in oil i.m. each day for 7 days and sacrificed 24 hr after the last injection. An equal number of female rats of the same age were injected on the same schedule with 0·9% NaCl solution. N.S., not significant.

in vitro after 7 weeks of administration of testosterone to female rats. Similar hormonally mediated changes in the effect and metabolism of pentobarbital have been reported.[20]

Age vs. size

Nutritional adequacy obviously influences body size and also is a determinant of demethylase activity.[7] A group of weanling rats obtained directly from the commercial suppliers had a body weight in excess of that of pups raised in our animal farm (Table 7), presumably reflecting improved nutrition and housing conditions. Demethylase activity per gram of liver was 2·5 times greater in the larger animals; on a whole liver basis, the activity was 7·6 times greater because of their heavier livers.

Another group of undersized animals was produced by maintaining sixteen pups with the mother rather than the customary eight. At 50 days of age, activity per unit of tissue and per liver were both diminished compared to age-matched controls (Table 4).

TABLE 7. EFFECT OF BODY SIZE (NUTRITION) ON AMINOPYRINE DEMETHYLASE ACTIVITY OF LIVER SUPERNATANT OF 24-DAY-OLD RATS

	No. of animals		Body wt.	Liver wt.	Demethylase activity per g liver	Demethylase activity per liver
	M	F				
Free-fed	4	2	69	3·234	1·09 ± 0·08	3·47 ± 0·29
Limited diet	3	2	27	1·051*	0·44 ± 0·05†	0·46 ± 0·07*
Ratio			2·6	3·1	2·5	7·6

* Free-fed vs. limited diet: $P < 0·001$.
† Free-fed vs. limited diet: $P < 0·05$.

DISCUSSION

The data obtained confirm certain previous observations of *N*-dealkylation by rat liver: (1) increased specific activity during the first week of life (from 25 to 45 per cent

of adult female values in our studies; from 17 to 30 per cent in those of Dallner *et al.*[13]); (2) a sex difference developing concurrent with sexual maturation,[6,10] with males having approx. twice the specific activity of females; (3) the important role of nutrition.[10]

Our data on pH optima suggest that the overall reaction "aminopyrine to formaldehyde" may not be the same in newborns and adults. LaDu *et al.*[5] and Gram *et al.*[21] have delineated two steps in the reaction, the rapid removal of one methyl group from the tertiary amine followed by a much slower demethylation of the product, monomethylaminopyrine. Gram *et al.*[21] could not determine if these reactions were catalyzed by different enzymes or enzymatic sites or if the second reaction is inhibited by aminopyrine. If the former situation is present, i.e. two enzymes are involved, we would postulate that it is the first step which may differ with age, since our studies mainly measure formaldehyde resulting from the initial demethylation. Differences in activity of enzyme from newborn or adult with changes in pH could result from differences in their pH optima or susceptibility to inhibition. Alternatively, if the enzymes do not differ with age, an inhibitor(s) may be present in the newborn whose activity or amount is pH dependent. Kinetic studies of the disappearance of aminopyrine and concurrent measurement of the appearance of monomethylaminopyrine, 4-aminoantipyrine and formaldehyde are necessary to solve this important problem.

The long duration of "immaturity" (about 30 days) and our inability to identify inhibitory factors or masked enzyme in the newborn period fail to answer the critical question as to the rate-limiting step in the development of aminopyrine demethylase activity. Histologically, the endoplasmic reticulum in the rat has developed completely by 3 days of age.[13] Dallner *et al.*[13] have also reported that the electron transfer enzymes and cytochromes of the mixed-function oxidase system have reached, or closely approached, adult levels by 7 days of age. Moreover, immature rabbits exhibit elevated demethylase activity when exposed to phenobarbital,[8] proving that at least in one species the system is responsive to inducing drugs and capable of increased activity. The defect appears to be in the hydroxylation step itself, which undergoes a gradual development to a basal level, which can be further increased by androgens or "inducing drugs" such as barbiturates.

Although no evidence for an inhibitor of aminopyrine metabolism was found, as previously reported for newborn rabbit homogenates,[12] such an explanation for apparent enzyme deficiency remains a possibility. If all inhibitor molecules were tightly bound to enzyme in the newborn rat liver, our technique of mixing homogenates would not detect their presence.

These studies attest to the fact that "maturation" only changes demethylation capacity 3–4 times whereas simple growth of liver size is the chief reason for the overall increase of 100–300 times. Since liver weight bears a rather constant relationship to body weight, sensitivity to a pharmacologic effect (from a dose comparable on a mg/kg body weight basis) greater than 3- to 4-fold from birth to adulthood is probably not explicable simply as lessened detoxification by the "immature" liver. Although the artificial conditions existing *in vitro* may distort the precise values for differences in rate with age, the principle is generally valid. Further studies are required to quantify these relationships for other drugs metabolized by enzymes localized in the hepatic endoplasmic reticulum.

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