DIETARY FACTORS RELATED TO LIVER XANTHINE OXIDASE*

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In a previous study (1) it was shown that dogs maintained on a purified diet developed a defect in acetaldehyde metabolism. Of the five known enzyme systems that could theoretically be involved in acetaldehyde metabolism in vivo (aldolase, mutase, carboxylase, aldehyde oxidase, and xanthine oxidase), the xanthine and aldehyde oxidases seemed most likely to be dependent upon a dietary factor not already incorporated in the purified rations used. Our attention was therefore directed to a study of the relationship between liver xanthine oxidase in rats and the dietary factors supplied to the animals. In this way it was observed that some unidentified factor had to be supplied in the diet in order to obtain normal levels of xanthine oxidase in the liver (2).

Previous studies have implicated both riboflavin (3) and protein (4) as essential dietary factors required by the rat for the establishment of normal xanthine oxidase levels in the liver. Inanition has also been shown to decrease liver xanthine oxidase in the rat (5). Keith et al. (6) reported that the incorporation of less than 1 mg. per cent of folic acid in a purified diet decreased the xanthine oxidase activity of chick livers, but as far as rat liver xanthine oxidase is concerned, the feeding of folic acid or 6-pteridylaldehyde is without effect.

EXPERIMENTAL

Xanthine oxidase activity was determined in rat livers by the method of Axelrod and Elvehjem (3). A study of this method was carried out simultaneously with the dietary experiments reported herein, and the results showed that the activity measurement reflected the true order of magnitude of the xanthine oxidase content of the liver (7). All manometric determinations were run in duplicate and the final values for xanthine

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oxidase activity were expressed in units (c.mm. of O₂ consumed per gm. of dry liver per hour).

21 day-old rats were obtained as needed from the Albino Farms and Sprague-Dawley, Inc.; all others were from our own stock, originally Albino Farms animals. Both males and females were used without any detectable difference in response, but males were used predominantly. Early in this study a few 21 day-old animals from each group were sacrificed immediately for analysis of liver xanthine oxidase. The remainder was placed on one of the diets to be described for varying periods of time before their livers were similarly analyzed. Groups of six rats were generally used initially for each point studied, although larger groups were required in many instances to establish a difference as statistically significant.

All of the purified diets used were based upon the 21 per cent purified casein diet shown in Table I; all such diets were identical except for the specific differences noted. The 8 per cent casein diet contained 81 per cent glucose. The peanut protein diet contained 8 per cent casein, 22 per cent defatted peanut meal (contributing 13 per cent peanut protein and 1.5 per cent fat), 0.5 per cent Wesson oil, and 60.5 per cent glucose. The albumin diet contained 21 per cent dried egg albumin, instead of the casein, plus 0.15 mg. per cent of biotin, 0.5 per cent Na₂HPO₄, and 67.5 per cent glucose. 10 per cent Wilson’s whole dried liver was added to the 21 per cent purified casein diet at the expense of glucose. Dried whole milk powder and whole fluid milk were supplemented with a daily intake

Table I

<table>
<thead>
<tr>
<th>Purified Diet For Xanthine Oxidase Studies</th>
<th>gm.</th>
<th>mg.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein (vitamin test)*</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>Crisco</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Wesson oil</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Cod liver oil</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Salts (Phillips and Hart†)</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>68</td>
<td></td>
</tr>
<tr>
<td>Choline chloride</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>Calcium pantothenate</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Riboflavin</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>Thiamine</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>Pyridoxine</td>
<td>0.4</td>
<td></td>
</tr>
</tbody>
</table>

* Obtained from General Biochemicals, Inc.
of 42 γ of Mn and Cu, and 420 γ of iron as appropriate salts. Purina
dog chow, containing 21 per cent protein, was also fed as a natural un-
purified ration.

All the purified 21 per cent protein diets gave similar growth curves of
about 3.0 gm. per day. Purina dog chow and the 10 per cent liver diets
gave 3.0 to 4.0 gm. growth gains per day, while an increase of approxi-
mately 2.5 gm. per day was obtained with the milk diets. Growth on the 8
per cent casein diet averaged 0 to 0.5 gm. per day in weanling rats, while
feeding the 8 per cent casein diet to adult rats resulted in appreciable
weight gains due to an obvious deposition of fat.

Results

The livers from twelve normal adult rats maintained on Purina dog
chow had an average xanthine oxidase activity of 1553 units with a stand-
ard error of the mean of ±68 units. At birth, rat livers were devoid of
xanthine oxidase. No activity was found in seven pooled samples, each of
which contained all the livers from an entire litter of six to ten rats. At
the same time, five of the livers of mother rats had an average xanthine
oxidase activity of 1247 ± 101 units. At 12 days of age small amounts of
activity were found (0 to 300 units), indicating that the necessary dietary
factors were supplied in the mother’s milk. At 21 days of age, the starting
point for the dietary experiments, the liver xanthine oxidase activity
averaged 717 ± 116 units.

The results of feeding various diets to 21 day-old rats are shown in
Fig. 1. The natural rations gave nearly normal levels of xanthine oxidase
within 2 weeks. Purina dog chow, fluid milk, whole milk powder, and the
10 per cent whole liver diet gave values of 1535 ± 97, 1385 ± 38, 1535 ±
140, and 1292 ± 72 units, respectively. Feeding a 21 per cent crude
casein diet resulted in a significant increase in liver xanthine oxidase to
1260 ± 65 units after 4 weeks, but not within 2 weeks. When the purified
21 per cent protein diets containing casein, casein plus peanut protein,
or albumin were fed, the starting level of liver xanthine oxidase remained
essentially unchanged (500 to 1000 units) for at least 6 weeks. Feeding
the purified diet for 7 weeks, followed by dog chow for 3 weeks, gave
1433 ± 44 units. The fluctuations observed on the three purified diets
during the first 6 weeks were not significantly different from the values
obtained with weanling rats or from each other. The 40 to 66 per cent
higher values obtained with dog chow, fluid milk, milk powder, and 10
per cent liver within 2 weeks were all significantly higher than the 921 ±
77 units obtained with the 21 per cent purified casein diet in 2 weeks
(\(P = <0.01\)).

Increasing the protein content of the purified diet to a total of 25 per
cent and 30 per cent did not give appreciably different results from those obtained with the 21 per cent protein diets. These diets contained 8 per cent casein and the remaining 17 per cent and 22 per cent protein were contributed by defatted peanut meal at the expense of glucose. The livers of weanling rats placed on these diets were analyzed in groups of six after 2, 4, and 6 weeks. No differences were found in the groups with increasing dietary periods, and all of the rats on the 25 per cent protein diet averaged 1031 ± 65 units of liver xanthine oxidase. The 30 per cent protein diet gave 1108 ± 67 units as compared with the value of

927 ± 40 for all rats on the 21 per cent casein diet during the first 6 weeks \((P = 0.2 \text{ and } 0.03)\). The results obtained with liver and milk could not be attributed to the higher protein content of these diets. Supplementing the 21 per cent purified casein diet with 0.5 per cent methionine per cent cystine insignificantly increased the control level by 4 per cent \((P = 0.8)\) and 15 per cent \((P = 0.3)\), respectively.

Additional results of feeding experiments are shown in Fig. 2. An 8 per cent casein diet depleted the liver of xanthine oxidase, irrespective of the initial xanthine oxidase activity (Fig. 2). It also depleted the xanthine oxidase activity of adult rat livers, but at a somewhat slower rate (8).
Rats depleted of liver xanthine oxidase by a low protein diet restored this enzyme when the dietary essentials were supplied. Xanthine oxidase activities of 0 to 100 units obtained with a low protein diet were restored to $1361 \pm 92$ units by feeding dog chow for 4 weeks (Fig. 2). The 21 per cent purified protein diets gave values of 1000 to 1100 units under similar circumstances, which represented a remarkably good but incomplete restoration of the liver xanthine oxidase.

![Graph](http://www.jbc.org/)

**Fig. 2.** Changes in liver xanthine oxidase activity that resulted from alterations in the diet. Weanling rats were fed Purina dog chow (Curve C) or Diet III for 2 and 4 to 6 weeks respectively; the diets were then changed as indicated. The figures at each experimental point refer to the number of rat livers averaged to obtain the point. Curve II, 21 per cent purified casein; Curve III, 8 per cent casein; and Curve IV, 8 per cent casein and 13 per cent peanut-protein.

Weanling rats brought to a normal level of xanthine oxidase activity by feeding dog chow for 2 weeks could be partially depleted of this enzyme by feeding the 21 per cent purified protein diets. The casein diet reduced the xanthine oxidase activity to $1055 \pm 81$ units in 8 weeks, while the peanut protein diet resulted in livers with an average activity of $832 \pm 84$ units in 4 weeks. These results were significant decreases from the dog chow level ($P = <0.01$).

Diets containing sucrose or starch instead of glucose were fed to three
groups of six weanling rats each for 2 weeks. The liver xanthine oxidase activities were 613 ± 98, 771 ± 94, and 822 ± 88 units respectively for the sucrose, glucose, and starch diets (P = 0.3 and 0.7 in comparison with glucose). Adding liver to the sucrose diet gave normal xanthine oxidase levels in 4 weeks (the earliest tested; Table II). Hence the nature of the carbohydrate in the diet did not appear to be of major importance in this relationship.

The fat content of the diet was increased 2- and 3-fold by proportional increases in the three lipid constituents. The fat was added at the expense of glucose, and in other tests the casein and salts were simultaneously increased so that the intake of these constituents per 100 calories of diet remained the same as in the original control diet. When fed for 2 weeks to weanling rats, none of the higher fat diets gave liver xanthine oxidase levels that were significantly different from the simultaneous control group. The values for the 21 per cent fat diet with and without adjustment of the casein and salts intake were 1025 ± 119 and 939 ± 116 units, respectively, in comparison with the simultaneous control values of 814 ± 88 (P = 0.2 and 0.4).

Test Supplements---The 21 per cent purified casein diet was supplemented with known dietary factors and fed to groups of six weanling rats for 2 weeks without obtaining significant increases in liver xanthine oxidase above the control level established by the purified diet alone. The supplements tested and the percentage increases (plus per cent) or decreases (minus per cent) from the control values were as follows: (a) biotin 0.25, inositol 10.0, p-aminobenzoic acid 10.0, rutin 2.0, ergostanyl acetate 2.0, and folic acid 1.0 mg. per cent gave +4 per cent (P = 0.8); (b) biotin 0.25, inositol 10.0, and p-aminobenzoic acid 10 mg. per cent gave -25 per cent (P = 0.2); (c) riboflavine 1.2, adenine 10.0, D-ribose 20.0 mg. per cent gave -14 per cent (P = 0.3); (d) I 1.0, Zn 10, Mn 20, Cu 10, and Co 5 mg. per cent as appropriate salts gave -27 per cent (P = 0.3); (e) Armour's pernicious anemia extract (≈100 gm. of fresh liver per 100 gm. of diet) gave -12 per cent (P = 0.5); (f) Armour's vitamin B12 concentrate (≈100 per cent fresh liver) administered orally and subcutaneously gave +14 per cent (P = 0.06) and +7 per cent (P = 0.6) respectively; and (g) 5.0 mg. per cent of folic acid gave -3 per cent (P = 0.8). The injection of 0.175 γ daily of Merck's crystalline vitamin B12 for 4 weeks while feeding the purified diet gave +13 per cent (P = 0.3).

Xanthopterin gave erratic results which could not be reproduced consistently. Xanthopterin was tested in twenty groups of six rats each, by adding it to the diet in amounts varying from 0.05 to 1.0 mg. per cent or injecting it subcutaneously in amounts varying from 0.002 to 0.1 mg. daily for total dietary periods ranging from 10 to 28 days. Of the twenty
tests, four were considered to be positive or highly suggestive responses, but a repetition of these tests, together with all the others, was clearly negative. One of the positive or highly suggestive tests was obtained by feeding 0.1 mg. per cent of xanthopterin for 19 days, followed by 9 days of control diet alone, before the livers were analyzed and found to contain 1301 ± 148 units of xanthine oxidase; the simultaneous control group averaged 866 ± 77 units. The probability of this being chance variation was about 1 in 40. While xanthopterin was the only pure compound tested that yielded positive or suggestive results, the erratic nature of the response and the preponderant weight of negative evidence suggest some indirect action of xanthopterin or an incomplete supplementation as the explanation for the few positive tests observed.

The following compounds related to xanthopterin were fed at a level of 0.2 mg. per cent in the diet for 2 weeks with negative results: 6-methylisoxanthopterin, α-dihydroxanthopterin, β-dihydroxanthopterin, isoxanthopterin, 7-methylxanthopterin, and 2-amino-4-hydroxypteridine; a simultaneous group fed 0.2 mg. per cent of xanthopterin was also negative.

Compounds Related to Folic Acid—The effect of adding folic acid and 6-pteridylaldehyde to the diet was studied. In vitro, the latter compound was a potent inhibitor of xanthine oxidase (9, 10), and the incorporation of folic acid in a purified diet has been reported (6) to decrease the liver xanthine oxidase activity in chicks. Both compounds were added to the purified 21 per cent casein diet and to the diets that otherwise gave normal xanthine oxidase levels in the liver; i.e., dog chow, 10 per cent liver, and whole milk powder. Any increase or decrease in liver xanthine oxidase could thereby be detected. In additional tests, the liver was wetted and autoclaved for 1 hour at 15 pounds before it was mixed with the other dietary components in order to destroy any enzymes that might possibly alter these pteridyl compounds.

The diets were fed to weanling male rats for 4 weeks; the results are shown in Table II. The following points may be noted. The liver xanthine oxidase activity of the rats on the purified diet fell between 800 and 1000 units in the presence or absence of folic acid and 6-pteridylaldehyde. Adding 1 per cent sulfasuxidine to the purified diet gave the same results as the purified diet alone. All of the groups of rats on Purina dog chow, liver, or milk averaged above 1300 units, and in no case did the addition of these pteridyl compounds significantly increase or decrease this activity. Heating the liver did not elicit a response from the pteridyl derivatives; nor did it destroy the factor in liver responsible for increasing the xanthine oxidase activity above the levels obtained with a purified diet. Feeding the rats Purina Startena for 4 weeks gave 1539 ± 119 units, a normal level of activity in rats, in contrast with the very low xanthine
oxidase activity observed in chicks on this diet (6). Obviously the xanthine oxidase in rat liver did not respond to these particular diets in the manner reported for chick livers.

Additional Observations—In confirmation of the work of Miller (5), significant decreases in liver xanthine oxidase were produced by fasting. Fasting for 6 days gave 1115 ± 60 units in comparison with 1540 ± 110 units observed simultaneously in a group of fed rats. A 50 per cent decrease appeared to be about the maximum effect of prolonged fasting, as observed in near fatal fasts of 12 days. In comparison, the 8 per cent

| TABLE II |
| Liver Xanthine Oxidase Activity in Rats Maintained for 4 Weeks Post-Weaning, on Diets Indicated |
| The numbers in parentheses indicate the number of animals used. |

<table>
<thead>
<tr>
<th>Diet</th>
<th>Xanthine oxidase activity: mean ± s.e.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unsupplemented diet</td>
</tr>
<tr>
<td></td>
<td>units</td>
</tr>
<tr>
<td>Purina dog chow</td>
<td>(6) 1639 ± 129</td>
</tr>
<tr>
<td>21% purified casein*</td>
<td>(12) 897 ± 90</td>
</tr>
<tr>
<td>10% liver*</td>
<td>(6) 1343 ± 159</td>
</tr>
<tr>
<td>10% heated liver*</td>
<td>(10) 1903 ± 195</td>
</tr>
<tr>
<td>Whole milk powder†</td>
<td>(4) 1728 ± 260</td>
</tr>
</tbody>
</table>

* Diet as given in Table I, except that sucrose was used instead of glucose. 10 per cent whole dried liver replaced an equal amount of sucrose. The heated liver was wetted and autoclaved for 1 hour at 15 pounds before mixing with the other constituents.

† Dried whole milk powder (Klim, Borden Company) supplemented with 8 mg. of pyridoxine, 8 mg. of thiamine, 30 mg. of MnSO$_4$·H$_2$O, 470 mg. of FeSO$_4$·7H$_2$O, and 38 mg. of CuSO$_4$·5H$_2$O per 2.26 kilos.

purified protein diet fed *ad libitum* removed practically all of the enzyme from the liver.

In general, livers with a low xanthine oxidase activity were pale in color, while the very active livers were much darker. Even adult rats that were changed from a chow diet to the purified 8 per cent casein diet lost some color from the liver concomitantly with the decreased xanthine oxidase activity. This correlation of color with activity may be significant or it may be chance association, since all of the diets giving little color and low xanthine oxidase activity to the liver were purified, while the dark, active livers were obtained on rations containing natural foodstuffs.

Over 1000 rat livers have been analyzed for xanthine oxidase activity in
connection with these and related experiments, and some idea of the consistency of the results has been obtained. Individual rats under identical dietary conditions show considerable variation in liver xanthine oxidase, with any one group of six identically treated rats often showing a spread of 1000 units from the lowest to the highest value. In spite of such wide individual fluctuations, the averages of such groups are reasonably reproducible. For example, many groups of six control rats fed the 21 per cent purified casein diet for 2 weeks have been analyzed; none of these averages ever exceeded 1100 units, and most of them fell between 800 and 1000 units. The average for the first fifteen rats analyzed was 921 ± 77, and an additional eighteen rats gave little change, the over-all average for the thirty-three rats being 877 ± 50. Similarly, many groups of rats maintained on Purina dog chow have all given averages above 1300 units, usually between 1400 and 1800 units. There can be no doubt that a real difference exists between the effects of these two diets (11), but it is obvious that no importance can be attached to the relatively minor fluctuations observed from group to group on the same diet or to small differences between groups on different diets. We have accepted differences between groups as significant only when they were run simultaneously and when a statistical analysis of the results showed the probability of chance variation to be less than 1 in 50.

Occasional groups of animals maintained on the purified 21 per cent casein or stock chow diets gave values for liver xanthine oxidase appreciably different from the usual ranges. For example, several groups of weanling rats fed the purified diet for 2 weeks had unusually low averages of 300 to 400 units instead of the 800 to 1000 units commonly obtained. Occasional groups of rats maintained on dog chow had activity values over 2000 units instead of the 1400 to 1800 units usually observed. Low values occurred more frequently during the summer months. The reasons for such wide fluctuations among similarly treated animals are not yet clear, but the results obtained with such atypical groups have generally been discounted. The need for simultaneous controls in any study of liver xanthine oxidase is evident, not only because of animal variation but also because of the change in the activity of the xanthine solution itself with aging (7).

Sprague-Dawley Rats—The results previously cited were obtained with rats from the Albino Farms. The limited data available indicate that Sprague-Dawley rats responded similarly, but with some quantitative differences. The average xanthine oxidase activity of eighteen rats at 21 days of age was 468 ± 30 units. Feeding dog chow for 2 and 4 weeks gave 1030 and 1356 units respectively. Feeding the 21 per cent purified casein diet for 4 weeks resulted in an activity of 1123 units. After 3 weeks
on the 8 per cent casein diet, the activity was 26 units; such values were restored to 1133 units after 2 weeks on dog chow, as compared with 479 units on the purified 21 per cent casein diet. In general it appeared that Sprague-Dawley rats required a longer dietary period to reach normal levels of liver xanthine oxidase and that the differences between the purified and natural foodstuffs may not have been as great as when the Albino Farms rats were used.

**DISCUSSION**

Preparations of xanthine oxidase from milk (12) and liver (13) contain an unknown substance in the prosthetic group in addition to flavin-adenine dinucleotide. The evidence for this can be summarized as follows: (1) The color of the enzyme is golden brown instead of the bright yellow that is typical of flavoproteins; (2) approximately one-third of the absorption at 450 mp is due to flavin, the remainder being due to the unknown component; (3) addition of hypoxanthine bleaches the flavin portion of the prosthetic group, but does not remove all of the color; (4) the activity of the apoenzyme, prepared by prolonged dialysis of the enzyme, can be restored by a coenzyme extracted from xanthine oxidase but not by flavin-adenine dinucleotide or flavin phosphate. The evidence presented in this paper shows that some unidentified factor must be supplied in the diet in order for the weanling rat to deposit and retain normal levels of xanthine oxidase in the liver. No evidence has yet been obtained that the required dietary factor is related to the unidentified component of xanthine oxidase, but the possibility is obvious.

Ball (14) has pointed out that more and more flavoproteins with atypical absorption spectra and presumably containing two or more prosthetic groups are being discovered. The list would include two yeast flavoproteins (14, 15), xanthine oxidase (12, 13), liver aldehyde oxidase (16), glucose oxidase from *Penicillium notatum* (17, 18), and quinine oxidase (19). In no case has the non-flavin portion of the prosthetic group been identified, but Ball (14) has indicated that in one of the yeast flavoproteins its absorption spectrum is reminiscent of the pteridine compounds.

**SUMMARY**

Rats are born without any detectable xanthine oxidase activity in the liver, even when the mother's liver contains normal amounts. About half of the normal adult level was present at weaning.

Feeding weanling rats a purified 21 per cent protein diet for 6 weeks gave little or no increase in the liver xanthine oxidase. Increasing the protein or fat content of the diet or changing the type of carbohydrate fed was similarly without effect. Incorporating 10 per cent whole liver
in the diet or feeding natural rations such as Purina dog chow and milk gave nearly normal levels of liver xanthine oxidase within 2 weeks. Supplementing the purified diet with the known crystalline vitamins was without effect, while xanthopterin gave erratic results.

A purified 8 per cent casein diet depleted the liver of xanthine oxidase when fed to rapidly growing or adult rats. The liver enzyme could be restored to normal by feeding the necessary dietary factors. Rapidly growing rats were also partially depleted of liver xanthine oxidase by feeding a purified 21 per cent protein diet.

Folic acid and 6-pteridylaldehyde neither increased nor decreased the liver xanthine oxidase in rats when incorporated in purified or natural diets.

**BIBLIOGRAPHY**

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