The Inhibition of Xanthine and Succinic Oxidases by Carbonyl Reagents*

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Previous studies have demonstrated the existence of three different types of enzymes that oxidize xanthine. The enzyme found in bird tissues is a dehydrogenase without significant ability to react directly with oxygen (1); it can be assayed manometrically by the use of methylene blue as an electron carrier between the reduced enzyme and oxygen. The enzyme found in mammalian tissues reacts directly with air and is therefore an oxidase like milk xanthine oxidase (2). These two oxidases can be differentiated by the fact that only the tissue enzyme can be inhibited by tetraethylthiuram disulfide (Antabuse) (3) or chalcones (4), while the milk enzyme is unaffected. This inhibition of the tissue enzyme by Antabuse or chalcones is limited to its oxidase activity, and no effect is observed on its dehydrogenase activity with methylene blue as the electron acceptor.

The three enzymes are therefore fundamentally alike in catalytic activities, but presumably differ in that portion of the molecule responsible for the reaction with air. All three enzymes have been obtained in highly purified form (1, 5, 6), and each contains Fe, flavin adenine dinucleotide, and Mo in the ratios 8:2:1, 4:1:1, and 8:1:1 for the milk, mammalian liver, and chicken liver enzymes, respectively. A comparison of these relative compositions cannot yet account for the differences in reactivity with oxygen (7). It is possible that the two xanthine oxidases contain an additional unidentified "oxidase" group, such as a quinone, which is absent from the dehydrogenase.

The present study demonstrates that the oxidase activity of the enzymes from milk and from rat liver can be inhibited selectively by the carbonyl reagents, phenylhydrazine, and semicarbazide; this inhibition can be reversed completely by 2-methyl-1,4-naphthoquinone. However, glutathione was also effective in overcoming the semicarbazide inhibition, and chelating agents (e.g. 8-hydroxyquinoline) were effective under certain conditions. The latter effects implicate the enzymatic iron as the point of attachment by the carbonyl reagents.

Numerous studies in recent years have implicated a quinone in the mitochondrial electron transport chain from DPNH or succinate to oxygen. Some of these studies point to a quinone related to vitamin K (8-11) and others to a quinone derived from α-tocopherol (12-14). A quinone related to vitamin K was recently isolated from Mycobacterium phlei, and was shown to restore oxidative phosphorylation to an irradiated bacterial preparation (15). The only quinone isolated (16, 17, 14) from mammalian mitochondria, Q-275, is not clearly related to either vitamin K or E (18), and its role in the electron transport mechanism has yet to be established. Substances other than quinones also show a stimulation of the solvent-extracted mitochondrial system which oxidizes succinate or DPNH (19, 20). The present study suggests that the oxidase activity of milk and rat liver xanthine oxidase is due to a structure which is also present in succinic oxidase since both are inhibited by the same substances.

EXPERIMENTAL

Xanthine oxidase activity was measured in the usual Warburg apparatus at 37° with air as the gas phase. The flask contained an amount of enzyme which gave an oxygen uptake of 20 to 30 c.mm. per 10 minutes with 0.15 ml. of 0.05 M hypoxanthine as the substrate; 0.2 ml. of 2.5 per cent crystalline bovine serum albumin was added to stabilize the activity, and sufficient 0.04 M phosphate buffer, pH 7.4, was added to give a total volume of 2.0 ml. in all experiments. An additional 0.2 ml. of 0.0113 M methylene blue was present in some of the flasks. Unless indicated otherwise, the details of the various tests were carried out as previously described (4).

Milk xanthine oxidase (21) and chicken liver dehydrogenase (1) were prepared as indicated. Crude rat liver oxidase was prepared from an homogenate by high speed centrifugation; the enzyme was recovered from the supernatant fraction by discarding the precipitate from the 30 per cent saturated ammonium sulfate mixture and collecting the precipitate produced by 60 per cent saturated ammonium sulfate. Beef heart mitochondria were prepared by differential centrifugation in sucrose (22), and the oxidation of succinate by this enzyme preparation was measured manometrically in a system containing 0.2 ml. of 0.5 M sodium succinate, 0.5 ml. of 0.24 M phosphate buffer, pH 7.4, and approximately 1 ml. of 8.5 per cent sucrose in a total volume of 2.0 ml.

RESULTS

Milk Xanthine Oxidase

Carbonyl Reagents—The inhibition of the oxidase or dehydrogenase activity of the milk enzyme obtained with hydroxylamine, phenylhydrazine, and semicarbazide (all neutralized to pH 7) is shown in Fig. 1. In confirmation of the results reported by Dietrich and Borries (23), 0.1 to 0.01 M hydroxylamine inhibited the dehydrogenase group of milk xanthine oxidase; the inhibition was not overcome by methylene blue. Incubation of the enzyme...
Semicarbazide and phenylhydrazine therefore inhibited the oxidase portion of milk xanthine oxidase without enzyme with these inhibitors, and was completely reversed by amine to the flask. Semicarbazide and phenylhydrazine were effective inhibitors of the milk enzyme at 10⁻⁶ M concentrations; gave more effective inhibition than simple addition of hydroxylamine at 37°C for 30 to 45 minutes before testing and air as the gas phase. One hundred per cent of the original activity represents an uptake of 39 and 24 c.mm. of O₂ per 10 minutes in the presence and absence of methylene blue, respectively.

The milk oxidase had the same absorption spectrum between 300 and 600 mg in the presence or absence of 0.001 M semicarbazide. The spectrum of the enzyme treated aerobically with hypoxanthine was the same with or without semicarbazide; the partial reduction of the flavin portion of the enzyme which was obtained by the addition of hypoxanthine was prevented by cyanide or 6-pteridylaldehyde, but not by semicarbazide.

**Reactivation of Semicarbazide-inhibited Milk Xanthine Oxidase**

Once the milk oxidase had been inactivated by 0.001 M semicarbazide, it was not reactivated by incubation for 4 hours with 0.002 M pyruvate, acetone, riboflavin, or Na₂MoO₄. Ferrous ammonium sulfate, 0.01 M, restored 30 to 40 per cent of the original activity, but the restored activity was not well sustained. Addition of 0.05 to 10 mg. of extra albumin per ml. or 1 × 10⁻⁴ mole of lecithin per ml. had no effect on the activity of the milk oxidase or on the semicarbazide inhibition. A hot water or alcohol extract of the milk enzyme did not restore the activity.

The addition of a boiled water extract of a variety of rat tissues (∼ 0.3 gm. tissue) allowed an oxygen uptake by the milk enzyme in the presence of semicarbazide and hypoxanthine. The most active extracts were prepared from intestine, liver and blood, and they restored 45 to 65 per cent of the original activity of the semicarbazide-inhibited enzyme; the effectiveness of the rat liver extract was lost on ashing. Further identification was not pursued because other studies established the non-specific nature of this response.

**Vitamin K**—Fig. 2 shows a 50 per cent stimulation of the original aerobic activity of milk xanthine oxidase by 2-methyl-1,4-naphthoquinone (menadione), and the complete restoration of the aerobic activity of the semicarbazide-inhibited enzyme by this quinone. 2-Methyl-3-pheryl-1,4-naphthoquinone in concentrations of 0.005 to 1 mg. per flask (2 ml.) had no effect on the activity of untreated or semicarbazide-inhibited enzyme. 300 µg. per ml. of hydroquinone restored 40 per cent of the original activity to the semicarbazide-inhibited enzyme. Mixed tocopherols and d-α-tocopherol (0.05 to 10 mg. per flask) were ineffective.

**Sulphydryl Compounds**—Glutathione in a concentration of 0.5 mg. per ml. increased the original aerobic rate of oxidation of hypoxanthine (0.1 to 10 mg. per flask) by less than 10 per cent, and the oxidation of p-hydroxybenzaldehyde as substrate (0.3 to 5.5 mg. per flask) by about 30 per cent. Glutathione concentrations of 5 to 20 mg. per ml. increased the aerobic activity of the original enzyme with hypoxanthine substrate by 70 to 90 per cent. Fig. 2 shows the effect of various concentrations of glutathione on the restoration of the aerobic activity of semicarbazide-inhibited milk xanthine oxidase; all of the initial activity was restored by 0.5 to 1 mg. per ml. of GSH. Cysteine exhibited a similar effect; 0.5 mg. per ml. restored 50 per cent, while 1 mg. per ml. restored 100 per cent of the original activity.

**Chelating Agents**—Fig. 2 shows the effect of 8-hydroxyquinoline in overcoming the semicarbazide inhibition of the milk enzyme; in low concentration it was without effect on the original oxidase activity of this enzyme with hypoxanthine or p-hydroxybenzaldehyde as substrate, and higher concentrations were inhibitory in the presence or absence of semicarbazide; an optimal concentration (0.05 mg. per ml.) effectively reversed the semicarbazide inhibition. 8-Hydroxyquinoline was without effect on the dehydrogenase activity of the enzyme in the presence of methyl-
ene blue. 1,10-Phenanthroline and Fe$^{+++}$-specific Versene (ethylenediaminetetraacetae) gave similar results with the milk enzyme. 2-Thenyltrifluoracetone,$^2$ 2-furoyl trifluoracetone,$^2$ disodium Versenate, and benzoyl acetone restored only about half of the semicarbazide-inhibited aerobic activity, but they also inhibited the methylene blue reaction about 50 per cent in the presence or absence of semicarbazide.

**Other Agents**—Thiosemicarbazide was less effective than semicarbazide as an inhibitor of the milk enzyme; a control rate with hypoxanthine substrate of 31 c.mm. of O$_2$ per 10 minutes was reduced to 6 by 0.001 m semicarbazide and to 19 by thiosemicarbazide in the same concentration. The effect of methylene blue, glutathione, and 8-hydroxyquinoline was the same with both the semicarbazide- and thiosemicarbazide-inhibited enzymes.

Q-275 had no effect on the aerobic activity of untreated milk xanthine oxidase, and could not restore any of the semicarbazide-inhibited activity when tested at a final concentration of 50 $\mu$g. per ml.

Six extractions of the milk enzyme with equal volumes of isooctane did not reduce the manometric activity of the enzyme with hypoxanthine substrate in the presence or absence of methylene blue; hence it did not alter the oxidase:dehydrogenase ratio by extracting an "oxidase" grouping.

Measurements of uric acid formation by the milk oxidase instead of O$_2$ uptake gave similar results. The rate of change of the optical density at 290 rnp was 0.021 per minute with hypoxanthine substrate, 0.007 in the presence of 0.001 carbazide, and 0.023 with semicarbazide plus 0.5 mg. per ml. of glutathione or 0.01 $\times$ 8-hydroxyquinoline.

**Rat Liver Xanthine Oxidase**

The rat liver enzyme responded to the carbonyl reagent inhibitors in the same manner as the milk enzyme, except that the rat liver enzyme was more crude, and 100-fold higher concentrations of semicarbazide and phenylhydrazine were required to achieve the same degree of inhibition. Hydroxylamine was about as active with the rat liver enzyme as with the milk oxidase, and with both enzymes the hydroxylamine inhibition was not overcome by methylene blue. Fig. 3A shows the effect of semicarbazide on the oxidase activity of rat liver xanthine oxidase with hypoxanthine as the substrate. Like the milk enzyme, the inhibition by semicarbazide was overcome completely by methylene blue.

Fig. 3B shows the effect of glutathione, menadione, and 8-hydroxyquinoline on the oxidase activity of the original untreated enzyme. In concentrations above 1 mg. per flask (2 ml. volume), glutathione stimulated this activity, and a maximal stimulation of about 50 per cent was obtained with 5 mg. of GSH per flask. Menadione (0.5 mg. per flask) increased the original activity by 25 per cent. In concentrations of 0.5 to 10 mg. per flask, 8-hydroxyquinoline progressively inhibited the oxidase activity of the rat liver enzyme.

Fig. 3C shows the effect of these same agents in overcoming the inhibition by 0.01 $\times$ semicarbazide. Approximately 40 per cent of the original aerobic activity was reattained at this concentration. Both glutathione and menadione restored this activity to about the same levels observed in the absence of semicarbazide; the effective concentrations were also the same as those required in the absence of semicarbazide. 8-Hydroxyquinoline was completely inactive; it did not restore the activity of the inhibited enzyme, nor did it produce any additional inhibition.

**Antabuse and Chalcones**—The inhibition of the oxidase activity of milk xanthine oxidase by semicarbazide and phenylhydrazine was comparable with the previously demonstrated effect of Antabuse and chalcones on rat liver xanthine oxidase. All of these agents inhibited the oxidase activity of the appropriate enzyme without inhibiting the dehydrogenase activity, but the carbonyl reagents were effective with both the milk and rat liver enzymes, while Antabuse and the chalcones exerted an effect only on the rat liver enzyme.

Figs. 4A and 5A show the inhibition of the oxidase activity of the rat liver enzyme by Antabuse and 3',3',4',4'-tetrahydroxychalcone. It was demonstrated previously that these inhibitions could be overcome completely by methylene blue. Figs. 4B and 5B show the restoration of this activity by menadione in the presence of either inhibitor. Glutathione also completely restored the activity of the chalcone-inhibited enzyme, but had relatively little effect on the Antabuse-inhibited enzyme. 8-Hydroxyquinoline was completely ineffective in overcoming either inhibition.

With a higher concentration of chalcone (0.02 mg. per ml.), the reversal by methylene blue was often only partial and sometimes negligible. Pig liver xanthine oxidase, prepared the same way as the rat liver oxidase, was completely inactivated by 0.02 mg. per ml. of chalcone, and this inhibition was not reversed by methylene blue; with lower concentrations of chalcone, the reversal by methylene blue was partial. Beef liver xanthine oxidase was partially inhibited by chalcone, and the inhibition was completely reversed by methylene blue. It is evident that...

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1 Bersworth Chemical Company, Framingham, Massachusetts.
2 Midcontinent Chemical Company, Chicago, Illinois.
FIG. 4. A, the inhibition of rat liver xanthine oxidase by Antabuse, and B, the effect of 2-methyl-1,4-naphthoquinone (K), glutathione (G or GSH), and 8-hydroxyquinoline (HQ) on the restoration of this activity. Concentrations are given as mg. per flask containing 2 cc. of fluid.

One hundred per cent of the original activity, as measured manometrically with hypoxanthine substrate, was equivalent to an uptake of 33 c.mm. of O₂ per 10 minutes. For the curves in B, the Antabuse concentration was 2 mg. per flask (2 cc.) except for the dotted line with GSH, wherein the antabuse concentration was 1 mg. per flask.

Fig. 6 shows the effect of menadione in linking the activity of the chicken liver dehydrogenase to oxygen. Increasing aerobic activity was obtained with concentrations of menadione from 0.01 to 10.0 mg. per flask; the highest concentration tested gave 75 per cent of the activity obtained with methylene blue. Neither menadione nor 8-hydroxyquinoline had any effect on the dehydrogenase activity measured in the presence of methylene blue, while higher concentrations of glutathione (5 mg. per ml.) markedly stimulated this dehydrogenase activity. No oxygen was consumed when 0.25 mg. per ml. of Q-275 replaced methylene blue in the usual assay procedure. Glutathione and 8-hydroxyquinoline were completely inactive in the absence of methylene blue, and were therefore unable to alter the dehydrogenase so that it would react directly with oxygen. Neither α-tocopherol nor 2-methyl-3-phytyl-1,4-naphthoquinone in concentrations of 0.05 to 10 mg. per flask had any effect on the activity of this enzyme in the presence or absence of methylene blue; unlike menadione, they were incapable of conferring oxidase activity upon the dehydrogenase enzyme.

**Succinic Oxidase**

The effect of semicarbazide on the aerobic oxidation of succinate by beef heart mitochondria is shown in Fig. 7A. The succinic oxidase was less sensitive to semicarbazide than the milk xanthine oxidase, but was roughly comparable with the rat liver oxidase since a final concentration of 0.1 M was required to produce essentially complete inhibition. Thiosemicarbazide gave the same inhibition curve as semicarbazide. Unlike both the xanthine-oxidizing enzymes, methylene blue gave only a slight reversal of the semicarbazide inhibition of succinic oxidase. Since methylene blue does not react directly with succinic dehydrogenase in particulate preparations (24), the site of the semicarbazide inhibition could be the dehydrogenase itself, but a possible effect elsewhere along the electron transport chain has not been excluded. Figure 7B shows a similar degree of inhibition of succinic oxidase by 0.01 M hydroxylamine (25) or 0.001 M phenylhydrazine.

**Chicken Liver Xanthine Dehydrogenase**

Since the chicken liver xanthine dehydrogenase has negligible oxidase activity and is tested in the presence of methylene blue for its dehydrogenase activity, no effect of semicarbazide was expected or obtained.

Fig. 5 shows the effect of menadione in linking the activity of the chicken liver dehydrogenase to oxygen. Increasing aerobic activity was obtained with concentrations of menadione from 0.01 to 10.0 mg. per flask; the highest concentration tested gave 75 per cent of the activity obtained with methylene blue. Neither menadione nor 8-hydroxyquinoline had any effect on the dehydrogenase activity measured in the presence of methylene blue, while higher concentrations of glutathione (5 mg. per ml.) markedly stimulated this dehydrogenase activity. No oxygen was consumed when 0.25 mg. per ml. of Q-275 replaced methylene blue in the usual assay procedure. Glutathione and 8-hydroxyquinoline were completely inactive in the absence of methylene blue, and were therefore unable to alter the dehydrogenase so that it would react directly with oxygen. Neither α-tocopherol nor 2-methyl-3-phytyl-1,4-naphthoquinone in concentrations of 0.05 to 10 mg. per flask had any effect on the activity of this enzyme in the presence or absence of methylene blue; unlike menadione, they were incapable of conferring oxidase activity upon the dehydrogenase enzyme.

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Fig. 7. The inhibition of the aerobic oxidation of succinate by beef heart mitochondria: A, by semicarbazide (SC) in the presence or absence of methylene blue (MB), and B, by hydroxylamine (HA) and phenylhydrazine (PH).

One hundred per cent activity = 36 c.mm. of O₂ per 10 minutes.

Fig. 8. The effect of various agents on the oxidation of succinate by beef heart mitochondria: A, in the absence of any inhibitors, and B, in the presence of a final concentration of 0.1 M semicarbazide (SC); glutathione (G or GSH), albumin (Alb), 8-hydroxyquinoline (HQ), 2-methyl-1,4-naphthoquinone (K), 2-methyl-3-phytyl-1,4-naphthoquinone (K-P).

The original activity equivalent to 100 per cent activity varied in different experiments from 37 to 55 c.mm. of O₂ per 10 minutes; 16 to 32 per cent of the original activity was retained in the presence of 0.1 M SC. All concentrations are recorded as mg. per 2 cc. of fluid in the flask. In B the scale for G also applies to HQ and K. The scale for Alb (not shown) is twice the KP scale.

The effect of various agents on the activity of the original uninhibited succinic oxidase is shown in Fig. 8A. Glutathione in low concentrations (0.01 to 0.02 mg. per ml.) produced some slight inhibition, but stimulated the activity of this system by 50 per cent when present at 5 mg. per ml. Albumin, 8-hydroxyquinoline, 2-methyl-3-phytyl-1,4-naphthoquinone, and methylene blue in concentrations of 10⁻³ to 10⁻⁶ M (not shown) were without major effect; increasing concentrations of menadione progressively inhibited the activity. When these same agents were added to the enzyme system that had been inhibited by 0.1 M semicarbazide, the results shown in Fig. 8B were obtained. Increasing concentrations of albumin and glutathione progressively overcame the semicarbazide inhibition. 8-Hydroxyquinoline was partially effective, while menadione and 2-methyl-3-phytyl-1,4-naphthoquinone were completely inert. A concentration of 0.2 mg. per ml. of Q-275 was strongly inhibitory to the untreated succinic oxidase activity (reducing the O₂ uptake from 30 c.mm. per 10 minutes to 5), and did not overcome the semicarbazide inhibition of this system.

Chalcone and Antabuse—Both 3,3',4,4'-tetrahydroxychalcone and Antabuse inhibited the succinic oxidase system, as shown in Figs. 9 and 10. The succinic oxidase system was more sensitive to low concentrations of Antabuse than was the rat liver enzyme, while the latter was more sensitive to the chalone. Glutathione completely reversed the chalone or Antabuse inhibition, and at a concentration of 10 mg. per flask, it gave the same 90 per cent stimulation observed with the uninhibited enzyme. 8-Hydroxyquinoline also completely overcame the chalone inhibition at an optimal concentration of 0.4 mg. per flask, but higher concentrations were inhibitory. Methylene blue partially overcame the chalone, but was relatively ineffective in overcoming the Antabuse inhibition. 8-Hydroxyquinoline had no effect on the Antabuse inhibition, and the naphthoquinones were without significant effect on either inhibitor.

d-Amino Acid Oxidase

None of the carbonyl reagents were very effective inhibitors of the metal-free flavoprotein, d-amino acid oxidase (Worthington), in the manometric O₂-uptake assay with alanine as substrate. A final concentration of 0.1 M semicarbazide, with or without incubation, left 90 per cent of the original activity; with 0.1 M hydroxylamine at least ½ of the original activity of this

Fig. 9A. The inhibition of the aerobic oxidation of succinate by beef heart mitochondria by 3,3',4,4'-tetrahydroxychalcone, and B, the effect of various agents on the inhibition produced by 0.04 mg. of chalcone per flask. The abbreviations are the same as in Fig. 8.

One hundred per cent activity = 38 to 48 c.mm. of O₂ per 10 minutes. Concentrations for all substances except MB are given in mg. per flask containing 2 cc. of fluid. In B, the scale for G also applies to HQ and K-P; The scale for K (not shown) is 1/8 of G. Methylene blue is given as the logarithm of the molar concentration.
enzyme remained even after preliminary incubation for 30 minutes. Phenylhydrazine did not inhibit at 0.001 M, but enzyme remained even after preliminary incubation for 30 minutes.

One hundred per cent activity = 23 to 32 c.mm. of O₂ per 10 minutes.

Concentrations for all substances except MB are given in mg. per flask (2 cc.); the scale for G also applies to HQ and K-P; the scale for K (not shown) is 1/5 of G. MB is given as the logarithm of the molar concentration.

Semicarbazide in vivo

Two groups of weanling male rats were fed Purina dog chow plus 0.1 per cent semicarbazide hydrochloride; each rat in the second group was also given 1 mg. of 2-methyl-1,4-naphthoquinone daily in 0.1 ml. of sesame oil by subcutaneous injection. After 5 weeks, the livers were analyzed for xanthine oxidase in the presence and absence of methylene blue (26), and compared with an untreated control group maintained on chow.

The results are shown in Table I. The semicarbazide feeding significantly lowered the liver xanthine oxidase, and menadione administration partially restored it. However, a comparison of the enzyme activities as measured in the presence and absence of methylene blue demonstrated that the semicarbazide feeding decreased the dehydrogenase as well as the oxidase activity of this enzyme. Semicarbazide did not exert its effect by specifically blocking the oxidase portion of the enzyme. Menadione did not prevent the retardation of growth or the osteolathyrism produced by semicarbazide-feeding. All of the rats in both semicarbazide-fed groups exhibited the skeletal exostoses and spinal curvature characteristic of osteolathyrism (27), and the extent of bone changes was as great in the menadione-treated rats as in those receiving semicarbazide alone.

### DISCUSSION

The inhibition of the oxidase activity of the xanthine enzymes by semicarbazide and phenylhydrazine might suggest a reaction with a carbonyl component of the enzyme. However, a semicarbazone derivative would not be expected to form under the mild neutral conditions of the enzyme tests, and once formed would not be disrupted by glutathione or hydroxyquinoline. Hence, any reaction between these inhibitors and the enzyme must be a loose complex irrespective of the nature of the component of the enzyme with which they react.

The iron present in the enzyme is undoubtedly involved in these inhibition effects. Thiosemicarbazide chelates with ferric ions to give a colored compound in acid solution; it acts like semicarbazide and phenylhydrazine in blocking the oxidase activity of the xanthine enzymes. The reversal of the semicarbazide inhibition by 8-hydroxyquinoline or ferric-specific Versene seems to be concerned with the chelation of the enzyme iron by these substances, and as a result of this chelation, the displacement of the inhibitor from the enzyme. Similarly, the reversal of the semicarbazide inhibition by sulfhydryl compounds, such as glutathione, can be attributed to a reaction with the iron and a displacement of the inhibitor from the iron. These carbonyl reagents also act like chelating agents in blocking the oxidation of homogentisic acid, and this inhibition can be reversed by ferrous ions (28). The oxidase activity of the enzyme is not apt to be due to the iron alone because the dehydrogenase also contains iron without being autooxidizable, and treatment with 8-hydroxyquinoline or glutathione does not convert the iron of the dehydrogenase into an oxidase grouping.

The oxidase activity of xanthine oxidase can therefore be attributed to a particular kind of Fe-PAD structure, or to a complex between the iron and another unknown group, such as a quinone. The inhibitions and reversals would apply equally with respect to either suggestion, since the point of attack is the iron rather than the associated group. While no evidence for the existence of a quinone in xanthine oxidase has been obtained, the results have demonstrated that a suitable quinone can fulfill the function of an oxidase group in all three xanthine enzymes. If such an iron-quinone structure were present in the milk and rat liver oxidases, it would have to function well as a link to oxygen, but would be a poor link to cytochrome c. Iron without the quinone in the chicken liver dehydrogenase would have to react poorly with oxygen and well with cytochrome c in order to explain the different activities of these enzymes (29). However, such a concept is not supported by the observation that the reduction of cytochrome c by the chicken liver dehydrogenase with hypoxanthine or DPNH substrates is stimulated 40 per cent and 600 per cent, respectively, by the addition of menadione.

### Table I

**Effect of feeding 0.1 per cent semicarbazide to weanling rats for 5 weeks on liver xanthine oxidase activity**

<table>
<thead>
<tr>
<th>Diet</th>
<th>Body weight</th>
<th>Liver xanthine oxidase*</th>
<th>No. of rats analyzed</th>
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<tr>
<td></td>
<td>Start</td>
<td>5 weeks</td>
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<td></td>
<td>gm.</td>
<td>gm.</td>
<td>c.mm. O₂/10 min./flash</td>
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<tr>
<td>Chow</td>
<td>41</td>
<td>174</td>
<td>34 ± 2.44±54 ± 3.62</td>
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<tr>
<td>Chow + 0.1 per cent</td>
<td>40</td>
<td>115</td>
<td>19 ± 1.78±88 ± 3.42</td>
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<tr>
<td>semicarbazide</td>
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<tr>
<td>Chow + 0.1 per cent</td>
<td>41</td>
<td>111</td>
<td>28 ± 3.32±44 ± 3.73</td>
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<tr>
<td>semicarbazide + menad-</td>
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<td>adione†</td>
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* Mean ± standard error of mean.

† 1 mg. of 2-methyl-1,4-naphthoquinone injected subcutaneously into each rat daily.
A comparison (7) of the metalloflavoproteins with respect to spectrum, iron content, and reactivity with oxygen provides a different possible explanation of the aerobic activity of some of these enzymes. d-Amino acid oxidase has a typical flavin spectrum, is free from metal (30), and reacts readily with oxygen to form $\text{H}_2\text{O}_2$. Milk and rat liver xanthine oxidases contain 4Fe:1 FAD, and have an atypical spectrum in which the flavin component is still clearly recognizable; both react with oxygen to form $\text{H}_2\text{O}_2$. The chicken liver dehydrogenase contains 8 Fe:1 FAD, and has a spectrum in which the flavin component can no longer be recognized. Succinic dehydrogenase from beef heart (31) or bakers’ yeast (32) contains 4Fe:1 FAD and has a spectrum in which the flavin component is difficult to recognize. None of the dehydrogenases react with oxygen. The presence of Mo in the xanthine enzymes has no effect on the spectrum. If these spectral differences are due only to the iron and the flavin, then it is evident that the prosthetic group in the dehydrogenase is an Fe-flavin complex rather than a simple flavin nucleotide. Moreover, the disappearance of the flavin absorption from the spectrum of these dehydrogenases can only be interpreted as an alteration in the isoalloxazine ring to such an extent that it no longer exists as the simple riboflavin structure. Any other effect of the iron on the spectrum would leave the flavin portion recognizable because it would be additive to whatever other changes did occur. The dehydrogenases therefore fail to react with oxygen because they contain a nonautoxidizable Fe-FAD prosthetic group, rather than an autoxidizable flavin nucleotide. When the flavin structure is sufficiently intact to be recognizable in the spectrum, as it is with the milk and rat liver enzymes, then the enzyme reacts directly with oxygen. A flavin “difference spectrum” can not be demonstrated for the reduction of the chicken liver dehydrogenase by its substrate because an unaltered flavin structure does not exist in the active prosthetic group. If the flavin spectral alterations in the dehydrogenases are due to some unidentified component of the enzyme rather than the iron, the same general considerations should apply, but the active prosthetic group would then be FAD-X*Fe instead of an Fe-FAD complex.

The gradations in spectra from “pure flavin” in d-amino oxidases to “partial flavin” in the xanthine oxidase to “no flavin” in xanthine dehydrogenase can be related to the presence of 0, 4, and 8 atoms of Fe per flavin, respectively. All of the iron in the enzyme need not be combined with the flavin in order to produce such spectral changes. A ratio of 2 Fe:1 flavin in succinic dehydrogenase is sufficient to alter the flavin characteristics of the spectrum (31). The milk enzyme could theoretically have one flavin present as Fe-FAD and the other present as a simple nucleotide to give the “mixed” spectrum actually observed; this concept is attractive because it could explain some of the puzzling features of the milk enzyme. However, the rat xanthine oxidase also has a spectrum similar to milk xanthine oxidase, and it has only one molecule of flavin present in the enzyme.

The combination of iron and flavin can not be a simple chelation because of the marked spectral changes and because of the inability of riboflavin to chelate with ferric iron (33), the form present in succinic dehydrogenase (34). As a structural part of the Fe-FAD prosthetic group, the Fe need not enter into electron transfers, though it obviously might do so with certain acceptors. The iron must also have an attachment to the protein which is independent of the flavin because the flavin can be removed from milk xanthine oxidase without removing the iron (7).

Fridovich and Handler (35) have postulated a mechanism of action for milk xanthine oxidase in which electrons are removed from the substrate by one FAD, passed to the other FAD via an Fe bridge, and then transferred to oxygen by the second FAD. Such a mechanism could not explain the oxidase activity of the rat liver enzyme because the latter contains only one FAD. This mechanism for the milk enzyme was based on studies which showed an inhibition of oxygen but not cytochrome c reduction by excess substrate. However, the dehydrogenation of hypoxanthine substrate by milk xanthine oxidase and the transfer of electrons to oxygen or methylene blue is at least 40 times faster than the transfer of electrons to cytochrome c by the same enzyme. Hence, the reaction with oxygen or methylene blue could be inhibited some 40-fold before any effect could be observed on cytochrome c reduction, irrespective of the mechanism of inhibition of the dehydrogenation reaction.

REFERENCES
7. Westerfeld, W. W., Richert, D. A., and Higgins, E. S.,


