MAGNETIC AND SPECTROPHOTOMETRIC STUDIES OF THE KINETICS OF THE CATALYSIS OF XANTHINE OXIDATION BY XANTHINE OXIDASE FROM COW'S MILK

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(Received May 3rd, 1961)

SUMMARY

The increase in paramagnetism upon reaction is, under certain conditions, greater than can be explained on the basis of changes in the electronic configurations of the prosthetic groups. The difference is attributed to the formation of substrate free radicals.

The effects of pH, temperature, and concentration of substrate and product on the rate of production of uric acid were studied. The kinetic data at pH 8.3 and 25° were described in terms of true Michaelis constants for xanthine and oxygen of $36 \cdot 10^{-6}$ *M* and $240 \cdot 10^{-6}$ *M*, respectively.

The degree of anaerobic reduction by xanthine of enzyme flavin, as estimated spectrophotometrically, depends upon the concentration of enzyme.

A mechanism is presented that embraces the manifold properties of this reaction.

INTRODUCTION

Xanthine oxidase is a widely distributed metalloflavoprotein enzyme. It is found in mammalian liver and skin, and in cream[1]. It catalyzes a wide variety of different oxidations; the oxidation of xanthine to uric acid in the presence of molecular oxygen is the most rapid. This reaction has been investigated both spectrophotometrically[2] and magnetically[3], but the details of the steps have not definitely been established. The data in the present paper were obtained in order to apply the instrumentation available to us toward the identification of the intermediate steps in this reaction.

Xanthine oxidase is a large protein, and its reactions are complicated by the presence of more than one possible prosthetic group. The xanthine oxidase from cow's milk[1] has been shown to have a molecular weight of about 300 000 and to contain one or two molybdenum atoms, two FAD groups, and eight iron atoms per molecule. Changes in the optical absorption spectra due to the FAD groups are observed during the reaction. It is not certain whether the molybdenum and iron are also active. If the iron atoms enter the reaction, sizable changes in magnetic susceptibility could occur. Our experiments were initiated with this in mind. However, free-radical intermediates are possible in two parts of the reaction. The conversion of xanthine to

Abbreviations: APR, "activity-protein"; AFR, "activity–flavin".

uric acid could occur in two one-electron steps. It is also possible that hydrogen peroxide, a product of the reaction, is formed from free-radical intermediates. Therefore we had to consider that magnetic changes within the enzyme might be masked by the magnetic susceptibility of such external free-radical intermediates. This situation does in fact occur. The magnetic evidence and supporting spectrophotometric measurements are described below.

MATERIALS AND APPARATUS

Xanthine oxidase was prepared from cow's milk by the method of AVIS *et al.*[1], with the following modifications. Instead of using pressure filtration to bring the enzyme into contact with a calcium phosphate gel column, we suspended the gel in enzyme solution, allowed the mixture to stand 30 min, and centrifuged it to bring down the gel (with adsorbed enzyme). The gel was then washed twice by suspending it in the appropriate buffer and centrifuging it. Three elutions were performed in the same way. The enzyme preparations were carried to the stage of purity designated M6 by AVIS *et al.*[1], at which time the APR and AFR ratios defined by these authors were in the ranges 2 to 8 and 20 to 80. In most of the experiments to be described, preparations with APR's from 6 to 8 were used. The preparations were not analyzed for iron, molybdenum, or flavinadenine dinucleotide, and it is assumed that these were present as found previously[4]. When the enzyme was stored, sodium salicylate was used as preservative. Prior to the experiments the enzyme was dialyzed against 0.033 *M* sodium pyrophosphate adjusted to the appropriate pH with hydrochloric acid, and dilutions were made with like buffer.

The catalase used was of bacterial origin (*Micrococcus lysodeikticus*), from a preparation of a purity value (which is defined as A 406 mμ/A 280 mμ) exceeding 0.7.

Solutions of xanthine and uric acid were analyzed spectrophotometrically. At 292 mμ we measured an extinction coefficient for uric acid of $11.5 \cdot 10^3\, M^{-1}\,cm^{-1}$ at pH 8.3. The maximal difference between the extinction coefficient for xanthine and that for uric acid at pH 8.3 was $9.5 \cdot 10^3\, M^{-1}\,cm^{-1}$ at 295 mμ.

The magnetic susceptometer, combined with a flow system, has been described previously in this journal[5] and, in more detail, elsewhere[6,7].

A split-beam recording spectrophotometer[8,9] was employed to measure over-all rates of production of uric acid and disappearance of xanthine. For the rapid-reaction measurements, a double-beam recording spectrophotometer[10] in conjuction with rapid-flow apparatus[11] was used.

MAGNETIC EXPERIMENTS

The flow system used with the susceptometer is shown schematically in Fig. 1. In these experiments the main solution was buffered xanthine oxidase, and the secondary solution was buffered xanthine that had been equilibrated with oxygen at a total pressure (gas plus water vapor) of 1 atmosphere. Fig. 2 is the record of one such experiment. At I, the secondary solution begins to be injected continuously into the flowing main solution, and a steady-state reaction mixture is set up in half cell 2. At O, both flows are brought to a stop; a transient artifact follows, and then the remainder of the

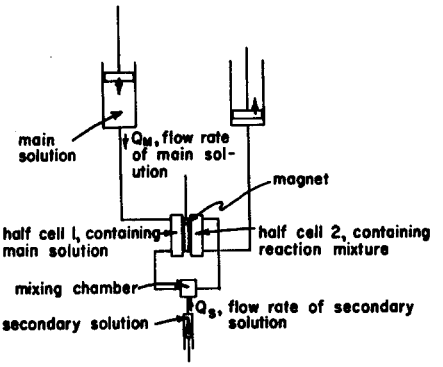


Fig. 1. Schematic representation of flow experiment after BRILL *et al.*[6]

Fig. 2. Recording of changes in magnetic susceptibility when buffered xanthine equilibrated with oxygen at a total pressure of 1 atm. is injected into buffered xanthine oxidase, 1.6 units/l, at 20.5°. Increased magnetic susceptibility is shown by an upward deflection, while time increases from left to right along the abscissa. At I, injection is started into flowing solution; at O, flows are brought to a stop.

reaction is recorded. It is also possible to make several measurements of the steady-state deflection by alternately starting and stopping the injection of secondary solution, as at I, S, and I in Fig. 3.

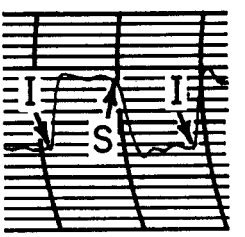


Fig. 3. Steady-state change in magnetic susceptibility resulting from alternately starting (I) and stopping (S) injection of buffered xanthine solution, oxygenated as in Fig. 2, into a flowing buffered solution of xanthine oxidase, 1.6 units/l, at 20.5°.

The steady states set up in the experiments shown in Fig. 2 and 3 correspond to reaction mixtures 0.1 sec after the initiation of the reaction. A large part of the deflection arises from the paramagnetism of the injected oxygen, of which a negligible amount has been used up in the 0.1 sec. The component produced by oxygen is readily recorded by injecting xanthine-free secondary solution, as shown in Fig. 4. This measurement also permits calculation of the concentration of oxygen in the main solution, with the following considerations.

Oxygen in the gaseous state has a molar susceptibility, χ_{O_2}, of $3380 \cdot 10^{-6}$ emu at 20°, corresponding to two unpaired electrons. THEORELL AND EHRENBERG[12] measured water saturated with oxygen against evacuated water and found that the

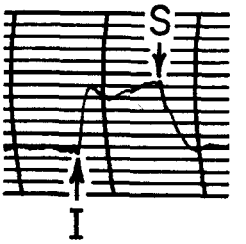


Fig. 4. Change in magnetic susceptibility due to injection of buffer, oxygenated as in Fig. 2, into xanthine oxidase, 1.6 units/l, at 20°. This is a control experiment that permits elimination of component of deflection in records of Fig. 2 and 3 that is due to oxygen.

contribution of physically dissolved oxygen is the same as that of the gas at the same concentration. We have found this relationship to hold at the low concentrations used in our experiments, and this circumstance has proved useful in computing the oxygen content of the main solution. If the main solution were oxygen-free, the volume magnetic susceptibility in half cell 2 would increase upon injection of the secondary solution by

$$\Delta \kappa_0 = 10^{-3} \chi_{0_2} \frac{Q_S}{Q_S + Q_M} [O_2]_S ,$$

in which Q_S is the flow rate, $[O_2]_S$ is the concentration of oxygen of the secondary solution, and Q_M is the flow rate of the main solution. The presence of oxygen at a concentration $[O_2]_M$ in the main solution reduces the change in magnetic susceptibility to

$$\Delta \kappa = \Delta \kappa_0 \left(1 - \frac{[O_2]_M}{[O_2]_S}\right) .$$

In the runs depicted in Fig. 2, 3 and 4, for example, $Q_M/Q_S = 23.0$, and the temperature was 20.5°, at which $\chi_{0_2} = 3370 \cdot 10^{-6}$ emu and $[O_2]_S = 1.34 \cdot 10^{-3} M$, so that $\Delta \kappa_0 = 1.88 \cdot 10^{-10}$ emu. The sensitivity was set at $1.76 \cdot 10^{-11}$ emu per scale division (on the basis of 10 scale divisions between heavy lines), and the deflection corresponding to $\Delta \kappa_0$ was 10.7 scale divisions. Fig. 4 shows an average deflection of 9.5 scale divisions, so that $[O_2]_M = 150 \cdot 10^{-6} M$. In all experiments the concentration of xanthine in the secondary solution, $[X]_S$, was adjusted to make it about equal to $[O_2]_S$. In this way all of the injected xanthine was used up in each run, while $[O_2]_M$ remained nearly constant.

The contribution of hydrogen peroxide, a product of the reaction, to the observed changes in magnetic susceptibility was found to be negligible. Other control experiments showed that in the absence of the enzyme neither xanthine nor uric acid caused any appreciable change in the measured magnetic susceptibility at the concentrations we used.

The data from magnetic experiments are summarized in Table I; activities recorded there and throughout this paper were determined by the method of AVIS *et al.*[1]. The changes in magnetic susceptibility were obtained by substracting the deflections observed for injecting oxygen alone from the deflections observed for the reaction mixtures. At 0.07 and 0.10 sec the amount of oxygen that had reacted was small. However, at 0.43 sec and for the higher activities, there was a magnetically detectable diminution of the concentration of molecular oxygen. This change served to make the values listed at 0.43 sec less than the true increase in paramagnetism above oxygen. The effect was greatest for the last entry in the column, which was then estimated to

TABLE I

CHANGES IN MAGNETIC SUSCEPTIBILITY DUE TO INJECTION OF XANTHINE (x) INTO AEROBIC SOLUTIONS OF XANTHINE OXIDASE*

[a] (Average activity, units per liter)	*Temperature*	*Concentrations at initiation of reaction*		*Increase in observed volume magnetic susceptibility above that due to injected oxygen, at following times:*		
		x	O_2	*0.07 sec*	*0.10 sec*	*0.43 sec*
0.65	23°	15 μM	215 μM	$-0.8 \cdot 10^{-11}$ emu	0	$+0.7 \cdot 10^{-11}$ emu
0.43	22°	49 μM	330 μM	—	$3.7 \cdot 10^{-11}$ emu	$-1.1 \cdot 10^{-11}$ emu
1.5	20.5°	46 μM	205 μM	—	$6.0 \cdot 10^{-11}$ emu	$+2.3 \cdot 10^{-11}$ emu
3.1	22°	49 μM	335 μM	—	$3.0 \cdot 10^{-11}$ emu	—
9.3	20.5°	46 μM	335 μM	$+4.6 \cdot 10^{-11}$ emu	$4.4 \cdot 10^{-11}$ emu	$+3.3 \cdot 10^{-11}$ emu

* Solutions buffered with 0.033 M sodium pyrophosphate, pH 8.2.

be 4.3 to $4.8 \cdot 10^{-11}$ emu, a correction of 1.0 to $1.5 \cdot 10^{-11}$ emu. The correction decreases to less than $0.35 \cdot 10^{-11}$ emu for the other entries in this column. For times greater than 0.43 sec we were not able to separate the magnetic change due to oxygen from that of the other constituents of the reaction mixture, and are therefore not able to comment quantitatively upon the susceptibility of the reaction mixture beyond this time.

Several conclusions can be drawn from the data of Table I, the primary one being that under many conditions the reaction mixture is paramagnetic compared with the "resting oxidized enzyme." At concentrations of oxygen of about $300 \cdot 10^{-6}$ M and concentrations of xanthine of about $50 \cdot 10^{-6}$ M, this increase in magnetic susceptibility, measured during the first few tenths of a second, varies by a factor of only 2. when the concentration of enzyme is varied 20-fold. At low concentrations of xanthine (less than $15 \cdot 10^{-6}$ M), the magnetic increase is small.

At lower concentrations of enzyme no increase in magnetic susceptibility was detected. In a set of experiments at pH 7 (not shown in Table I) there was no detectable change in magnetic susceptibility. The average activity of the enzyme in the experiments at pH 7, as measured at pH 8.3, was 0.7 unit/l. At pH 7.0 the maximal rate of reaction was almost three times slower.

There are several possible sources of the observed increase in magnetic susceptibility of the reacting system above that of the nonreacting enzyme. Xanthine oxidase contains iron (Fe), flavin (fl), and molybdenum (Mo) in the ratio of about 8 : 1.9 : 1.4, as shown by AVIS *et al.*[4]. These authors found that

$$[\mathrm{Fe}] = 1.41 \cdot 10^{-6} [a],$$

in which $[a]$ is the number of activity units/l of solution. The theoretical maximum change in susceptibility of the enzyme can be calculated by assuming (we do not propose that each of these changes is occurring) that all the iron goes from the low-spin ferrous form (diamagnetic) to the high-spin ferric form (five unpaired electrons), that all the flavin goes to a semiquinone free radical, and that all the molybdenum goes from a diamagnetic form (such as hexavalent or covalent tetravalent) to the trivalent form (three unpaired electrons). (Monovalent molybdenum could have the greatest magnetic susceptibility of all molybdenum valence states, but no monovalent compounds have been found. We are also excluding intramolecular co-operative

TABLE II

COMPARISON OF OBSERVED MAXIMAL PARAMAGNETIC INCREASES WITH THEORETICALLY COMPUTED VALUES

[a] (*Activity, units per liter*)	$\Delta \kappa^{max}_{obs}$	$\Delta \kappa^{max}_{Fe,\ fl,\ Mo}$*	$\Delta \kappa^{max}_{fl,\ Mo}$	$\Delta \kappa^{max}_{obs} - \Delta \kappa^{max}_{fl,\ Mo}$
0.43	$3.7 \cdot 10^{-11}$ emu	$0.9 \cdot 10^{-11}$ emu	$0.1 \cdot 10^{-11}$ emu	$3.6 \cdot 10^{-11}$ emu
1.5	$6.0 \cdot 10^{-11}$ emu	$3.3 \cdot 10^{-11}$ emu	$0.3 \cdot 10^{-11}$ emu	$5.7 \cdot 10^{-11}$ emu
3.1	$3.0 \cdot 10^{-11}$ emu	$6.8 \cdot 10^{-11}$ emu	$0.6 \cdot 10^{-11}$ emu	$2.4 \cdot 10^{-11}$ emu
9.3	$4.6 \cdot 10^{-11}$ emu	$2.0 \cdot 10^{-10}$ emu	$1.8 \cdot 10^{-11}$ emu	$2.8 \cdot 10^{-11}$ emu

* Fe = iron; fl = flavin; Mo = molybdenum.

effects among the iron atoms.) The paramagnetic increase is then given, at 20°, by

$$\Delta \kappa^{max}_{Fe,\ fl,\ Mo} = 2.2\ [a] \cdot 10^{-11}.$$

In Table II are listed values of $\Delta \kappa^{max}_{Fe,\ fl,\ Mo}$, calculated from the experimental activities per liter, and the greatest observed values of $\Delta \kappa$, $\Delta \kappa^{max}_{obs}$. At the activity concentrations of 0.43 and 1.5, the paramagnetic increase exceeds that theoretically possible from the enzyme constituents. We attribute the excess to free radicals outside the enzyme, such as might arise from the xanthine and in the production of hydrogen peroxide. Less likely is the existence of triplet state intermediates along the paths from substrates to products.

Because of the complexity of the system it is not apparent how to separate the various contributions to the magnetic susceptibility. BRAY *et al.*[3] have stated that the iron remains in a diamagnetic ferrous state and that only the molybdenum and flavin undergo cycling. In the 4th column of Table II are given the magnetic changes associated with this scheme, in which we have once again taken all the molybdenum from a diamagnetic even-valent state to the spin 3/2 trivalent state, and all the flavin to free radical. The fifth column lists the contributions above the reduced molybdenum and semireduced flavin necessary to account for the observed values. The concentration of free radical corresponding to the average of the figures in column five is $30 \cdot 10^{-6}\ M$; this is so large a fraction of the initial concentration of xanthine as to suggest either that the changes in electronic configuration of all the metal atoms have not been properly described or that additional free radicals arise in the production of hydrogen peroxide, or both. In any case, there is a substantial free-radical concentration varying nonlinearly with the concentration of enzyme, and a possible mechanism predicting this circumstance is presented in the discussion.

SPECTROPHOTOMETRIC OBSERVATIONS ON SUBSTRATE AND PRODUCT

These observations were made in the ultraviolet region of the spectrum, from 350 mμ to 250 mμ. Most measurements were performed at 295 mμ, at which uric acid has a peak at pH 8 to 10. However, in studies of the effect of pH on the reaction, for observations of the disappearance of xanthine, and in the search for possible intermediates it was necessary to carry out measurements throughout the entire region from 350 to 250 mμ as well as time measurements at individual wavelengths. For the observations of uric acid at pH 8, 295 mμ was not completely satisfactory, because Beer's law was found to be no longer valid above absorbancies of about 1.0.

At these high concentrations the measurements were then made at 305 to 315 mμ, at which the much smaller absorbancies are linearly related to the concentration of uric acid.

pH dependence of the rate of formation of uric acid: Recordings were made, in the split-beam spectrophotometer, of the uric acid formed and of the xanthine consumed as a function of time for a concentration of oxygen of $240 \cdot 10^{-6}$ *M* and for various pH's. With the low concentrations of enzyme used, the protein absorption did not block the spectrophotometer. To permit interpretation of the spectrophotometric recordings at varying pH it was necessary to have spectra for xanthine and uric acid. These are approximately constant above pH 8, but shift continuously toward shorter wavelengths as the pH is decreased to pH 5. Spectra at pH 8 and pH 5 are shown in Fig 5.

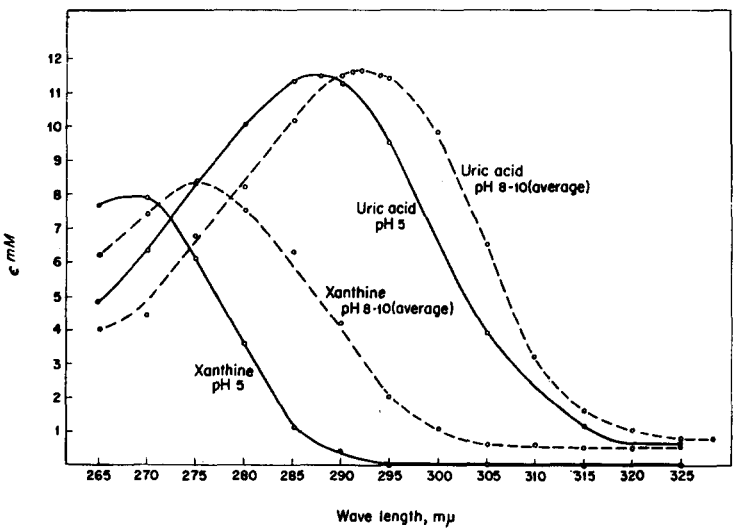


Fig. 5. Absorption spectra of xanthine and uric acid.

Typical spectrophotometric traces are shown in Fig. 6. These were analyzed by computing the rate (v) for various concentrations of xanthine (x). A Lineweaver-Burke plot was then made of x/v against x, and, provided the initial concentration of xanthine was not too high (see page 406 for quantitative description of inhibition by xanthine), the points fell along a straight line. From the slope and the intercept were obtained v'_{max}, the maximum rate at the fixed concentration of oxygen, and K'_x, the concentration of xanthine at which the rate is half of v'_{max}. The relationships between v'_{max} and v_{max}, the latter being the rate when neither xanthine nor oxygen is limiting, and between K'_x and K_x, the true Michaelis constant for xanthine, will be developed in the discussion. However, we emphasize here that the parameters obtained from these Lineweaver-Burke plots are functions of the concentration of oxygen, and are comparable only because this concentration is the same in all the plots.

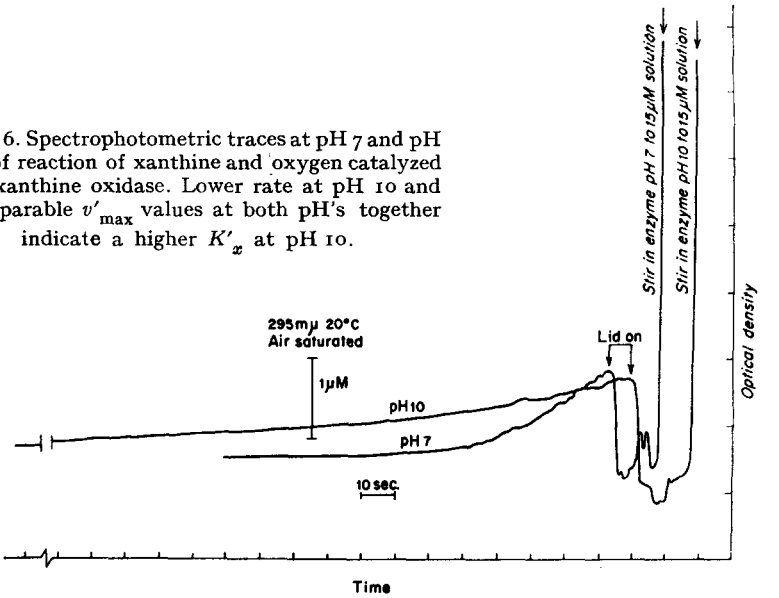


Fig. 6. Spectrophotometric traces at pH 7 and pH 10 of reaction of xanthine and oxygen catalyzed by xanthine oxidase. Lower rate at pH 10 and comparable v'_{max} values at both pH's together indicate a higher K'_x at pH 10.

Fig. 7 summarizes these data, showing v'_{max} and K'_x from the Lineweaver-Burke plot. The values for v'_{max} were confirmed by direct measurement. With $240 \cdot 10^{-6}$ M oxygen at pH 8.3 and 25°, the value for K'_x was $15 \pm 3 \cdot 10^{-6}$ M. The pH dependence suggests that the form of the xanthine probably limits the reaction rate below pH 8 and that changes in the enzyme itself limit the rate above this pH. At pH 8 the rates in pyrophosphate–HCl and "Sorensen" phosphate buffer were compared. No difference was observed. Similar results were obtained at pH 7. Pyrophosphate–HCl was used for all other measurements reported herein.

Temperature studies: Measurements of the rate of formation of uric acid at pH 8.3, $pO_2 = 0.2$ atm,* and $100 \cdot 10^{-6}$ M xanthine, from 2° to 35°, emphasize the complexity of the mechanisms involved. The rates do not lie on a straight line in an Arrhenius plot; the Q_{10} changes continuously. At 5° the Q_{10} was 2.5, at 15° it had decreased to 1.4, and at 20° it was 1.1. The rate at 35° was identical with that at 30°. These data should not be represented in terms of an energy of activation. Rather they indicate that several different effects are entering into the temperature dependence of the rate of formation of uric acid.

Wavelength studies: Since the magnetic data indicate free-radical intermediates, it appeared possible that spectrophotometric studies might also show an intermediate between xanthine and uric acid. Unfortunately, at the concentrations of enzyme at which the greatest magnetic changes occurred, it was impossible to measure the changes of xanthine and uric acid because of the high protein light absorption. On the possibility that such a free-radical intermediate could be detected spectrophotometrically at lower concentrations of enzyme, time studies at 290, 295, 280, and 268 mμ were made. These showed only smooth variations and gave no hint of an intermediate

* The solubility of oxygen in water decreases by a factor of 2 from 2° to 35°. For the Michaelis constants at 25°, this change in concentration decreases the rate by only 25 %.

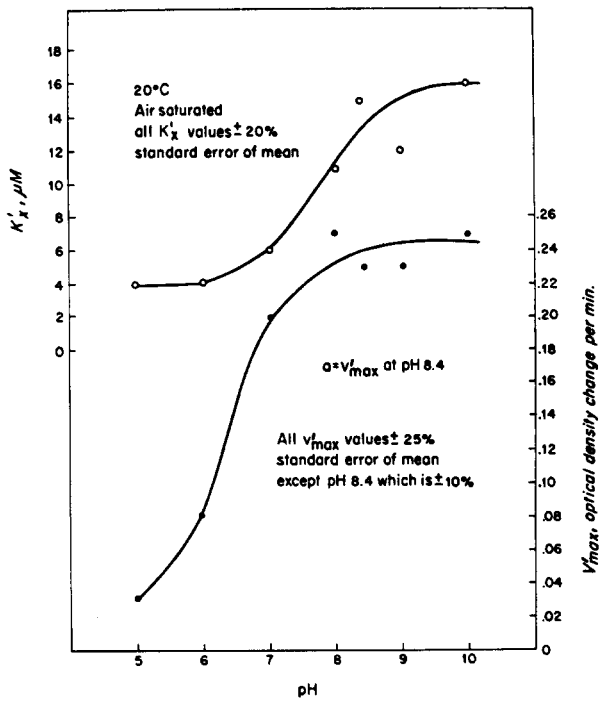


Fig. 7. Values of K'_x and v'_{max} at various pH's. Values of K'_x and v'_{max} were determined from Lineweaver-Burke graphs. Values of v'_{max} were also determined directly and averaged with those from Lineweaver-Burke graphs.

between xanthine and uric acid. To test this further, a series of 12 scans of 260–300 mμ were recorded, one every 20 sec. The curves showed a constant isobestic point and a continuous change from all xanthine to all uric acid. These negative results at low concentrations of enzyme do not rule out the possibility of appreciable concentrations of an intermediate between xanthine and uric acid, since there may be small differences or none at all between the absorption spectrum of xanthine and that of the intermediate in this region.

Measurements at varying oxygen concentrations: Buffered (pH 8.2±0.1) xanthine solutions at 25±2° were saturated with oxygen at a pressure of 1 atmosphere to give a concentration of $1200 \cdot 10^{-6}$ *M*. Small amounts of enzyme were then added, and the formation of uric acid was measured spectrophotometrically at 295 mμ. A comparison of the rate data at this concentration of oxygen with that at $240 \cdot 10^{-6}$ *M* showed a doubling of K'_x, while v'_{max} increased by only about 50 %. The rates of formation of uric acid at concentrations of oxygen less than $240 \cdot 10^{-6}$ *M* were also determined; they were found to be linearly proportional to the concentration of oxygen. These experiments are in accord with a true Michaelis constant for oxygen of about $240 \cdot 10^{-6}$ *M* at 25°, as will appear from the discussion.

Production of hydrogen peroxide: For the purpose of examining the production of hydrogen peroxide during the reaction, catalase was added to various reaction mixtures. In several experiments the reaction of $500 \cdot 10^{-6}$ *M* xanthine and $240 \cdot 10^{-6}$ *M* oxygen was catalyzed by xanthine oxidase at concentrations of about 0.03 activity units/l. The reaction was carried out in stoppered Beckman cuvettes, and it proceeded until $240 \cdot 10^{-6}$ *M* of the xanthine was converted to uric acid. When the catalase was stirred into the cuvette, the reaction started again. Since a small amount of oxygen

was unavoidably stirred in with the catalase, one would expect a slight further oxidation to occur, even if no hydrogen peroxide were present. The observed conversion of xanthine to uric acid amounted to between $120 \cdot 10^{-6}$ M and $180 \cdot 10^{-6}$ M, far in excess of that expected from the stirred-in oxygen. While this amount of conversion confirmed the presence of hydrogen peroxide, it was less than the $240 \cdot 10^{-6}$ M that would have been converted if the concentration of hydrogen peroxide had been equal to the oxygen used up in the first part of this experiment. Either hydrogen peroxide was not formed in stoichiometric amounts or else coupled peroxidations were catalyzed by the catalase.

Inhibition studies: HOFSTEE[13] and earlier workers found that xanthine at high concentrations inhibits the reaction. We have extended these experiments in a series of studies at $240 \cdot 10^{-6}$ M oxygen and initial concentrations of xanthine from 1.0 to $1000 \cdot 10^{-6}$ M, with the following conclusions. In the range 1.0 to $10 \cdot 10^{-6}$ M of xanthine and over a wide range of concentrations of enzyme, the disappearance of xanthine and the formation of uric acid strictly followed Michaelis-Menten kinetics. From 25 to about $500 \cdot 10^{-6}$ M the reaction was of zero order in xanthine. Finally, above $500 \cdot 10^{-6}$ M the initial reaction rates were noticeably decreased.

By contrast, neither $1000 \cdot 10^{-6}$ M uric acid nor $500 \cdot 10^{-6}$ M hydrogen peroxide had any noticeable effect on the measured rate of formation of uric acid when the initial concentration of xanthine was $50 \cdot 10^{-6}$ M and that of oxygen was $240 \cdot 10^{-6}$ M.

SPECTROPHOTOMETRIC OBSERVATIONS ON THE ENZYME

The enzyme was studied from 750 mμ to 350 mμ in the split-beam and double-beam spectrophotometers in a search for intermediates in addition to reduced flavin. While no consistent changes could be found of the nature discussed by BEINERT[14, 15] for other metallo-flavoproteins, pertinent results were obtained on the kinetics and amount of reduction of flavin under various conditions.

Rate of reduction of enzyme flavin: GUTFREUND AND STURTEVANT[2] found that for concentrations of xanthine of 50 to $500 \cdot 10^{-6}$ M the anaerobic reduction of xanthine oxidase is a first-order reaction with a rate of 10.5 sec^{-1}. For aerobic reductions to the steady state, they found, for an initial concentration of xanthine of $250 \cdot 10^{-6}$ M and concentrations of oxygen exceeding $200 \cdot 10^{-6}$ M, a first-order reaction rate of 32 sec^{-1}. Our measurements, which, like theirs, were made with a stopped-flow technic but with a double-beam spectrophotometer, support the latter data. For an enzyme solution of 6 activity units/l in the presence of $9 \cdot 10^{-6}$ M xanthine and $240 \cdot 10^{-6}$ M oxygen, at $25 \pm 2°$ and pH 8.3, the time for half-completion of the reduction of the FAD to the steady state was 0.04 ± 0.02 sec (maximum deviation in 20 determinations). The corresponding first-order rate constant is 12–35/sec., with a most probable value of 17 sec^{-1}. A lower rate for $9 \cdot 10^{-6}$ M as compared with $250 \cdot 10^{-6}$ M xanthine would be expected, since the former falls below the K'_x value of $15 \cdot 10^{-6}$ M. Limitations of equipment prohibited an accurate exploration of the dependence of of the rate upon the concentration of xanthine.

Amount of flavin reduction: MORELL[16] observed a rapid reaction followed by a slow one when xanthine oxidase was reduced by xanthine under anaerobic conditions. The final decrease in absorbancy was nearly as great as that obtained with dithionite, while the rapid phase accounted for a third to a half of the total change. GUTFREUND

AND STURTEVANT[2], in their anaerobic experiments, confirmed the work of MORELL, and in their aerobic experiments found that for initial concentrations of $600 \cdot 10^{-6}$ *M* oxygen and $250 \cdot 10^{-6}$ *M* xanthine, at 25° and pH 8.0, the steady-state reduction of flavin was about a third that of the rapid anaerobic reduction or about 15 % that obtained with dithionite.

We measured the changes in absorbancy accompanying the reduction of the enzyme to the steady state for an initial concentration of oxygen of $270 \cdot 10^{-6}$ *M*, at $20 \pm 1°$ and pH 8.3. Forty determinations at different concentrations of enzyme and initial concentrations of xanthine in excess of $25 \cdot 10^{-6}$ *M* but less than $100 \cdot 10^{-6}$ *M* gave changes in absorbancy ranging from 25 to 50 % of the maximal change due to dithionite, the precise average and standard error of the mean being 37 ± 3 %. The increase over the value of GUTFREUND AND STURTEVANT is somewhat greater than can be explained by the difference in concentration of oxygen, indicating that the temperature dependence of the reduction and oxidation reactions should be investigated separately.

In the experiments described in the preceding paragraph an excess of oxygen was present, and the absorbancy always returned to the value corresponding to oxidized enzyme. When, however, an excess of xanthine was present ($300 \cdot 10^{-6}$ *M* or higher initial concentration), so that the solution became anaerobic, pronounced differences in the absorbancy were noted, depending upon the concentration of enzyme, as the system approached equilibrium. When the activity exceeded 3.5 units/l (enzyme concentration greater than $0.55 \cdot 10^{-6}$ *M*), the change in absorbancy at the termination of the slow reduction was within 20 % of the total change with dithionite. This result is in agreement with MORELL'S measurements, referred to earlier, which were made at high concentrations of enzyme. But at activities of 1–1.5 units/l, the long-term change in absorbancy with $300 \cdot 10^{-6}$ *M* initial xanthine was not more than 20 % greater than the steady-state change with $70 \cdot 10^{-6}$ *M* xanthine. Between 1.5 and 3.5 units/l there was a transition in the observed absorbancy of the anaerobic solutions between the two levels. These comparisons were made in cuvettes in the dual beam spectrophotometer. Identical results were obtained at 438, 450, 530 and 550 mμ.

DISCUSSION

The experiments reported in this paper define more clearly than before the requirements of a reaction mechanism for xanthine oxidase activity. For example, the kinetic scheme proposed by GUTFREUND AND STURTEVANT[2] and shown below does not include an explanation of the magnetic data presented above.

$$\underset{\text{Oxidized enzyme}}{\text{E-FAD}} + \underset{\text{Xanthine}}{XH_2} \longrightarrow \text{E-FAD-}XH_2$$

$$\text{E-FAD-}XH_2 + H_2O \longrightarrow \underset{\text{Reduced enzyme}}{\text{E-FA}DH_2} + \underset{\text{Uric acid}}{XH_2O}$$

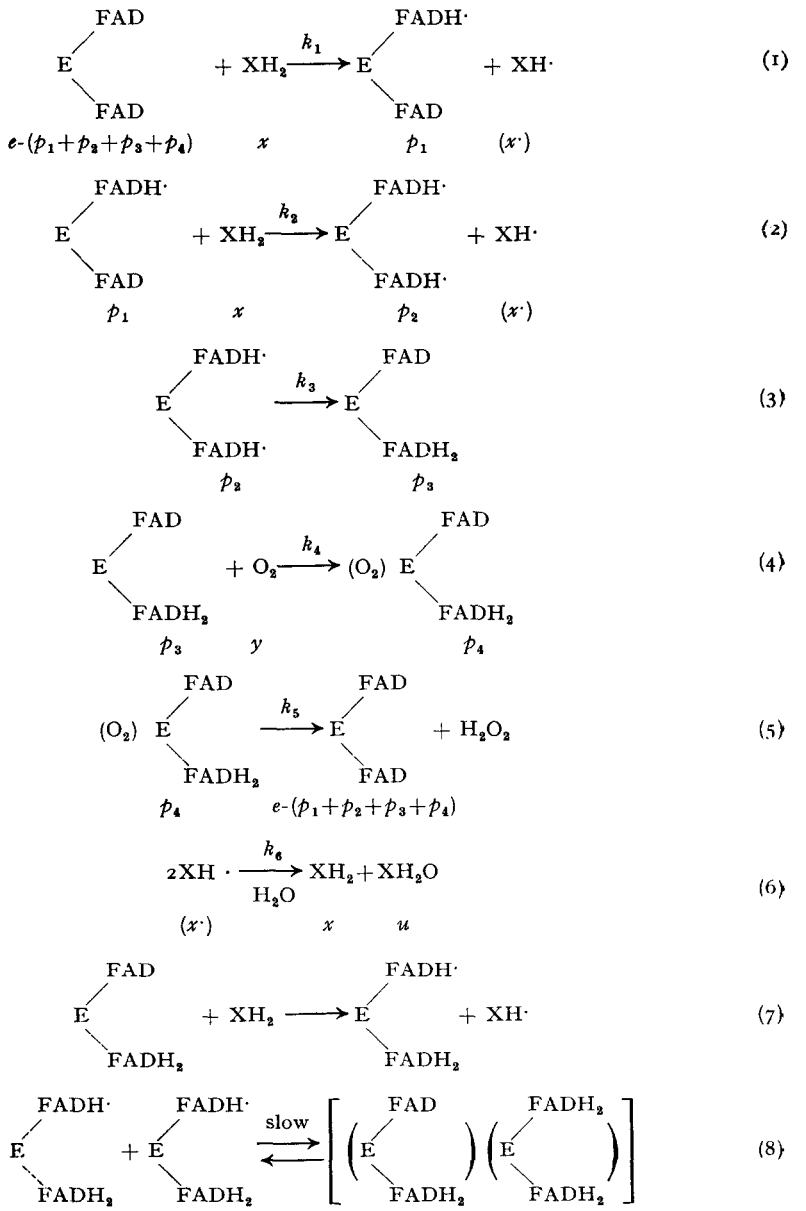
$$\text{E-FA}DH_2 + O_2 \longrightarrow O_2\text{-E-FA}DH_2$$

$$O_2\text{-E-FA}DH_2 \longrightarrow \text{E-FAD} + H_2O_2.$$

Nor does this scheme provide for the well-confirmed observation that under anaerobic conditions xanthine rapidly reduces, at most, half of the flavin reduced by dithionite. MORELL[16] and GUTFREUND AND STURTEVANT[2] have taken the immediate

reduction as a measure of the active enzyme, and they attributed theslow subsequent decrease in absorbancy to reduction of flavin bound to *inactive* enzyme. This explanation would mean that in spite of refined purification the best xanthine oxidase preparations have been at least 50 % inactive. Additionally, it accounts neither for our observation that the fraction of the dithionite change reached by the slowly decreasing absorbancy depends upon the enzyme concentration, nor for thein hibition at high concentrations of xanthine.

We propose the following mechanism:



but $\left[\left(\begin{smallmatrix}\text{FAD}\\ \text{E}\\ \text{FADH}_2\end{smallmatrix}\right)\left(\begin{smallmatrix}\text{FADH}_2\\ \text{E}\\ \text{FADH}_2\end{smallmatrix}\right)\right]$ cannot dissociate to $\begin{smallmatrix}\text{FAD}\\ \text{E}\\ \text{FADH}_2\end{smallmatrix} + \text{E}\begin{smallmatrix}\text{FADH}_2\\ \text{FADH}_2\end{smallmatrix}$. In these equations XH_2 is xanthine and XH_2O is uric acid. The second lines give the concentrations.

The pertinent kinetic equations are:

$$dp_1/dt = k_1 x(e-\sum_i p_i) - k_2 x p_1 \tag{9}$$

$$dp_2/dt = k_2 x p_1 - k_3 p_2 \tag{10}$$

$$dp_3/dt = k_3 p_2 - k_4 y p_3 \tag{11}$$

$$dp_4/dt = k_4 y p_3 - k_5 p_4 \tag{12}$$

$$dx/dt = -x\left[k_1(e-\sum_i p_i) + k_2 p_1\right] + k_6(x\cdot)^2 \tag{13}$$

$$d(x\cdot)/dt = x\left[k_1(e-\sum_i p_i) + k_2 p_1\right] - 2k_6(x\cdot)^2. \tag{14}$$

Features of the *rapid reduction phase* (Eqns. 1–3) are as follows. (a) Xanthine reacts with the two flavins independently to form both enzyme and substrate free radicals. (b) A Michaelis-Menten type of intermediate—that is, one which "dissociates" at a rate that is limiting when the concentration of substrate is high—is included, as required by the kinetic data of GUTFREUND AND STURTEVANT[2]. This intermediate, quite different from the enzyme–xanthine complex of these authors, is the biradical $\begin{smallmatrix}\text{FADH}\cdot\\ \text{E}\\ \text{FADH}\cdot\end{smallmatrix}$, the rearrangement of which to form $\begin{smallmatrix}\text{FAD}\\ \text{E}\\ \text{FADH}_2\end{smallmatrix}$ is a first-order reaction with a time constant of about 0.1 sec. (c) Only half of the total flavin can be reduced by xanthine in these three fast steps. (Dithionite will, of course, reduce all the flavin.) (d) D_2O will not affect the rate of anaerobic reduction, as required by the data of GUTFREUND AND STURTEVANT. (e) The free-radical enzyme intermediates are assumed to have the same absorption spectra (in the regions investigated) as the resting oxidized enzyme.

The *oxidative phase* is shown in Eqns. 4 and 5. By Eqn. 4 we do not wish to imply that oxygen can be bound to the enzyme only when it is in the form $\begin{smallmatrix}\text{FAD}\\ \text{E}\\ \text{FADH}_2\end{smallmatrix}$. There are no data that indicate what fraction of the cycle oxygen remains bound to the enzyme. However, there is just one Michaelis-Menten intermediate in the oxidative phase, namely $(O_2)\ \begin{smallmatrix}\text{FAD}\\ \text{E}\\ \text{FADH}_2\end{smallmatrix}$. It is of the usual enzyme–substrate complex type. Free radicals may be involved in the dissociation of this complex, but there is no evidence that bears directly upon the reaction. The experiments in which catalase was used to indicate the amount of hydrogen peroxide (H_2O_2) produced would

be explained if free hydroxyl radicals were formed but not converted stoichiometrically to hydrogen peroxide. On the other hand the extreme reactivity of hydroxyl radicals makes them unlikely to be intermediates.

The production of uric acid from xanthine radicals is shown in Eqn. 6. We assume that this is the only mechanism for the disappearance of xanthine free radicals, and we find from steady-state analysis in a manner analogous to that of YAMAZAKI *et al.*[17] for the peroxidase system that the concentration of xanthine radicals is proportional to the square root of the concentration of enzyme. This is in accord with the nonlinear nature of the dependence of the changes in magnetic susceptibility on concentration of enzyme.

Eqns. 1–6 represent the mechanism by which xanthine oxidase catalyzes the production of uric acid from xanthine under aerobic conditions, such as in activity tests. Only when the concentration of xanthine is high is reaction 7 operative under aerobic conditions. The enzyme free radical formed in this reaction is catalytically inactive, and it is responsible for the inhibition observed when the concentration of xanthine exceeds $500 \cdot 10^{-6}$ *M*. We suppose that the oxidation of the fully reduced flavin group is somehow hindered by the semireduction of the other group. In the absence of oxygen, xanthine rapidly converts all of the oxidized enzyme to $E\begin{matrix}FAD\\ \diagup\\ \diagdown\\ FADH_2\end{matrix}$ and then reacts with the latter to form the enzyme free radical just mentioned, $E\begin{matrix}FADH\cdot\\ \diagup\\ \diagdown\\ FADH_2\end{matrix}$. There is evidence for reaction 7 in the work of BRAY *et al*[3], in which they obtained a $g = 2.0$ electron spin resonance signal after adding xanthine to the enzyme anaerobically. Since rapid-flow apparatus was not used in the Bray group's experiments, the system on which the measurement was made was beyond the rapid-reduction phase. The xanthine free radicals produced in Eqn. 7 can react as before to form uric acid.

Eqn. 8 represents the slow formation of a nonmagnetic bimolecular enzyme complex in which three fourths of the flavin groups are reduced. The equilibrium degree of association of this complex would increase with the initial concentration of enzyme. The light absorption of a reduced flavin group in this complex is probably not the same as that in free xanthine oxidase, since the flavin groups are implicated in the binding. If the extinction were not much different, we would predict that at high concentrations of enzyme the final decrease in absorbancy upon anaerobic reduction with xanthine would be three fourths of that obtained with dithionite. In support of this viewpoint, our data and those of MORELL[16] give higher values than this, but they are certainly less than those for dithionite reduction. A troublesome point is the apparent transition from dissociated to associated enzyme with only a threefold increase in concentration of enzyme. The well-known dilution law calls for a much smaller dependence of association upon concentration. This question may have its answer in the kinetics of the process and the problems of measurement, for, at the lowest concentrations of enzyme, the terminal reduction phase is a very slow second-order reaction, and the final readings are subject to cumulative errors. The reaction (after Eqn. 8) that is not allowed would otherwise permit the reduction of all the flavin at any concentration of enzyme.

From steady-state analysis of the kinetic Eqns. 9–14 we find for the rate of appearance of uric acid,

$$v = -(\mathrm{d}x/\mathrm{d}t)_{ss} = v_{max}/(1 + K_x/x + K_{O_2}/y)$$

in which the rate with neither xanthine nor oxygen limiting is

$$v_{max} = e/(1/k_3 + 1/k_5),$$

and the true Michaelis constants for xanthine and oxygen are

$$K_x = (1/k_1 + 1/k_2)/(1/k_3 + 1/k_5)$$

and

$$K_{O_2} = 1/k_4(1/k_3 + 1/k_5).$$

A Lineweaver-Burke plot for varying xanthine is the function

$$x/v = K_x/v_{max} + x(1 + K_{O_2}/y)/v_{max}.$$

The inverse slope is not the absolute maximum rate, v_{max}, but the greatest rate possible when the oxygen concentration is y. This rate we call v'_{max}.

$$v'_{max} = v_{max}/(1 + K_{O_2}/y).$$

Similarly, the Michaelis "constant," K'_x, calculated from the quotient of the intercept and the slope, is not the true Michaelis constant, K_x, but the following function of the concentration of oxygen:

$$K'_x = K_x/(1 + K_{O_2}/y).$$

It is apparent that K'_x is the concentration of xanthine such that the rate is half of v'_{max}.

At pH 8.3 and 25° the true Michaelis constants that best fit the data mentioned above are $K_x = 36 \cdot 10^{-6}\ M$ and $K_{O_2} = 240 \cdot 10^{-6}\ M$. (It is coincidental that K_{O_2} is the solubility of oxygen in water for $p_{O_2} = 0.2$ atm at 25°.) In confirmation of this analysis is the Michaelis "constant" for xanthine of $20 \cdot 10^{-6}\ M$ that GUTFREUND AND STURTEVANT[2] obtained for a concentration of oxygen of $600 \cdot 10^{-6}\ M$ at 25° and pH 8.0. Our studies of pH dependence indicate that the latter decrease in pH reduces K_x by about 15 %, to $31 \cdot 10^{-6}\ M$. Using the following formula derived from the condition $v/v'_{max} = 1/2$,

$$\frac{1 + K_{O_2}/y}{1 + K_x/K'_x + K_{O_2}/y} = \frac{1}{2},$$

we obtain $K'_x = 22 \cdot 10^{-6}\ M$, in excellent agreement with the experimental value of GUTFREUND AND STURTEVANT. These authors have also "estimated" a Michaelis "constant" for oxygen, but this has been done under experimental conditions so unfavorable to its evaluation as to produce a result that is misleading, as reflected in the following discussion.

In our kinetic scheme and that of GUTFREUND AND STURTEVANT, the maximum turnover is related to the rates of "dissociation" of the Michaelis-Menten intermediates by

$$\text{max. turnover} = v_{max}/e = 1/(1/k_3 + 1/k_5).$$

For this equation to hold, the concentrations of xanthine and oxygen must be well above the true Michaelis constants. Because the concentration of oxygen ($600 \cdot 10^{-6}\ M$) at which GUTFREUND AND STURTEVANT obtained the turnover of 8 (± 0.5) sec^{-1} is closer to K_{O_2} than they expected, their rate is lower than the maximum turnover by

about 40 %. Furthermore, the apparent agreement that these authors have found between the observed turnover and that computed as above from observed rates is the result of an error in that calculation. In fact, a calculation based on their data yields 7.05 sec^{-1}, not 7.88 sec^{-1}. While we are concerned about the disparity between the turnover numbers evaluated in different ways, we attribute it to the inaccuracies involved in estimating the concentration of enzyme used in calculating the turnover from the rate of production of uric acid, and not to an error in the relationship given above.

With respect to our estimations of concentration of enzyme, a few points should be mentioned briefly. As the preparations of xanthine oxidase aged they became less active, and both the activity-to-protein and activity-to-flavin ratios decreased. However, for reactions lasting only a few minutes, the rate determinations were not affected, provided that the changes in flavin absorption were computed in terms of the absorption changes that occurred with dithionite rather than in terms of total absorbancy at 450 mμ. Likewise, in measuring production of uric acid and utilization of xanthine, solutions were always compared on the basis of their enzymic activity, as assayed at pH 8.3 in dilute solutions of enzyme. Finally, it should be recalled that since no assays for flavin, molybdenum, and iron were conducted, we did not measure an independent turnover number; rather the one obtained by AVIS *et al*[1]. was used by us to determine the concentration of active enzyme.

ACKNOWLEDGEMENTS

This Investigation is based on work done at the Johnson Research Foundation, the University of Pennsylvania. The advice and technical assistance of Dr. BRITTON CHANCE and his staff are gratefully acknowledged. This work was supported in part by the National Heart Institute of the United States Public Health Service under Grant H-3038.

The authors wish to thank the Dairy Department of the Pennsylvania State University for supplying unpasteurized milk and for carrying the preparation through the step of casein removal.

REFERENCES

1 P. G. AVIS, F. BERGEL AND R. C. BRAY, *J. Chem. Soc.*, (1955) 1100.
2 H. GUTFREUND AND J. M. STURTEVANT, *Biochem. J.*, 73 (1959) 1.
3 R. C. BRAY, B. G. MALMSTRÖM AND T. VÄNNGÅRD, *Biochem. J.*, 73 (1959) 193.
4 P. G. AVIS, F. BERGEL AND R. C. BRAY, *J. Chem. Soc.*, (1956) 1219.
5 A. S. BRILL, A. EHRENBERG AND H. DEN HARTOG, *Biochim. Biophys. Acta*, 40 (1960) 313.
6 A. S. BRILL, H. DEN HARTOG AND V. LEGALLAIS, *Rev. Sci. Instr.*, 29 (1958) 383.
7 A. S. BRILL, H. DEN HARTOG AND V. LEGALLAIS, *Rev. Sci. Instr.*, 29 (1958) 242.
8 C. C. YANG AND V. LEGALLAIS, *Rev. Sci. Instr.*, 25 (1954) 801.
9 C. C. YANG, *Rev. Sci. Instr.*, 25 (1954) 807.
10 B. CHANCE, *Rev. Sci. Instr.*, 22 (1951) 634.
11 B. CHANCE AND V. LEGALLAIS, *Rev. Sci. Instr.*, 22 (1951) 627.
12 H. THEORELL AND A. EHRENBERG, *Arch. Biochem.*, 41 (1952) 442.
13 B. H. HOFSTEE, *J. Biol. Chem.*, 216 (1955) 235.
14 H. BEINERT, *Biochim. Biophys. Acta.*, 20 (1956) 588.
15 H. BEINERT, *J. Biol. Chem.*, 225 (1957) 465.
16 D. B. MORELL, *Biochem. J.* 51 (1952) 657.
17 I. YAMAZAKI, H. S. MASON AND L. PIETTE, *J. Biol. Chem.*, 235 (1960) 2444.