PRELIMINARY NOTES

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Improved xanthine oxidase purification

In earlier work it was concluded from indirect evidence that active milk xanthine oxidase a is normally contaminated by two inactive modifications, xanthine oxidase i_1 and xanthine oxidase i_2, the last of these being devoid of molybdenum[1,2]. In this communication we present further data on the occurrence of these forms. It is concluded that xanthine oxidase i_1 is a preparation artefact whose formation can be minimized by rapid working and by the appropriate use of chelating agents. Xanthine oxidase i_2 on the other hand may be secreted by cows along with xanthine oxidase a but can be removed by selective denaturation with high concentrations of sodium salicylate.

Buttermilk, pancreatin digestion, *n*-butanol and ammonium sulphate steps of the purification were as described earlier[3]. Xanthine oxidase activity was measured spectrophotometrically[4,5] and molybdenum analyses were carried out by a modified dithiol procedure (L. I. HART, unpublished). Unless otherwise stated, salicylate (1 mM) and EDTA (1 mM) were present during all purification steps while storage was in the presence of salicylate (30 mM) and EDTA (1 mM)[4].

For 4 samples at the above stage of purification prepared using 1–10 mM Na_2-EDTA and 1 mM sodium salicylate in the buttermilk, the ratio activity (units/l) to Mo concn. (μM) was 5.8 $\pm$ 0.1 (mean deviation). However, when the concentration of the chelating agents was raised, higher activity/Mo ratios were obtained, indicating that less xanthine oxidase i was present. With buttermilk concentrations of about 30 mM EDTA and 10 mM salicylate (the additions being made to the cream, or to the milk and then to the cream) the mean value of the activity ratio for 8 samples rose to 6.5 $\pm$ 0.2. The highest activity/Mo value obtained was 6.9. This is to be compared with earlier values of 4.1 and 5.5 calculated from data in refs. 2 and 6, respectively, for samples prepared without, or using only low concentrations, of the chelating agents. Further increases in the ratio were not obtained on using still higher EDTA concentrations or when the cows were milked directly into glass containers and subsequent operations carried out in the laboratory to avoid contact with metals which normally occurred at the dairy. Some decreases in activity/Mo sometimes occurred during purification and storage of the enzyme despite the precautions taken.

The secretion of xanthine oxidase i_2 was studied by measuring the ratio FAD/Mo on enzyme samples isolated from the milks of individual cows. FAD analyses were carried out fluorimetrically[7]. The ratio was determined on samples prepared as above, then further purified by a single batch-wise calcium phosphate adsorption step. (In separate experiments on a number of samples it was found that neither this step, nor subsequent adsorption or gel filtration steps in the purification, changed the ratio significantly. In confirmation of earlier work[8] apparently homogeneous enzyme could be obtained showing non-integral FAD/Mo ratios.) 12 samples from 3 Friesian cows obtained over a 7 month period showed FAD/Mo ratios of from 1.3 to 2.9. Ratios over 1.0 were taken to indicate the presence of xanthine oxidase i_2. Variations

from one cow to another on a given day were relatively small, while it seemed that variations with time were of a nutritional origin, since the highest ratios were recorded during a period of abnormally low milk yield in the late winter.

Separation of xanthine oxidase a, xanthine oxidase i_1 and xanthine oxidase i_2 from one another has not been achieved but we have found conditions under which selective denaturation of xanthine oxidase i_2 occurs. Sodium salicylate is the denaturant used[6]. The conditions employed were as follows. 0.5–1% solutions of xanthine oxidase in 0.1 M sodium pyrophosphate buffer (pH 7.0) containing 0.6 M sodium salicylate were heated at 37° for 24 h. Precipitated material was centrifuged off and salicylate was removed by gel-filtration through Sephadex G-25. In a large number of experiments the product always contained greatly diminished amounts of xanthine oxidase i_2 (FAD/Mo decreased, usually to about 1.0) and while there were substantial losses of both xanthine oxidase a and xanthine oxidase i_1 in the process these went parallel to one another (activity/Mo unchanged or only very slightly decreased).

TABLE I

COMPOSITION AND PROPERTIES OF XANTHINE OXIDASE PREPARED USING SALICYLATE DENATURATION

FAD/Mo (molar ratios)	1.00
FAD/protein (molar ratios)*	1.65
Fe/FAD (molar ratios)	4.4
Activity (units/l)**/Mo concn. (μM)	5.7
Activity (units/l)**/$A_{450\ m\mu}$	153
$A_{420\ m\mu}/A_{450\ m\mu}$	0.89
Ultracentrifugation***; $s_{20,w}$§ (S)	10.4
Ultracentrifugation***; impurities: 7 S	<2%
14 S	9%
Approx. mol. wt. (Sephadex G-200)§§	310 000

* Biuret protein determination, taking mol. wt. 275 000.

** Ref. 5.

*** Data on a second sample of enzyme prepared in the same manner.

§ The corresponding value at the same concentration (1.25%) on material prepared by the earlier method is about 10.8 S (ref. 12).

§§ The elution volume (ref. 12) was (2 ± 2)% lower than that of a sample prepared by the earlier method.

By following salicylate treatment with gel filtration on Sephadex G-200, using 0.1 M pyrophosphate buffer (pH 7.0) and a 5 cm × 66 cm column for 0.21 g of enzyme, we have obtained material with the properties shown in Table I. The specific activity is higher than any previously published but since activity/Mo on this particular sample was still only 5.7 compared with the higher values quoted above for cruder material, we conclude that activity/$A_{450\ m\mu}$ ratios of up to about 180 or perhaps even higher might be obtainable. This means that many xanthine oxidase samples used hitherto in this laboratory and elsewhere must have contained no more than 50% xanthine oxidase a.

Xanthine oxidase prepared by the salicylate method comes close to containing 2 Mo, 2 FAD and 8 Fe per molecule as predicted for the pure milk enzyme[1]. Apart from the higher specific activity and molybdenum content it appears identical with earlier preparations. The continued presence of iron[9] is of interest in view of reports

that the metal may be removed by dialysis against dithionite *plus* dipyridyl or phenanthroline[10,11].

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1 R. C. BRAY, in P. D. BOYER, H. LARDY AND K. MYRBÄCK, *The Enzymes*, Vol. 7, Academic Press, New York, 1963, p. 533.
2 R. C. BRAY, R. PETTERSSON AND A. EHRENBERG, *Biochem. J.*, 81 (1961) 178.
3 G. PALMER, R. C. BRAY AND H. BEINERT, *J. Biol. Chem.*, 239 (1964) 2657.
4 F. BERGEL AND R. C. BRAY, *Biochem. J.*, 73 (1958) 182.
5 P. G. AVIS, F. BERGEL AND R. C. BRAY, *J. Chem. Soc.*, (1955) 1100.
6 R. C. BRAY, A. J. CHISHOLM, L. I. HART, L. S. MERIWETHER AND D. C. WATTS, in E. C. SLATER, *Flavins and Flavoproteins* (BBA Library, Vol. 8), Elsevier, Amsterdam, 1966, p. 117.
7 H. B. BURCH, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Academic Press, New York, 1957, p. 960.
8 P. G. AVIS, F. BERGEL AND R. C. BRAY, *J. Chem. Soc.*, (1956) 1219.
9 C. E. JOHNSON, P. F. KNOWLES AND R. C. BRAY, *Biochem. J.*, 103 (1967) 10C.
10 E. BAYER AND W. VOELTER, *Biochem. Biophys. Acta*, 113 (1966) 632.
11 M. UOZUMI, R. HAYASHIKAWA AND L. H. PIETTE, *Arch. Biochem. Biophys.*, 119 (1967) 288.
12 P. ANDREWS, R. C. BRAY, P. EDWARDS AND K. V. SHOOTER, *Biochem. J.*, 93 (1964) 627.

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Preparation of an adrenal steroid 21-hydroxylating system exhibiting activity in the absence of the carbon monoxide-binding pigment, cytochrome P-450

Cytochrome P-450 has been implicated as the oxygen-activating component in adrenal steroid hydroxylation using various mitochondrial and microsomal preparations[1–3]. We have been unable to confirm the necessity for the presence of cytochrome P-450 in active, purified preparations of steroid 21-hydroxylase (EC 1.14.1.8).

Sheep adrenal glands were processed, fractionated and assayed for 21-hydroxylating activity, using 17-hydroxyprogesterone as substrate, as previously described[4]. The most active dialyzed ammonium sulfate precipitate (0.3 S) was frozen and thawed once, and centrifuged for 20 min at 10 000 × *g*. About 50% of the activity remained in the supernatant, which was further fractionated by gel filtration on a column (5 cm × 100 cm) of Sephadex G-200 at 4°. The column was equilibrated with 0.1 M phosphate buffer (pH 6.8) containing reduced glutathione, 1 mg/ml, and