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The Composition of Milk Xanthine Oxidase

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The composition of milk xanthine oxidase has been reinvestigated. When the enzyme is prepared by methods that include a selective denaturation step in the presence of sodium salicylate the product is obtained very conveniently and in high yield, and is homogeneous in the ultracentrifuge and in recycling gel filtration. It has specific activity higher than previously reported preparations of the enzyme and its composition approximates closely to 2mol of FAD, 2g-atoms of Mo and 8g-atoms of Fe/mol of protein (molecular weight about 275000). In contrast, when purely conventional preparative methods are used the product is also homogeneous by the above criteria but has a lower specific activity and is generally comparable to the crystallized enzyme described previously. Such samples also contain 2mol of FAD/mol of protein but they have lower contents of Mo (e.g. 1.2g-atom/mol). Amino acid compositions for the two types of preparation are indistinguishable. These results confirm the previous conclusion that conventional methods give mixtures of xanthine oxidase with an inactive modification of the enzyme now termed 'de-molybdo-xanthine oxidase', and show that salicylate can selectively denature the latter. The origin of de-molybdo-xanthine oxidase was investigated. FAD/Mo ratios show that it is present not only in enzyme purified by conventional methods but also in 'milk microsomes' (Bailie & Morton, 1958) and in enzyme samples prepared without proteolytic digestion. We conclude that it is secreted by cows together with the active enzyme and we discuss its occurrence in the preparations of other workers. Studies on the milks of individual cows show that nutritional rather than genetic factors determine the relative amounts of xanthine oxidase and de-molybdo-xanthine oxidase. A second inactive modification of the enzyme, now termed 'inactivated xanthine oxidase', causes variability in activity relative to E_{450} or to Mo content and formation of it decreases these ratios during storage of enzyme samples including samples free from de-molybdo-xanthine oxidase. We conclude that even the best purified xanthine oxidase samples described here and by other workers are contaminated by significant amounts of the inactivated form. This may complicate the interpretation of changes in the enzyme taking place during the slow phase of reduction by substrates. Attempts to remove iron from the enzyme by published methods were not successful.

Milk xanthine oxidase has been widely studied and numerous methods of purification have been described. Many of these give products that are apparently homogeneous or nearly so by various criteria (Avis, Bergel & Bray, 1955, 1956*b*; Avis, Bergel, Bray, James & Shooter, 1956*a*; Gilbert & Bergel, 1964; Palmer, Bray & Beinert, 1964; Nelson & Handler, 1968; Massey, Brumby, Komai & Palmer, 1969). The enzyme is known to contain molybdenum, iron and FAD (Bray, 1963) but there are a number of serious discrepancies in previous reports of the proportions of these constituents and their roles in the enzymic reaction. One discrepancy is whether the enzyme contains two mol of FAD/g-atom of molybdenum (Totter, Burnett, Monroe, Whitney & Comar, 1953; Green & Beinert, 1953; Mackler, Mahler & Green, 1954; Richert & Westerfeld, 1954) or one (V. Massey, results presented at 3rd Internat. Conf. on Magnetic Resonance in Biological Systems, Warrenton, Virginia, U.S.A., 1968; see also Massey *et al.* 1969). Avis *et al.* (1956*b*) explained non-stoicheiometric FAD/Mo ratios and variable specific activities of their crystallized enzyme by postulating the presence of two inactive forms of the enzyme. However, other workers were reluctant to admit that these might

be present in their own preparations (Nelson & Handler, 1968; Massey *et al.* 1969). A second discrepancy concerns the iron of the enzyme. Bray, Palmer & Beinert (1964) obtained kinetic e.p.r.* data indicating that iron was involved in the catalytic action. However, other workers claimed that iron could be removed without destroying activity (Bayer & Voelter, 1966; Uozumi, Hayashikawa & Piette, 1967). Since a proper understanding of the mechanism of action of the enzyme is impossible without resolving these problems, we have carried out the investigations reported below.

MATERIALS AND METHODS

General. All preparative operations, unless otherwise stated, were carried out in a cold room at 0–5°C. Glass-distilled water was used throughout and analytical grade reagents were generally employed. Pyrophosphate buffers were prepared by adding HCl to $Na_4P_2O_7,10H_2O$. 1M-Phosphate, pH5.8, was prepared from 0.8M-KH_2PO_4 and 0.2M-Na_2HPO_4. Phosphate buffers for hydroxylapatite chromatography and for calcium phosphate gel purification procedures were prepared by diluting the 1M solution, causing a slight increase in pH. Unless otherwise stated, 1mM-sodium salicylate and 1mM-EDTA were added to buffers to increase the stability of the enzyme (Bergel & Bray, 1959).

Enzyme activity measurements. Oxidase activity with xanthine as substrate was measured spectrophotometrically, following uric acid production at 295nm with a Gilford model 220 optical-density converter (Gilford Instruments, Oberlin, Ohio, U.S.A.) coupled to a Unicam SP.500 spectrophotometer and a strip-chart recorder. The calibration of the instrument was checked regularly with KNO_3 solution, which has ϵ_{295} 6.6. The reaction was carried out at 23.5±0.1°C in 1cm cells with 2.5ml of 0.05M-pyrophosphate buffer (containing 1mM-EDTA but no salicylate), 25μl of 10mM-xanthine (in 20mM-NaOH) and 25μl of enzyme. The final pH was 8.2. The enzyme was generally diluted to give ΔE_{295}/min 0.025–0.1. Units were as defined by Avis *et al.* (1955). Thus 1 unit of activity in the assay cell gave ΔE^{1cm}_{295}/min 393. Conversion into international units (at 23.5°C and pH8.2) was carried out by multiplying by 104, this factor being based on $\Delta\epsilon_{295}$ 9600 for oxidation of xanthine to uric acid (Avis *et al.* 1956*b*). Activity/E_{450} ratio was calculated as ΔE^{1cm}_{295}/min divided by E^{1cm}_{450} for the enzyme sample at the overall dilution used in the activity measurement.

Dehydrogenase activity with 2,6-dichlorophenol-indophenol as acceptor and xanthine as substrate was measured aerobically by a method similar to that used for the oxidase activity. The procedure was based on that of Avis *et al.* (1956*b*) and the assay mixture contained 0.1mM-xanthine, 0.0125mM-indophenol and 50mM-pyrophosphate buffer, pH8.2, containing 1mM-EDTA, at 23.5°C. Measurements were made at 600nm with a 4cm light-path by using $\Delta\epsilon_{600}$ 21000 for indophenol reduction.

* Abbreviation: e.p.r., electron paramagnetic resonance.

Analyses. FAD was determined fluorimetrically on thoroughly dialysed samples of xanthine oxidase by the procedure of Burch (1957). The measurement of the fluorescence of unhydrolysed FAD was omitted, and the flavin content was calculated by comparison of the fluorescence of the acid-hydrolysed sample (10min at 100°C in 10% trichloroacetic acid) with that of riboflavin standards carried through the entire procedure. Internal riboflavin standards confirmed that there were no systematic errors. Separate determinations on a given enzyme sample generally agreed within 3%.

Molybdenum was determined colorimetrically by an improved dithiol method based on those of Clark & Axley (1955) and Bingley (1959, 1963). The procedure was as follows: samples of the enzyme (containing about 10ng-atoms of Mo) were wet-ashed in 25ml borosilicate glass tubes by adding 0.5ml of a mixture of 2 vol. of $HClO_4$ (sp.gr. 1.54) and 3 vol. of H_2SO_4 (sp.gr. 1.84) and heating the open tubes until all samples were colourless. The samples were cooled and 15ml of 4.8M-HCl was added to each tube, followed by 0.5ml of 5% (w/v) $FeSO_4,7H_2O$ in 0.4M-H_2SO_4 and 0.5ml of 50% (w/v) NaI respectively. After 10min the brown colour was discharged with 10% (w/v) $Na_2SO_3,7H_2O$. Then 2ml of 10% (w/v) thiourea and 0.5ml of 50% (w/v) tartaric acid were added, followed by 2ml of dithiol reagent [0.2% (w/v) toluene-3,4-dithiol in 1% (w/v) NaOH containing 1.4% (v/v) thioglycollic acid], and the mixtures were shaken vigorously for 30s and left for 30min. They were then shaken with 2.5ml of isoamyl acetate and the extinctions of the extracts were measured at 680nm in 4cm micro-cells on a Unicam SP.500 spectrophotometer. The method was standardized with MoO_3 (dried, then dissolved in dilute NaOH and adjusted to about pH3), carried through the entire procedure. Internal standards confirmed that there were no systematic errors. Separate determinations on a given enzyme sample generally agreed within 2%.

Iron was determined by the method of Seven & Peterson (1958) modified by substitution of the wet-ashing procedure described above, and by adding extra sodium acetate equivalent to the H_2SO_4 remaining after digestion. The method was standardized with ferrous ammonium sulphate solutions carried through the entire procedure. Internal standards confirmed that there were no systematic errors. Buffers used in the attempts to remove iron from xanthine oxidase by the method of Uozumi *et al.* (1967) were analysed for iron either as described above, or when *o*-phenanthroline was present, by adding iron-free hydroxyammonium chloride solution directly and measuring the resulting extinctions at 510nm in 4-cm microcells (Fortune & Mellon, 1938).

Total protein was determined by the biuret method (Layne, 1957) with albumin as standard (crystallized bovine plasma albumin, from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K.; dried at 100°C *in vacuo* over P_2O_5 in a drying pistol). Interference by pyrophosphate buffer was prevented by precipitating the enzyme with trichloroacetic acid before analysis (Robinson & Hogden, 1940). The mean protein recovery for a number of samples by this procedure was 90%. According to Massey and co-workers, the biuret extinction coefficient of albumin is 84% of that of xanthine oxidase (Massey & Williams, 1965; Massey *et al.* 1969). Since these two factors roughly cancel one another out, we have applied

no correction to our observed results. These were obviously less precise than our other analyses. Mol.wt. 275000 (Andrews, Bray, Edwards & Shooter, 1964) was assumed for converting protein weights to molar ratios.

Amino acid analyses were carried out by the method of Moore, Spackman & Stein (1958) using an EEL analyser, after hydrolysing for 24h in 6M-HCl. Ultracentrifuge studies were carried out in a Beckman Spinco model E analytical ultracentrifuge. Samples were dialysed against 20mM-pyrophosphate buffer, pH7.0 (containing 1mM-salicylate and 1mM-EDTA), and if necessary concentrated by vacuum dialysis (see below). They were adjusted to a concentration (based on E_{450}) of about 43μM.

Chromatographic methods. Perspex columns with low dead spaces (Wright Scientific Ltd., Kenley, Surrey, U.K.) were employed. Solutions were pumped into the columns with a peristaltic pump (Bühler micropump type MP1, from Northern Media Supply Ltd., Hull, U.K.). Light-absorption at 254nm was monitored continuously with a Uvicord 1 flow analyser (LKB Instruments Ltd., South Croydon, Surrey, U.K.). Samples were collected in a time-operated fraction collector. The outputs of the u.v.-absorption monitor and of an event marker actuated by the fraction collector were taken to a multi-channel strip-chart recorder. For recycling chromatography a special valve (type 4911B, LKB Instruments Ltd.) was employed. All chromatographic operations, including packing the columns, were carried out at 0–5°C.

Hydroxylapatite was prepared, packed and the packing tested as described by Levin (1962) except that during packing the column was fitted with an extension tube (55cm long) and a funnel connected to the top, the diameter of the extension tube and the funnel neck being the same as that of the column. The whole was filled with the buffer to be used for chromatography and a mechanical stirrer was fitted to operate in the funnel. When packing appeared to be complete (about 16–24h) the extension was disconnected and excess of hydroxylapatite was carefully removed, leaving a gap of several cm at the top of the column to allow for expansion of the bed. After the bed had settled, a glass fibre disc (Whatman GF/B) was placed on top of it and it was thoroughly washed by pumping buffer through. During chromatography a constant flow rate was maintained from a pump inserted between the buffer reservoir and the top of the column. The flow rate (usually 0.1–0.2 column vol./h) was chosen so as to be approximately equal to the unpumped flow rate of the column at a 200cm head of buffer.

Sephadex G-200 [Pharmacia (G.B.) Ltd., London W.5, U.K.] was first shaken on a 300-mesh sieve to remove fine particles and was then soaked in buffer (7 days at 0–5°C or 5h at 90–100°C). The de-aerated suspension was packed into a column fitted with an adjustable plunger as described by Porath & Bennich (1962). The flow rate was about 3.5ml/cm^2 per h when packing started, and finally about 7ml/cm^2 per h. The operating flow rate (upward flow) was 1.6–2.0ml/cm^2 per h. The performance of the column was checked with Blue Dextran 2000 [Pharmacia (G.B.) Ltd.].

Preparation of xanthine oxidase. The method is based on those described earlier (Gilbert & Bergel, 1964; Palmer *et al.* 1964; Hart & Bray, 1967) and consists of steps numbered 1, 2*a*(i), 2*a*(ii), 2*b* and 3. Steps 2*a*(i), 2*a*(ii) and 2*b* are alternative to each other.

Step 1. The initial step of the preparation differed from that of Palmer *et al.* (1964) only in that salicylate and EDTA were added at an earlier stage and the ammonium sulphate limits were narrowed. Cream was separated from fresh milk without cooling below about 23°C. Salicylate and EDTA were then added immediately as a concentrated solution to the cream to give the following final concentrations: sodium salicylate, 5mM; K_2H_2–EDTA, 10mM; tetrasodium EDTA, 10mM. The cream was then churned at about 9°C and the resulting buttermilk (pH6.0–6.6) was processed, usually in batches of about 20l. It was warmed to 36–38°C and the following additions were made/l of buttermilk: $NaHCO_3$, 17g; pancreatin (from British Drug Houses Ltd.), 1.6g; cysteine hydrochloride, 0.3g. The mixture was stirred and the temperature maintained for 3.5h. It was then cooled to about 3°C and 143ml of butan-1-ol, pre-cooled to −10°C, and 200g of ammonium sulphate were added/l of mixture. The mixture was centrifuged (1500$g_{av.}$ for 30min) 15min after the ammonium sulphate had dissolved, and the pale brown lower aqueous layer was sucked off from below a bulky precipitate, which was rejected. More ammonium sulphate (70g/l of aqueous layer) was added and dissolved. The mixture was left for about 16h. The enzyme precipitated and, on standing, collected on top of the clear yellow solution, which was run off and rejected. The remainder was centrifuged (4200$g_{av.}$ for 45min) and the precipitate was dissolved in a minimum volume of 0.1M-pyrophosphate, pH7.0. The turbid brown solution was clarified by centrifuging (40000$g_{av.}$ for 30min) and ammonium sulphate and butanol were removed by gel filtration on Sephadex G25 with the same buffer.

Step 2*a*(i). The concentration of xanthine oxidase in the solution in 0.1M-pyrophosphate buffer, pH7, obtained from Step 1 (determined from E_{450}), was usually 16–33μM. If not it was diluted with buffer to this range, which was suitable for the salicylate denaturation step, or concentrated (see below). Solid sodium salicylate (100g/l of enzyme, i.e. 0.6M) was then added and dissolved. The solution was heated at 37°C for 16h. After cooling, precipitated protein was centrifuged off (40000$g_{av.}$ for 60min) and the clear solution was passed through Sephadex G25 to remove salicylate, liberated FAD etc.

Step 2*a*(ii). Alternatively, exactly the same procedure as described under Step 2*a*(i) was carried out except that sodium salicylate was added at the rate of 16g/l (0.1M) and the heating at 37°C was continued for 7 days.

Step 2*b*. As a further alternative, the product from Step 1 was purified by chromatography on a column of hydroxylapatite (40cm×3.2cm diam.). For chromatography, 1mM-salicylate with 1mM-triethylenetetramine (Ralph N. Emanuel Ltd., London S.E.1, U.K., technical grade redistilled at 0.15mmHg pressure before use) was added to the phosphate buffers (see above). Triethylenetetramine resembles EDTA in its ability to sequester heavy metals, but unlike EDTA it has negligible affinity for Ca^{2+} (Reilley & Vavoulis, 1959). The enzyme was found to be stable on chromatography in this medium. The column was equilibrated with 0.3M-phosphate and the enzyme sample was dialysed against this buffer before application. The load was 3–5mg of total protein/

ml bed volume and the sample was usually applied at a protein concentration of 10–20mg/ml (maximum 50mg/ml). The enzyme from Step 1 could usually be applied directly to the column after dialysis, though a considerable number of runs were required for the product from 20l of buttermilk. Elution was accomplished by increasing the buffer concentration stepwise up to a maximum phosphate concentration of 1.0M. The enzyme generally came off in the 0.5M-phosphate fraction and activity and E_{450} were coincident in all eluted fractions. Contaminating proteins (devoid of activity and 450nm absorption) were eluted both before and after the enzyme fractions. After washing with 1.0M buffer the column could be re-used.

Step 3. The product either from Step 2*a*(i) or 2*a*(ii) or from Step 2*b* was purified by gel-filtration on Sephadex G-200 by the recycling technique (Porath & Bennich, 1962). The enzyme sample was concentrated (see below), dialysed against 0.1M-pyrophosphate buffer, pH7.0, then applied to the column (67cm×5cm diam.; bed vol. 1.3l). Up to 3g of enzyme could be processed on this column provided that sample volume was less than 3% of bed volume, and viscosity relative to buffer was less than 2. Three or four cycles were usually required. Results from the final cycle for two typical batches (the ones detailed in Table 1) are recorded in Fig. 1.

Mild methods for the early stages of xanthine oxidase isolation from milk. Three alternative procedures were used.

Milk 'microsomes'. Buttermilk containing 1mM-sodium salicylate was prepared from bulk Friesian herd milk by the procedure of Palmer *et al.* (1964) and taken to the Chester Beatty Research Institute. 'Microsomes' were isolated from this by the method given in Fig. 2 of Bailie & Morton (1958) except that the washing and suspension medium was 0.15M-NaCl containing 1mM-sodium salicylate. The procedure was taken to stage 'M_1 and M_2' of their method.

Method A. The procedure was generally as described in Step 1 above up to the second addition of ammonium sulphate, except that addition of pancreatin and subsequent incubation at 36–38°C were omitted. The first addition of ammonium sulphate was at the rate of 190g/l and the second at the rate of 110g/l of aqueous layer. The resulting precipitate was removed, suspended in 1M-phosphate buffer, pH5.8, containing 30mM-sodium salicylate and 1mM-EDTA, and dialysed against this buffer. The final solution was concentrated by vacuum dialysis and clarified by centrifuging at $100000g_{av.}$.

Method B. Whole milk was treated with 0.2M-EDTA–20mM-sodium salicylate–NaOH, pH6.7 (50ml/l). Buttermilk was obtained from this as described in Step 1 above except that no further additions of EDTA–salicylate were made either to the cream or the buttermilk. The buttermilk was cooled to about 2°C, cysteine hydrochloride (0.3g/l) and butan-1-ol (400ml/l) at −2°C were added and the mixture was stirred for 15min. The pH of the serum was then adjusted to 4.95 (at 2°C) with 0.25M-acetic acid to precipitate casein, which was discarded (Morton, 1953). The pH was adjusted to 7.5 with M-NaOH. On standing overnight a white gelatinous precipitate formed which was centrifuged off and discarded. The aqueous phase was fractionated with ammonium sulphate and the crude xanthine oxidase fraction obtained as in Method A above.

Xanthine oxidase samples from individual cows. Milk samples were obtained from individual cows from October 1965 to July 1966. The cows were milked by hand directly into glass or plastic containers. To each litre of milk was added 50ml of 0.2M-EDTA–0.1M-sodium salicylate–NaOH, pH6.7. Cream was obtained as in Step 1 above and a further 50ml/l of the same EDTA–sodium salicylate mixture was added to it. Buttermilks were then obtained as in Step 1 above. For the first set of milk samples in Fig. 4 an EDTA–sodium salicylate mixture containing only half the above amount of EDTA was used. Buttermilks were processed at once by digesting with pancreatin and treating with butanol and ammonium sulphate as described by Palmer *et al.* (1964). Each sample was further purified by a single batchwise adsorption and elution step on calcium phosphate gel, as follows. Enzyme dissolved in 0.1M-phosphate, pH6.2, was adsorbed on calcium phosphate gel (Singer & Kearney, 1950). This was centrifuged down and washed with 0.15M-phosphate, pH6.2. The enzyme was eluted with 1.0M-phosphate, pH5.8.

Concentration and storage of xanthine oxidase samples. Small samples were conveniently concentrated by vacuum dialysis in collodion thimbles (Sartorius-Membranfilter GmbH, Göttingen, Germany). Larger samples were concentrated by precipitating with ammonium sulphate (0.4g/ml), centrifuging ($40000g_{av.}$ for 15min), redissolving the precipitate in 0.1M-pyrophosphate, pH7.0 (containing 1mM-salicylate and 1mM-EDTA), and dialysing against the same buffer. This buffer was used for storage of enzyme samples in the present work in preference to the 1M-phosphate, pH5.8, containing 30mM-sodium salicylate and 1mM-EDTA that Bergel & Bray (1959) used for storage, because the solubility of the enzyme is greater at the higher pH (see also the Results section for long-term effects of the latter medium on the enzyme when prepared by Step 2*b*).

Anaerobic reduction with xanthine. Samples of xanthine oxidase were reduced under argon in tubes with taps (Beinert & Sands, 1961) and extinction and e.p.r. measurements were made in these tubes. Enzyme samples in 50mM-pyrophosphate buffer, pH8.2 (containing 1mM-EDTA but no salicylate), were first degassed in Thunberg tubes and 200μl portions were transferred to the e.p.r. tubes with syringes. Extinctions were measured at 450nm with a special adaptor. Xanthine (20μl) was added anaerobically and mixed with the enzyme with a wire. The extinction of each sample was redetermined 2min after mixing and the contents were frozen 1min later in liquid N_2. E.p.r. spectra were recorded at about −150°C on a Varian V4502 9GHz spectrometer. Tubes were then incubated in a water bath at 25°C for 1h, and finally the extinctions were remeasured before refreezing and rerunning e.p.r. spectra. Corrections were made for very small concentration differences between the different enzyme samples used. Measurements were made in duplicate or triplicate and results were averaged.

Attempts to dissociate iron. Methods were as described by Bayer & Voelter (1966) and Uozumi *et al.* (1967). To confirm that the treatment with *o*-phenanthroline by the latter procedure was carried out on the reduced enzyme, checks were made that dithionite was still present in the dialysis buffers after each period of dialysis, by observing

Table 1. *Purification of xanthine oxidase and properties of the final, apparently homogeneous, product*

Results of the full purification procedures described in the Materials and Methods section are given for three different batches. The results of three partial purifications by mild methods that avoided the use of pancreatin are also given. Values given under 'Total activity' refer to the yield of enzyme obtained from 1 litre of buttermilk as starting material. Units of activity (u.) are those of Avis *et al.* (1955) measured at 23.5°C. Specific activities are given as i.u./mg of protein at 23.5°C and pH 8.2; protein was determined by the biuret method as described in the Materials and Methods section. Protein molarities are calculated on the basis of mol.wt. 275000 (Andrews *et al.* 1964). Values of dehydrogenase/oxidase ratio correspond to the relative activities (on a molar basis) in assays with xanthine as substrate and 2,6-dichlorophenol-indophenol and oxygen respectively as acceptors, as described in the Materials and Methods section. For the first batch (with 0.6M-salicylate), the procedure described in the Materials and Methods section was modified by omitting the addition of tetrasodium EDTA to the cream.

	Full purification procedures giving apparently homogenous products (Step 3)												Partial purifications by mild methods		
Type of purification ...	Salicylate (0.6M)				Salicylate (0.1M)				Hydroxylapatite						
Properties after	Buttermilk	Step 1	Step 2*a*(i)	Step 3	Buttermilk	Step 1	Step 2*a*(ii)	Step 3	Buttermilk	Step 1	Step 2*b*	Step 3	Milk microsomes	Method A	Method B
Total activity (u.)	12.7	9.8	4.8	3.1	7.7	5.2	3.3	1.5	11.1	6.8	4.3	3.2	—	—	—
Specific activity (i.u./mg)	0.06	1.8	—	3.5	0.02	1.1	2.2	3.6	—	1.2	2.0	2.4	—	—	—
Activity (u./l per E^{1cm}_{450}nm)	—	119	147	142	—	92	143*	140	—	92	90	92	—	83	91
Activity (u./μg-atom of Mo)	—	5.4	5.3	5.1	—	5.6	5.5	5.5	—	6.0	5.5	5.6	4.6	6.0	6.1
Dehydrogenase/oxidase (%)	—	—	—	—	—	47	—	46	—	—	—	—	—	—	—
FAD/Mo (μmol/μg-atom)	—	1.42	0.93†	1.03	—	1.64	1.08	1.09	—	1.67	1.69	1.70	1.6	1.6	1.8
Fe/FAD (μg-atom/μmol)	—	—	—	3.5	—	—	—	3.5	—	—	—	4.0	4.4	—	—
FAD/protein (μmol/μmol)	—	—	—	1.9	—	—	—	1.9	—	—	—	1.9	—	0.4	—
E^{1cm}_{280} nm/E^{1cm}_{450} nm	—	—	5.8‡	5.1§	—	—	8.7	5.2	—	10.1	5.3	5.0§	—	—	—
ϵ^{mM}_{450}/2 FAD	—	—	—	68	—	—	—	72	—	—	—	72	—	—	—
$E^{1\%}_{1cm}$ protein (450nm)	—	—	—	2.4	—	—	—	2.4	—	—	—	2.5	—	—	—

* On other batches at this stage values for this ratio of up to 166 were obtained.

† FAD/Mo ratios determined on four other batches at this stage ranged from 0.96 to 1.00.

‡ This value was obtained on another batch. Purity at this stage varied from batch to batch. In several cases recycling gel filtration indicated high purities, but on another sample 27% impurities were detected by the ultracentrifuge.

§ Values determined on a Cary model 11 spectrophotometer. Measurements on a Unicam SP. 500 spectrophotometer gave smaller values. Gel-filtration and ultracentrifuge data on these samples are given in Figs. 1 and 2.

the ability of the buffer to bleach aqueous riboflavin. Since iron could have been removed from the enzyme then taken up on subsequent dialysis against buffers containing the metal as an impurity, precautions were taken to select buffer constituents with low iron contents (see the Results section).

RESULTS

Preparation of the enzyme. All of the methods described under 'Preparation of xanthine oxidase' gave good yields of highly purified material. Although all methods gave enzyme homogeneous by various criteria, enzyme prepared by the salicylate methods differed in composition from that prepared by the more conventional hydroxylapatite method, and its specific activity was higher. A considerable number of batches was prepared by all the methods and details for typical ones are summarized in Table 1. Overall recovery of enzyme activity (about 20–30%) was comparable in all cases, and the yield of the final product in some cases exceeded 100mg/l of buttermilk. In practice the salicylate methods were simpler than the hydroxylapatite method, particularly on a large scale (20l of buttermilk). The product from Step 2*a*(i) or 2*a*(ii) was often used without further treatment, the overall procedure then being very convenient. We have used enzyme prepared by Step 2*a*(i) in all e.p.r. studies on the enzyme in this laboratory over the last 2 years (e.g. Bray, Knowles, Pick & Vänngård, 1968; Knowles, Gibson, Pick & Bray, 1969; Bray & Vänngård, 1969; Pick & Bray, 1969).

On some batches the purification was followed in the ultracentrifuge. After Step 1 substantial contamination of the enzyme by faster- and slower-sedimenting impurities was always observed. Step 2*a*(i) or 2*a*(ii) gave decreases in the amounts of slow-sedimenting (about 7S) contaminants, and the alternative Step 2*b* eliminated these completely. Step 3 removed residual fast- and slow-sedimenting impurities (see below).

Properties of the purified enzyme common to all preparative procedures. The enzyme prepared by either of the salicylate methods (Steps 1, 2*a*(i) or 2*a*(ii) and 3) or by the hydroxylapatite method (Steps 1, 2*b* and 3) appeared homogeneous by two criteria. Thus single symmetrical peaks were present at the end of recycling gel filtration, with enzyme activity and light-absorption at 254nm coinciding accurately (Fig. 1), and in the ultracentrifuge (Fig. 2) single symmetrical peaks only appeared. The sedimentation coefficient found for enzyme prepared by the salicylate method [Step 2*a*(i)] was $s_{20,w}$ 11.0S at a concentration of about 1.3%, in good agreement with the value given by Andrews *et al.* (1964) for material prepared by a calcium phosphate method. Results of amino acid analyses are presented in Table 2. Agreement between analyses on samples prepared respectively by salicylate and hydroxylapatite methods is striking.

Enzyme prepared by the salicylate method (Fig. 3) gave a spectrum in the visible region very similar to that of enzyme prepared by the hydroxylapatite

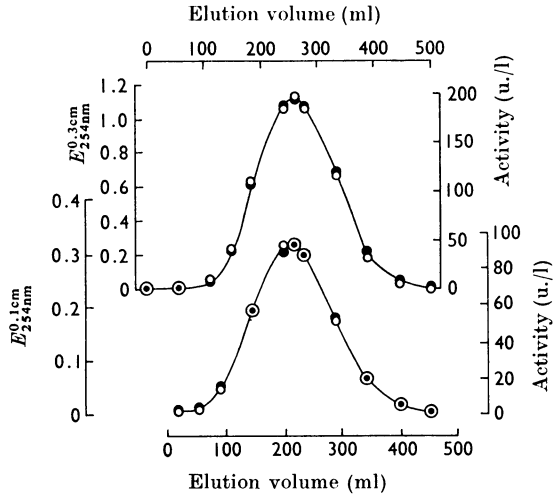

Fig. 1. Recycling gel filtration of purified xanthine oxidase samples on Sephadex G-200. ●, Extinction at 254nm; ○, xanthine oxidase activity. Values are from the final product after a number of cycles (see Materials and Methods, Step 3, for details) and appropriate rejection of unwanted fractions (zero elution volume is arbitrary). The salicylate method [Steps 1 and 2*a*(i)] was used for the sample on the upper curve and the hydroxylapatite one (Steps 1 and 2*b*) for that on the lower curve. The values correspond to the fourth and third cycles respectively for the two samples.

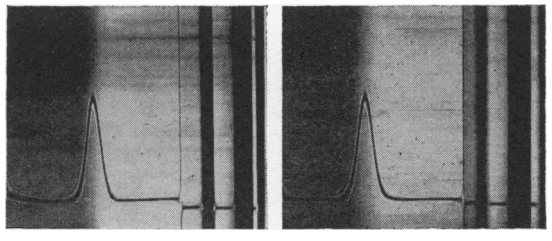

Fig. 2. Ultracentrifuge results on samples of purified xanthine oxidase (for details see the Materials and Methods section). The salicylate purification method [Steps 1, 2*a*(i) and 3] was used for the sample on the left and the hydroxylapatite method (Steps 1, 2*b* and 3) for that on the right. The direction of sedimentation is from right to left.

Table 2. *Amino acid composition of homogeneous xanthine oxidase samples*

Analyses were carried out on samples prepared by alternative procedures described in the Materials and Methods section. The samples were homogeneous in the ultracentrifuge and on recycling gel filtration. Content of amino acid residues is expressed as a percentage (by weight) of the total found; values for tryptophan and ammonia are not included. Single determinations only were carried out on samples hydrolysed for 24h in 6M-HCl, at 110°C. Data from more careful analyses on less-highly purified material by Bray & Malmström are included for comparison.

Amino acid	Salicylate method [steps 1, 2*a*(i) and 3]	Hydroxylapatite method (steps 1, 2*b* and 3)	Data from Bray & Malmström (1964)
Lys	8.2	8.3	8.0
His	3.1	3.1	2.9
Arg	7.1	7.1	6.3
Asp	9.3	9.4	8.8
Thr	6.7	6.7	6.6
Ser	5.0	4.9	5.2
Glu	12.1	12.3	12.1
Pro	4.7	4.7	4.9
Gly	4.4	4.4	5.3
Ala	5.1	5.0	4.9
Val	6.2	6.2	6.3
Met	1.3†	1.6†	2.4
Ile	5.5	5.4	5.2
Leu	9.5	9.4	9.0
Tyr	3.0	3.0	3.9
Phe	6.9	6.9	6.7
Cys	1.9*	1.9*	2.4
Total	100	100	100

* Sum of cysteic acid and cystine.
† Sum of methionine and oxides.

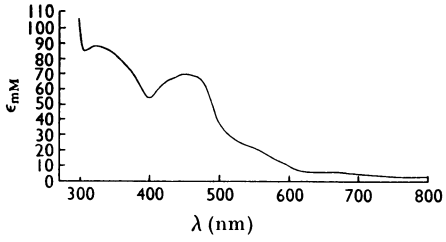

Fig. 3. Visible absorption spectrum of xanthine oxidase. The sample was homogeneous, prepared by the salicylate method [Steps 1, 2*a*(ii) and 3]. The vertical scale refers to the millimolar extinction coefficient/2mol of FAD, of the enzyme.

method, and to spectra obtained by Mackler, Mahler & Green (1954) and Massey *et al.* (1969). Our value of ϵ_{450}^{mM} of about 70 (Table 1) agrees with earlier data (cf. Bray, Malmström & Vänngård, 1959).

The final product obtained by the salicylate method showed about the same ratio of oxidase to dehydrogenase activities with xanthine as substrate (Table 1) as was found by Avis *et al.* (1956*b*) for the crystallized enzyme. Further, the salicylate process clearly did not affect this ratio despite the other changes that it produced (see below). The effect of xanthine concentration on enzyme activity (Bray, 1959) differed little from one sample to another in the oxidase assay. With the other conditions described in the Materials and Methods section, final samples prepared by either type of method showed optimum activity with 0.05–0.10mM-xanthine, the activity falling to about 70% of this with 1.0mM-xanthine.

Our final product always contained iron (Table 1) and some attempts were made to dissociate this. Partially purified samples of xanthine oxidase (prepared by Step 1, followed by batchwise treatment with calcium phosphate gel as described under 'Xanthine oxidase samples from individual cows') were dialysed against 2,2′-bipyridyl or *o*-phenanthroline, both in the presence of dithionite, under conditions reported to give essentially complete removal of iron from the enzyme, accompanied by only partial loss of activity. Results are summarized in Table 3. About half the activity of the enzyme was lost relative to E_{450}, and this agrees well with the values reported by Bayer & Voelter (1966) and by Uozumi *et al.* (1967). However, iron

Table 3. *Attempts to remove iron from xanthine oxidase*

In Experiments I and II samples of partially purified xanthine oxidase were dialysed against the chelating agents under the conditions described by Bayer & Voelter (1966) and by Uozumi *et al.* (1967) respectively, and analyses and activity measurements were carried out on the starting materials and products. Initial xanthine oxidase concentrations were 0.02mM and 0.1mM respectively in the two experiments. The buffer used in Experiment II was 5mM-phosphate–4mM-*o*-phenanthroline–H_2SO_4, pH6.8. Ratios are expressed as in Table 1.

Expt. no.	Method		Activity/E_{450}	FAD/Mo	Fe/FAD	Fe/protein
I	Bipyridyl+dithionite, pH5.4	Before	108	1.4	5.0	6.1
		After	51	1.5	4.2	5.7
II	Phenanthroline+dithionite, pH6.8	Before	102	1.5	4.1	4.7
		After	49	1.6	4.1	4.5

loss in our experiments was minimal, in sharp contrast to the original results of both the other groups. The discrepancy was not due to contamination of our buffers with extraneous iron, since in Expt. II analysis indicated that the total quantity of iron impurities in all reagents and buffers which came into contact with the enzyme sample in the entire treatment was less than 11% of the xanthine oxidase iron. In confirmation of these findings, samples of xanthine oxidase prepared by Step 2*a*(i) and treated as described by Bayer & Voelter (1966) also failed to give any active iron-free xanthine oxidase. These latter experiments were carried out by Dr P. F. Knowles and Dr P. Kraus in Professor E. Bayer's laboratory.

Properties of the purified enzyme dependent on the purification method. The composition (Table 1) of enzyme prepared by the hydroxylapatite method was comparable to that reported by Avis *et al.* (1956*b*) for crystalline material. The FAD/Mo ratio did not change during the purification (Table 1) and was 1.70 in the final product. We believe that our analytical methods gave ratios correct to ±5%. In contrast enzyme prepared by the salicylate method (especially when using Step 2*a*(i); see below) came very close to a stoicheiometric composition (Mo/FAD ratio 1.03). Salicylate treatment by either Step 2*a*(i) or 2*a*(ii), gave a decrease in the FAD/Mo ratio of the samples accompanied by an increase in activity/E_{450} ratio, but with little change in activity/Mo ratio (Table 1). As with conventional purification methods, enzyme prepared by the salicylate method tended to lose activity during storage or processing. Partially purified samples with substantially higher activities than those recorded in Table 1 were obtained on a number of occasions. Our highest activity/E_{450} ratio, 166, was recorded after Step 2*a*(ii) but the highest activity/Mo ratio remains 6.9 (Hart & Bray, 1967). This latter value refers to a sample that was rapidly and only partially purified on a small scale from milk obtained by hand-milking (thus avoiding contact with metals) and EDTA was added both to the milk and to the cream. We have not obtained such high activity/Mo ratios on samples prepared on a larger scale.

Anaerobic reduction of samples of the enzyme was studied spectrophotometrically (Morell, 1952) and by e.p.r. spectrometry (Bray, Pettersson & Ehrenberg, 1961). Results are given in Table 4. Three samples were examined. Two (I and III) were freshly prepared by the salicylate [Step 2*a*(i)] and hydroxylapatite (Step 2*b*) methods respectively, whereas the third (II), prepared by the salicylate [Step 2*a*(i)] method, had been stored for some time and had lost activity. Though there were substantial differences among the samples, under our conditions all showed small but significant 'slow phase reduction', i.e. decreases in E_{450} between 2 and 60min, and all also showed substantial changes in the e.p.r. signal intensities of molybdenum and free radical during the same period.

Salicylate treatment. Salicylate is a protein denaturant (Putnam, 1953) and it seems that its effect on xanthine oxidase preparations under our various conditions can be separated into a number of clearly defined phases. Full studies were not carried out but preliminary experiments were done (see below) before we decided on the conditions defined in Steps 2*a*(i) and 2*a*(ii).

We found that salicylate-mediated increases in activity/E_{450} ratio, with concomitant decreases in FAD/Mo ratio, could take place slowly under extremely mild conditions. We noticed this during long-term storage at 0–5°C of samples prepared by Step 1, in the medium containing 0.03M-salicylate recommended for storage by Bergel & Bray (1959) (i.e. 1M-phosphate, pH5.8, containing 1mM-EDTA). Activity/E_{450} ratios increased during such storage, in one case from 99 to 126 in 11 months. At the same time the FAD/Mo ratio of this sample (measured after dialysis) decreased from 1.48 to 1.16.

In the final preparative methods of Steps 2*a*(i)

Table 4. *Reduction of xanthine oxidase samples with xanthine*

Samples of the enzyme (0.05mM, from E_{450}) were treated anaerobically for 2min with xanthine (9 molar proportions) at pH8.2, E_{450} values were measured, the samples were frozen and their e.p.r. spectra recorded. These measurements were repeated after thawing and further incubation at 25°C. Details of the technique are given in the Materials and Methods section. E.p.r. signals were measured as described by Palmer, Bray & Beinert (1964), with intensities in one set of arbitrary units for molybdenum and radicals and in another for iron.

Sample	Preparation	Activity/ E_{450}	Activity/ Mo	Time (min)	ΔE_{450} (% decrease)	E.p.r. signal intensities		
						Mo†	Radical	Fe
I	Salicylate	144	5.3	2	52	85	25	—
				60	55	180	55	—
II*	Salicylate	112	4.1	2	40	31	15	16
				60	41	70	120	16
III	Hydroxylapatite	90	5.5	2	31	31	16	17
				60	35	85	130	14

* This sample lost activity in prolonged storage.

† The height of the β peak of the molybdenum signal was measured (Palmer, Bray & Beinert, 1964). The signals all appeared to be of the 'Rapid Complex formed, type 1' plus 'type 2' form and no differences in signal type were noted (Bray & Vänngård, 1969).

or 2*a*(ii), apart from the effects on specific activity and FAD/Mo ratio, salicylate treatment evidently provides a useful purification of the enzyme because impurities are apparently more sensitive to denaturation by this treatment than the enzyme. This was particularly marked with Step 2*a*(i), which gave products with low E_{280}/E_{450} ratios (Table 1). As shown in Table 1 and above, either Step 2*a*(i) or 2*a*(ii) lowered the FAD/Mo ratio to values close to unity. However, under the milder conditions of Step 2*a*(ii) losses of total activity were lower than they were in Step 2*a*(i), and at the same time, in Step 2*a*(ii), the FAD/Mo ratio remained significantly greater than unity.

Salicylate concentrations greater than 0.6M had a more destructive effect on the enzyme. When a sample with activity/E_{450} ratio 120 after Step 1 was treated with about 0.8M-salicylate at 37°C for 16h, the product had the extremely low FAD/Mo ratio of 0.30. After purification by recycling gel filtration (Step 3), the product, obtained in low yield, had activity/E_{450} ratio 105 and FAD/Mo ratio 0.80. This result and the observation of an FAD/Mo ratio as low as 0.93 after Step 2*a*(i) (Table 1) might suggest that FAD loss from xanthine oxidase is a continuous process that can continue indefinitely, and that a product with an FAD/Mo ratio close to unity is obtained merely by stopping salicylate treatment at the right moment. However, strong evidence against this was provided by experiments in which two successive treatments with 0.6M-salicylate were applied to the enzyme. In these, the second treatment, like the first, produced some overall loss of enzyme activity but had relatively little effect on the FAD/Mo ratio, which

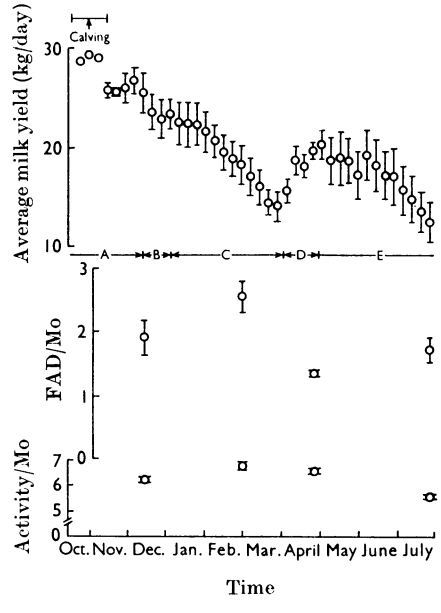

Fig. 4. Properties of partially purified xanthine oxidase samples isolated from the milks of individual cows at different times. The enzyme was purified from milk obtained on the days shown and was analysed as described in the Materials and Methods section. Three cows were used and individual results were averaged; error bars indicate average deviations from the means. FAD/Mo ratios are given as μmol/μg-atom and activity/Mo ratios are given as in Table 1. Average daily milk yields and dates of calving are also shown, and the different diets used were: A, kale, hay and concentrates; B, silage, hay and concentrates; C, straw, hay and concentrates; D, grass and concentrates; E, grass only.

always remained near, though sometimes slightly less than unity. Thus in the case of the batch recorded in Table 1, the initial Step 2*a*(i) treatment lowered the FAD/Mo ratio from 1.42 to 0.93. When a sample of the material, after purification by gel filtration (Step 3), was submitted to a second cycle of salicylate treatment under the original conditions, the product had FAD/Mo ratio 0.91.

Mild methods for the early stages of xanthine oxidase isolation from milk. Since the preparative procedures employed to obtain the highly purified enzyme involve treatments potentially damaging to the enzyme molecules, we compared the products obtained by them with those obtained by alternative milder isolation methods. We devised three mild methods for obtaining crude xanthine oxidase from whole milk samples as detailed under Materials and Methods. None of these methods involved the pancreatin digestion step frequently used in xanthine oxidase purification and originally introduced by Ball (1939). The method described under 'milk microsomes' was a particularly mild one as it involved no treatments more drastic than centrifugation. Analyses on the products obtained by these methods are summarized in Table 1. The products are rather crude, but FAD/Mo ratios substantially greater than unity are clearly obtained for xanthine oxidase prepared without the use of pancreatin. These mild methods were not used generally because either they were unsatisfactory on a large scale ('milk microsomes method') or they gave a low yield (Methods A and B).

Xanthine oxidase samples from individual cows. Partially purified xanthine oxidase (see the Materials and Methods section) was obtained from each of three cows on several occasions during lactation. FAD/Mo ratios, activity/Mo ratios and milk-yield data are recorded in Fig. 4. Activity/Mo ratios were generally high but showed some relatively small variations. We assume that these are due to variations in the extent to which inactivation occurred during the isolation procedures. FAD/Mo ratios were all significantly greater than unity but were, on the other hand, distinctly variable. It is noteworthy that variations in this ratio from cow to cow on a given day (expressed by the error bars in Fig. 4) were less than the variations with time in the average ratio. In some cases FAD/Mo ratios were also determined on the corresponding cruder samples before calcium phosphate-gel purification. Values obtained in this way did not differ significantly from those reported in Fig. 4.

DISCUSSION

Specific activity and purity of the present xanthine oxidase preparations. There were substantial differences between the final products obtained by the salicylate and hydroxylapatite methods. Whereas the latter gave enzyme generally comparable to the best crystallized samples of Avis *et al.* (1956*b*), the former appeared to give a product both purer and of higher specific activity than other preparations in the literature. In the ultracentrifuge it was devoid of all detectable impurities and was possibly slightly better by this criterion than the crystallized enzyme of Avis *et al.* (1956*a*). In contrast, the preparation claimed by Nelson & Handler (1968) to be '95–100% pure', shows, from their ultracentrifuge data, substantial amounts of impurities. These workers were apparently confused by a typographical error (interchange of symbols for activity and E_{230}) in Fig. 3 of Andrews *et al.* (1964) from which they assumed that the high molecular weight contaminant often present in xanthine oxidase samples is enzymically active. This impurity has only very low xanthine oxidase activity (Bray, Chisholm, Hart, Meriwether & Watts, 1966). Massey *et al.* (1969) do not report ultracentrifuge data on their preparations of xanthine oxidase. For our samples prepared by the salicylate procedure (Table 1), the E_{280}/E_{450} ratio is within the range (5.0–5.2) given by Avis *et al.* (1955) for the crystallized enzyme. This range is slightly lower than the values reported by Nelson & Handler (1968) and lower than that of Massey *et al.* (1969).

Our final samples prepared by the salicylate method have activity/E_{450} ratios at 23.5°C comparable to those of Massey *et al.* (1969) but have slightly higher activity/protein ratios at the same temperature. For the preparations of Nelson & Handler (1968) no activity/E_{450} ratios are given but calculation from their data gives values little more than half of those reported here (a 'specific activity' of 15 reported by these workers appears to refer to activity/E_{280} ratio and not to units/mg).

FAD and molybdenum content and in active forms. The literature on the FAD/Mo ratio of xanthine oxidase is confusing. Several groups (see the introduction) reported a FAD/Mo ratio of 2, for the bovine milk enzyme. However, Mahler & Green (1954) suggested that the true value in the native enzyme might be 1, and this value was accepted in a review by Handler, Rajagopalan & Aleman (1964), though they do not cite any supporting evidence or give reasons for discounting the previous work.

Work from this laboratory on the crystallized enzyme showed that this had a non-integral and slightly variable FAD/Mo ratio and variable specific activity (Avis *et al.* 1956*b*). This was explained (cf. Bray *et al.* 1961) by postulating that the active enzyme, 'xanthine oxidase-*a*', was contaminated by two inactive forms, 'xanthine oxidase-i_1' and 'xanthine oxidase-i_2'. In this paper we refer to xanthine oxidase-i_1 (which contains molybdenum) as 'inactivated xanthine oxidase' and to

xanthine oxidase-i_2 as 'de-molybdo-xanthine oxidase'. Salicylate treatment to lower FAD/Mo ratios of xanthine oxidase preparations, was introduced by Bray *et al.* (1966) and elaborated by Hart & Bray (1967), who used it to obtain milk enzyme with probably the first genuine reported FAD/Mo ratio of 1. The preparation, by conventional methods, of milk enzyme with a well authenticated FAD/Mo ratio of 1 was only recently described (Massey *et al.* 1969).

The present results show conclusively that FAD/Mo ratios greater than unity and non-integral values of the ratio in xanthine oxidase preparations are due to the presence of inactive 'de-molybdo-xanthine oxidase'. The preparation, by alternative salicylate and hydroxylapatite procedures, of apparently homogeneous samples of the enzyme, with indistinguishable FAD/protein ratios and amino acid analyses but very different Mo/protein ratios, leaves little room for doubt on this point. The data exclude alternative possibilities such as contamination of xanthine oxidase with extraneous flavoproteins, or the binding of extra FAD molecules. Further, our results could not be explained by contamination with a hypothetical variant of the enzyme with 1 molybdenum atom/mol and a low specific activity. We conclude that our preparation of the enzyme by the hydroxylapatite method gives a mixture of xanthine oxidase and de-molybdo-xanthine oxidase. Suitable salicylate treatment eliminates [Step 2*a*(i)] or largely eliminates [Step 2*a*(ii)] the de-molybdo enzyme, with a corresponding increase in specific activity.

The mol.wt. of the enzyme based on ultracentrifuge and gel-filtration data has been given as 275000 (Andrews *et al.* 1964). Within the limits of experimental error, the FAD content of the enzyme prepared by either type of method agrees well with this value, assuming 2mol of FAD/mol. We conclude that active xanthine oxidase free from the de-molybdo form has 2mol of FAD and 2g-atoms of Mo/mol.

Routine availability, with the salicylate method, of enzyme samples devoid of de-molybdo-xanthine oxidase also clarified the evidence relating to 'inactivated xanthine oxidase'. In previous samples from this laboratory it could only be detected from variations in activity/Mo ratio but, in the absence of demolybdo-xanthine oxidase, it would show up by variability in activity/E_{450} ratio, and for samples devoid of extraneous proteins, by variability in activity/protein ratio. Since variability in activity/E_{450} ratio still occurred even for the salicylate-treated enzyme and since this ratio tended to fall during storage, we conclude that formation of inactivated xanthine oxidase was not eliminated. This is of particular significance in studies on the mechanism of action of the enzyme because Morell (1952) claimed that inactive xanthine oxidase molecules are slowly reducible.

Combining the maximum activity/Mo ratio reported by Hart & Bray (1967) with data in Table 1 suggests that these preparations still contain about 20% of inactivated xanthine oxidase. Thus values of activity/E_{450} ratio up to at least 180 at 23.5°C ought, in theory, to be attainable. This value is higher than our best ratio of 166 recorded on a crude sample, higher than the values given by Massey *et al.* (1969) and much higher than values calculated from Nelson & Handler (1968). We conclude that the preparations of both groups are probably contaminated by inactivated xanthine oxidase despite the claim by Nelson & Handler (1968) that their samples are 'fully active'.

Origin of de-molybdo-xanthine oxidase. The constancy of the FAD/Mo ratio during the steps of routine preparation of the enzyme by the hydroxyl apatite method (Table 1) suggests that de-molybdo-xanthine oxidase occurs naturally in milk and is not a preparation artifact. On a large number of xanthine oxidase samples subjected to routine manipulation and examined over a considerable period of time in this laboratory we have never observed significant changes in FAD/Mo ratio except during salicylate treatment. The possibility that de-molybdo-xanthine oxidase is an artifact produced in the early stages of our purification seems excluded by the finding of non-stoicheiometric FAD/Mo ratios in crude xanthine oxidase samples prepared by special mild methods (Table 1). Further evidence that de-molybdo-xanthine oxidase is present in milk is provided by variations with time in the FAD/Mo ratios of enzyme samples from individual cows. Since a constant and carefully controlled purification method was used, it is unlikely that the large variations in this ratio could arise from causes other than variations in the milk. Fluctuations in the de-molybdo-xanthine oxidase content clearly do not arise from genetic causes as the products from different cows on a given day always differed little from one another.

The results strongly suggest that nutritional factors determine the amount of the de-molybdo form present. The obvious inference is that dietary molybdenum is the important factor and that when this is not adequately available cows make the xanthine oxidase molecule but, because they fail to incorporate the metal, the de-molybdo enzyme appears. The seasonal dip in the milk-yield curve (Fig. 4) from February to April is presumably nutritional in origin and FAD/Mo ratios seem to correlate with this. Thus the lowest ratios were obtained in spring when the milk yield was increasing, and extremely high FAD/Mo ratios, averaging 2.5, were obtained from milk samples in late winter. A value greater than 2 could not be accounted for

by any mixture of mono- and di-molybdenum forms of the enzyme. Hence de-molybdo-xanthine oxidase definitely exists, and for simplicity we have elsewhere in this paper ignored the possibility of the simultaneous occurrence of the mono-molybdo and de-molybdo forms. Although we never obtained FAD/Mo ratios as high as 2.5 from bulk-milk samples a batch of xanthine oxidase purified on a large scale at about the same time as the February individual samples had the rather high FAD/Mo ratio of 1.9. Five other samples prepared from bulk milk at other times during the period covered in Fig. 4 had ratios in the more usual range of 1.3 to 1.6 (cf. Avis *et al.* 1956*b*). The nutritional conditions that prevail in late winter and give abnormally high FAD/Mo ratios may be regarded as unusual.

In view of our findings, the fact that Massey *et al.* (1969) obtained xanthine oxidase with an FAD/Mo ratio 1 from milk by purely conventional procedures, requires explanation (a sample of their enzyme kindly supplied by Dr V. Massey was analysed in this laboratory and gave an FAD/Mo ratio 1.01). Since Massey's group uses pasteurized rather than fresh milk as starting material, the pasteurization process may bring about selective denaturation of de-molybdo-xanthine oxidase analogous to that produced by salicylate. However, in view of variability in milk samples (see above) and of pasteurization methods, salicylate treatment may provide a more general procedure for obtaining xanthine oxidase free from the de-molybdo form than does Massey's method.

Iron content of the enzyme. Analysis of our most highly purified samples showed a content of 7–8 iron atoms/mol. Removal of iron from the enzyme could not be achieved under the conditions either of Bayer & Voelter (1966) or of Uozumi *et al.* (1967). This is in keeping with a functional role for the metal in the catalytic cycle (Bray, 1961; Bray *et al.* 1964).

Use of salicylate and of pancreatin in the preparation. The mode of action of sodium salicylate seems to be primarily one of denaturation (cf. Putnam, 1953). Xanthine oxidase and also inactivated xanthine oxidase are apparently less sensitive to denaturation in the presence of salicylate than is the de-molybdo form. This may be a result of specific binding of salicylate to the active centre of the enzyme (Bergel & Bray, 1959). Under the conditions specified, FAD loss (relative to molybdenum) invariably stopped at or very close to the stoicheiometric FAD/Mo ratio of 1. It is particularly significant that two successive salicylate treatments had little more effect on this ratio than did a single treatment. However, under only slightly more vigorous conditions than those of Step 2*a*(i) a further action of salicylate is to remove some FAD from the active enzyme in a manner analogous to the action of calcium chloride (Komai, Massey & Palmer, 1969), and of thiocyanate (Uozumi, Piette, Orme-Johnson & Beinert, results reported at 3rd International Conference on Magnetic Resonance in Biological Systems, Warrenton, Virginia, U.S.A., 1968). The deflavo-enzyme has dehydrogenase but no oxidase activity (Komai *et al.* 1969). Our finding that samples with FAD/Mo ratios less than 1 show some increases in this ratio during purification by gel filtration indicates that the deflavo enzyme is less stable than the native enzyme. To confirm that the two types of action by salicylate are distinct, dehydrogenase activity was measured before and after the standard preparative treatment with salicylate [Step 2*a*(ii)]. No change in dehydrogenase/oxidase ratio was observed, in agreement with the expectation that under the conditions used no de-flavo-xanthine oxidase would be produced.

Our routine preparative procedures involve treatment with pancreatin, and it has been suggested that occurrence of inactive forms of xanthine oxidase may be related to the use of this step (Handler, 1966). Our results provide no support for this suggestion. It was further reported that pancreatin causes proteolytic degradation of xanthine oxidase (Carey, Fridovich & Handler, 1961; Nelson & Handler, 1968; Massey *et al.* 1969). Although some limited proteolysis may be produced, comparison of amino acid analyses indicates that degradation of the enzyme must be minimal. Nelson & Handler (1968) compared the analysis of a non-digested enzyme preparation with that reported by Bray & Malmström (1964) for xanthine oxidase prepared by the pancreatin method. Differences are negligible and this is difficult to reconcile with the conclusion of Nelson & Handler (1968) that 10% of the molecule is removed by pancreatin. The 10% molecular weight difference that they found may be partly due to experimental errors.

Rapid and slow reduction of xanthine oxidase. Rapid and slow phases in the reduction of xanthine oxidase were first noted by Morell (1952) and their cause has been the subject of controversy (Gilbert, 1963). In general our present results, which include some on our best preparations, are in agreement with earlier ones, though interpretation is complicated by contamination with inactivated xanthine oxidase. In agreement with Morell (1952), the three samples in Table 4 show good parallelism between activity/E_{450} ratio and the extent of 'immediate' (i.e. 2 min) bleaching at 450nm by xanthine. They all also show a 'slow' phase in the bleaching, though under the conditions used (9 molar proportions of xanthine) its extent was not large. The parallel e.p.r. results provide additional information. In

agreement with Bray *et al.* (1961) molybdenum and free radical signals increased in intensity during the 'slow' phase, while in agreement with Ehrenberg & Bray (1965) the iron signals did not change significantly. The former authors suggested that all the free radical signals observed under our conditions, i.e. employing excess of substrate, were due to flavin in the inactive forms. This conclusion receives support from some the current work, since the slow phase radical signal development is less marked on the best enzyme preparation (sample I) than it is on the other two samples (particularly relative to the molybdenum signals). Sample II must contain substantially more inactivated xanthine oxidase than the other samples, while sample III contains de-molybdo-xanthine oxidase. Bray *et al.* (1961) concluded that the slow phase changes in molybdenum signals also were related to the inactive forms. However, it seems here that this interpretation may be incorrect, since the best preparation has the largest molybdenum signal both at 2min and at 60min (Table 4). Though further work on the slow phase of reduction is required, there seems to be no evidence for inactivated xanthine oxidase giving distinguishable e.p.r. signals of its own.

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