Cellular Constituents. The Chemistry of Xanthine Oxidase. Part I. The Preparation of a Crystalline Xanthine Oxidase from Cow's Milk.

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A procedure for the preparation of a crystalline metallo-flavoprotein from buttermilk is described. It possesses high xanthine oxidase activity ($Q_{O_2} = 2300$; spectrophotometric assay with xanthine at 23·5°) and a protein/flavin ratio (*i.e.*, E_{280}/E_{450}) of 5·0—5·2, the lowest reported for material with such enzymic properties.

Xanthine oxidase controls biologically the last stages of nucleic acid catabolism by removal of hypoxanthine and xanthine in the form of uric acid from the general pool of purines. Its presence in excess or its absence, its inhibition or stimulation, might possibly reflect on the chemistry of normal or abnormal cellular growth. When examining such a hypothesis Haddow *et al.* (*Brit. Emp. Cancer Camp. Ann. Report*, 1953, **31**, 35) found, first, that xanthopterin and some related compounds which induce hypertrophy of the kidney in rodents inhibit bovine xanthine oxidase *in vitro* and, secondly, that injections of xanthine oxidase concentrates from cow's milk into mice bearing spontaneous mammary tumours produce, apparently, antitumour effects. Although exceptions to the hypertrophy-inhibition correlation have been encountered and the preliminary results in the mouse need further confirmation, we were encouraged to embark on a thorough study of the chemistry of this enzyme.

The preparation of highly active material from cow's milk has been achieved in a number of laboratories, *e.g.*, by Corran, Dewan, Gordon, and Green (*Biochem. J.*, 1939, **33**, 1694), Ball (*J. Biol. Chem.*, 1939, **128**, 51), Horecker and Heppel (*ibid.*, 1949, **178**, 683), Morell (*Biochem. J.*, 1952, **51**, 657), and Mackler, Mahler, and Green (*J. Biol. Chem.*, 1954, **210**, 149). Their products were not crystalline and none of the authors claimed a homogeneous product. The identity, or otherwise, of the bovine milk enzyme with the xanthine oxidases of animal and human somatic tissues has not so far been ascertained.

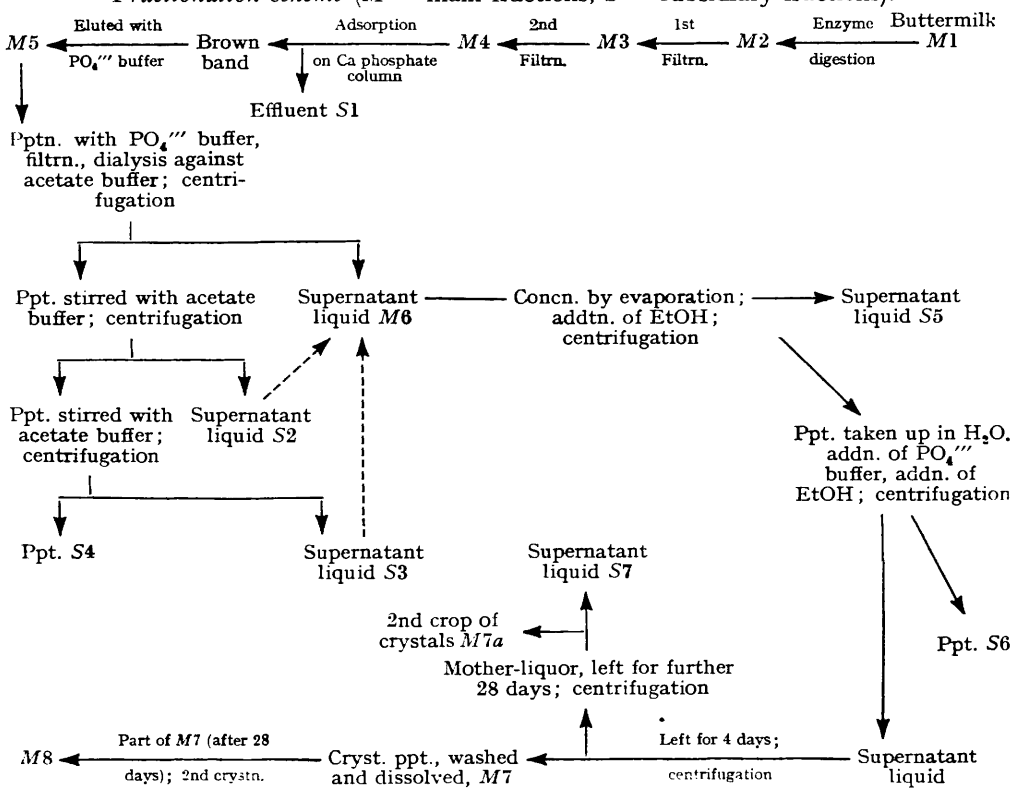
Using improved preparative methods we have obtained from buttermilk an active crystalline metallo-flavoprotein as reported in a preliminary communication (Avis, Bergel, Bray, and Shooter, *Nature*, 1954, **173**, 1230). In the present paper we describe our preparative procedures in detail and give the relevant assay figures.

The progressive purification of the enzyme was followed by means of the following measurements: optical densities at 280 mμ ("protein") and 450 mμ ("flavin") (cf. Corran *et al.*, *loc. cit.*), and the slope $\Delta E_{295\ m\mu}/\Delta t_{(min.)}$ in the presence of xanthine ("enzyme activity") (Kalckar, *J. Biol. Chem.*, 1947, **167**, 429; Morell, *loc. cit.*). Details of these assay methods and definitions of the unit of activity are given in the Experimental section, where also evidence can be found that solutions of the enzyme, unlike certain other proteins (cf. Champagne, *J. Chim. phys.*, 1950, **47**, 693; Rosenberg *et al.*, *Bull. Soc. Chim. biol.*,

1953, **35**, 521), obey Beer's law over the range of concentrations employed. From the three sets of figures obtained, the ratios Activity (units/l.)/E_{280} = " APR," E_{280}/E_{450} = " PFR," and Activity (units/l.)/E_{450} = " AFR " were calculated for each fraction. These ratios give a fair picture of the composition of any fraction containing xanthine oxidase. " APR " is directly proportional to activity per unit weight of protein and therefore to the conventional Q_{O_2} (cf. Morell, *loc. cit.*) if variations in the absorption coefficient ($E^{1\%}_{1\,cm.}$) of different proteins at 280 mμ are ignored and if this assumption is accepted as coming nearer the truth with increasing purity of the preparations. " PFR " depends largely on the relative amounts of coloured material and total protein; thus once xanthine oxidase forms the only constituent of the preparation absorbing at 450 mμ, a decrease in " PFR " must represent a higher purity. The third ratio " AFR " has been little used by other workers except that Corran *et al.* (*loc. cit.*) reported that their Activity/" apparent flavin phosphate " ratio for most but not all preparations remained constant from an early stage in their purification. In our case we have taken an increase of " AFR " as an indication of the removal of inactive coloured impurities, while a decrease during the later stages of purification was considered as a sign of increase in the proportion of inactive to active enzyme (cf. Morell, *loc. cit.*).

From the annexed scheme, it can be seen that the main steps of our procedure are these: production of whey from buttermilk by removal of casein through the action of

Fractionation scheme (M = main fractions, S = subsidiary fractions).



pancreatin (cf. Ball, *loc. cit.*), followed by pressure filtration; chromatographic adsorption on a calcium phosphate column (cf. Polis and Shmukler, *J. Biol. Chem.*, 1953, **201**, 475, with peroxidase), and elution with phosphate buffer (cf. Horecker and Heppel, *loc. cit.*); precipitation of impurities at low salt concentrations (acetate buffer), and fractionation and crystallisation in presence of ethanol and very dilute phosphate buffer. This scheme has

been followed with a considerable number of batches, all giving generally the same results. The assay figures of all fractions of a typical batch are given in the Table.

Fraction	Volume (l.)	Total Units X.O.	" AFR "	" APR "	" PFR "	Remarks
*M*1	25	277	—	0·14 *	—	Buttermilk
*M*2	25	275	—	—	—	After pancreatin treatment
*M*3	21	96	—	—	—	1st filtrate
*M*4	20	85	35	0·53	65	2nd filtrate (whey)
*M*5	1·22	96 †	70	7·4	9·4	Eluate
*M*6	0·45	43	88	11·1	8·0	" Starting concentrate "
*M*7	0·050	17	79	15·2	5·2	Crystalline
*M*7*a*	0·015	3	66	10·4	6·3	,,
*M*8	—	—	71 ‡	14·2 ‡	5·0 ‡	,,
*S*1	20	0·2	0·2	0·001	170	Effluent, see *M*5
*S*2	0·16	10	99	12·5	7·9	Supernatant, see *M*6
*S*3	0·13	3	83	12·2	6·9	Supernatant, see *M*6
*S*4	0·15	20	37 *	4·2 *	8·8 *	Precipitate, see *S*3
*S*5	0·32	4	73	6·8	10·7	Supernatant
*S*6	0·022	6	81	6·2	13·1	Precipitate
*S*7	0·095	1·7	63	4·6	13·7	Supernatant, see *M*7 and *M*7*a*
A	—	—	32	6·1	5·3	Crystalline (cf. Avis *et al.*, *loc. cit.*)
B	—	—	53	10·4	5·1	Crystalline (cf. Avis *et al.*, *loc. cit.*)

* Value only approx. owing to turbidity of solution. † Apparent increase in activity, probably due to experimental error. ‡ *M*7 solution before 2nd crystallisation gave " AFR " 71, " APR " 13·3, " PFR " 5·3.

As the details are given fully in the Experimental part, it should suffice to stress only the following points: While the use of pressure filtration facilitates the preparation of milk whey, the losses of activity during this operation were more severe than in any of the subsequent stages and greater than those reported for similar steps by other workers. We do not know yet the reasons for this; however, the loss was offset by the great advantages of the novel chromatographic treatment on solid calcium phosphate, leading to *M*5. A fourteen-fold increase in purity as measured by " APR " and a sixteen-fold decrease in volume were gained with full recovery of activity. During the dialysis (*M*5 ⟶ *M*6) coloured impurities were removed, together with some active material. A proportion of the latter could have been recovered from fractions *S*2 and *S*3 by combination with the main concentrate *M*6. This was not done in working up the run presently described.

Both the final product, *M*7, and the recrystallised sample, *M*8, appeared to be wholly crystalline under a polarising microscope. Often these coloured crystals occurred as small thin needles (*ca.* 10 μ long), sometimes bunched together in rosette-like formations (Plate 1); on other occasions larger plates were observed (Plate 2). The values for " PFR," 5·2 for *M*7 and 5·0 for *M*8 (not altered on further recrystallisation), are suggestive of a high purity, especially if compared with the best values reported in the literature, 6·15 (Morell, *loc. cit.*) and 6·2 (Corran *et al.*, *loc. cit.*, who measured E_{275}/E_{450}). There remains the question of activity. Considering the process as a whole the recovery of activity in the crystalline fraction, as represented by *M*7 was *ca.* 6% of that originally measured in the buttermilk. There is little doubt that this could be increased substantially by reworking various rejected fractions. The " APR " values are very encouraging, especially if one converts that for *M*7 into Q_{O_2} (see Experimental part), arriving at 2300 (23·5°), and compares it with Morell's figure of 694 (19°). However, the " AFR " values are not yet entirely satisfactory. They show variations between crystallisate and crystallisate (see in Table, *M*7, *M*8, and materials A and B, as previously described by Avis *et al.*, *loc. cit.*) and a slight drop from the non-crystalline *M*6 to the solution of crystalline material *M*7. As this reflects on the proportion of inactive metallo-flavoprotein to active enzyme, explained above, one has to assume that the crystals still consist of mixtures of fully potent and probably closely related inactivated xanthine oxidase, but that additional impurities have been removed to a very large extent. However, the fact that we have obtained crystalline material regularly with the properties described above shows that we have achieved a major step towards the isolation of a fully active enzyme. The questions of

PLATE 1.

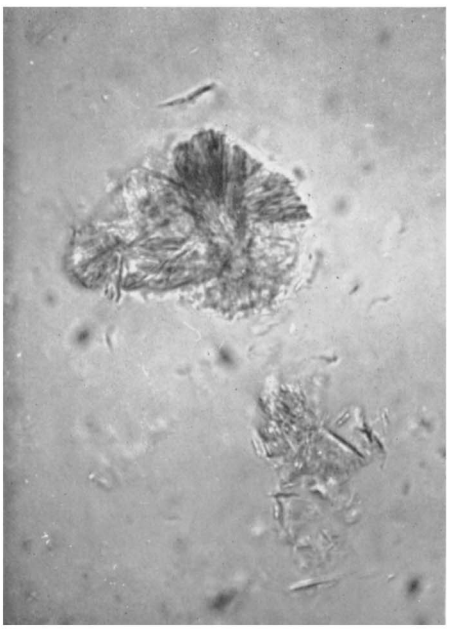
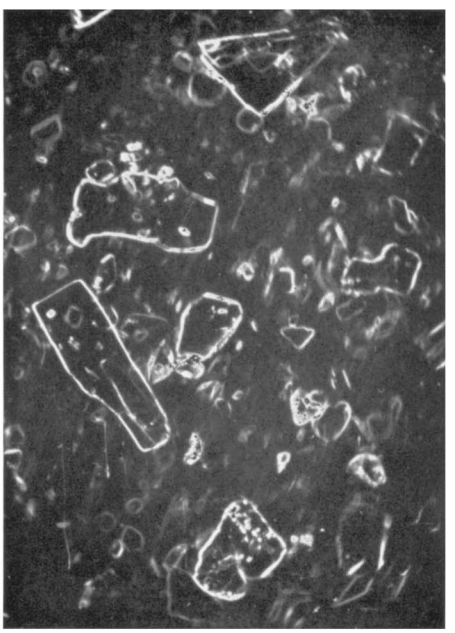


PLATE 2.



Crystalline forms of xanthine oxidase.

[*To face p.* 1102.

homogeneity of our purest fractions and their composition and catalytic properties will be treated in Parts II and III. It can be briefly stated here that sedimentation experiments with crystalline material yielded diagrams which showed an increasing degree of simplicity in comparison with those of preceding (less pure) fractions (cf. Avis *et al.*, *loc. cit.*) and with those of Corran *et al.* (*loc. cit.*). Secondly, fraction *M*7 contained FAD, iron, and molybdenum in the ratios 2·0 : 8·1 : 1·4 per unit of protein. The molybdenum ratio is higher than that reported in recent literature (cf. Richert and Westerfeld, *J. Biol. Chem.*, 1954, **209**, 179; Mackler *et al.*, *loc. cit.*) and is therefore nearer to that predicted for native xanthine oxidase (Mahler and Green, *Science*, 1954, **120**, 7). The iron ratio confirms the results of Richert and Westerfeld (*loc. cit.*).

Experimental

Purification of Milk Xanthine Oxidase (*see Scheme*, p. 1101).—(a) *Materials* (all chemicals were "AnalaR"; water was distilled and de-ionized). The buttermilk was prepared by the National Institute for Research in Dairying, Reading, by separating the cream from fresh milk; the latter was cooled overnight to 5°, then churned in the usual manner, and the resulting buttermilk passed through a cream-separator to remove any fat carried over from the churning. On one batch the buttermilk was found to contain approx. 50% of the enzyme activity originally present in the whole milk.

Calcium phosphate (cf. Polis and Shmukler, *loc. cit.*) was prepared by adding, simultaneously and with stirring, a solution of hydrated calcium chloride (1640 g.) in N/25-ammonia (5 l.) and a solution of disodium hydrogen phosphate (710 g.) in N/25-ammonia (5 l.) to water (20 l.), the solutions and water being at 70°. The precipitate was washed free from Cl ions with water by decantation during 14 days, filtered off by suction, and dried in an oven at 70°. The dried cake was gently ground to pass through a 40-mesh sieve. The adsorption power of the calcium phosphates varied from batch to batch. Analysis of three samples gave a Ca/P ratio of 1·2 [Calc. for $Ca_3(PO_4)_2$, 1·5; for $CaHPO_4$, 1·0]. One batch contained about 15% of water but did not show different adsorption power from one which was anhydrous. The final product would appear to be a mixture of $Ca_3(PO_4)_2$ and $CaHPO_4$. Buffer solutions were: (i) Phosphate (1M): from KH_2PO_4 (108·8 g.) and Na_2HPO_4 (28·36 g.) in water (to 1 l.): pH 5·8. (ii) Phosphate (0·02M): from KH_2PO_4 (2·18 g.) and Na_2HPO_4 (0·57 g.) in water (to 1 l.): pH 6·2. (iii) Phosphate (4M): from KH_2PO_4 (544 g.) and KOH (224 g.) in water (to 1 l.): pH *ca.* 9. (iv) Acetate (0·011M): from N-acetic acid (370 ml.) and sodium acetate trihydrate (99·4 g.) diluted with water to 1 l. and subsequently as required—1 vol. to 100 vols.: pH 5·1.

(b) *Enzymic precipitation of casein.* Buttermilk (25 l.) (*M*1) was warmed to 34° in a thermostat-controlled enamel-lined cast-iron vessel fitted with a pressure-tight lid and adapted as a pressure-vessel for subsequent filtration. Calcium chloride (0·5M; 250 ml.) and pancreatin (from British Drug Houses Ltd.) (40 g.) were added. After 15 min. a dense precipitate of casein was formed which was removed by straining through Nylon net (corresponding to butter muslin) (*M*2).

(c) *Filtration.* A pressure filter, of type PF40 (British Filters Ltd.), was used. The press was fitted with 13 Sterimats of grade SF/C/2, pre-coated with Hyflo Super Cel (650 g.) from a pressure vessel (British Filter No. V18) at about 5 lb./sq. in. Hyflo Super Cel (325 g.) was mixed with the material (*M*2). A starting pressure of 7 lb./sq. in. from a nitrogen cylinder was applied. The first 3 l. of the filtrate were colourless and were discarded. The rate of filtration decreased after about 10 l. had passed and a final pressure of about 28 lb./sq. in. was required before the filtration was complete (total volume 21 l.) (*M*3). The cloudy filtrate was stored for 16 hr. at 5° to allow for further hazing and refiltered through PF40 and 11 Sterimats of grade GS. The filtrate (*M*4; 20 l.) was clear and brown-yellow.

(d) *Separation of the enzyme fraction by chromatography* (all operations were at, or slightly below, 20°). A column, consisting of a piece of 6-in. Pyrex pipeline 1-ft. long, was fitted with 6 in. to $\frac{5}{8}$ in. standard adaptors at either end. A plate of stainless steel with $\frac{1}{8}$ in. perforations was clamped between the adaptor and the pipeline. In order that only the parallel-sided centre part of the pipeline should be used for the adsorbent column, a stand in the form of a crosspiece was made from $\frac{1}{4}$ in. Tufnol, $4\frac{1}{2}$ in. high; on this stand a perforated disc of $\frac{1}{4}$ in. Tufnol was placed; finally, as a support for the adsorbent a disc was cut from a Ford KC9 resin-bonded mat to fit. Calcium phosphate (*ca.* 600 g.), prepared as described above, was mixed with an equal solid volume of Celite 545 in a 5-l. beaker, and washed several times by decantation to remove any fine particles. Sufficient water was left after the final washing to prevent air bubbles

being retained. The slurry was then poured into the glass column and allowed to settle. Water was carefully layered on top of the mixture and allowed to run through. This was continued until the packing of the column was reasonably firm. Finally water was passed through at increasing pressures from 1 to 4 lb./sq. in. until a flow rate of about 4 l./hr. was obtained. Any cracks that appeared were carefully pressed out; this gave finally an adsorption column, *ca.* 6 cm. long. A layer of cotton wool was soaked in water and placed on top of the column, the whole being kept in place with a piece of stainless-steel gauze (20 mesh). The filtrate (*M*4) was cooled to 5°, adjusted to pH 6·2 (glass electrode) with 0·1N-acetic acid, and passed through the calcium phosphate column at a pressure of 4 lb./sq. in., from the pressure vessel. This gave again a flow rate of approx. 4 l./hr. The enzyme fraction developed as a brown band at the top of the column. This band diffused somewhat at the front and was not allowed to develop beyond 2 cm. from the bottom of the column, to prevent active material being lost in the effluent (*S*1). After this stage the column was washed with phosphate buffer (0·02M), until the washings gave a negative protein-test with trichloroacetic acid.

The enzyme fraction was then eluted with phosphate buffer (M), yielding fraction (*M*5) (1·22 l.). In this batch and in some others, it was necessary to subdivide filtrate (*M*4) into two parts and, after washing the adsorbent column with water until it was free from phosphate ions, to repeat the above process with the second half of fraction (*M*4). The column has been used repeatedly for chromatography of further batches, after being washed with water.

The eluate (*M*5) was precipitated with an equal volume of phosphate buffer (4M), and the precipitate filtered off in a sintered-glass funnel with the aid of Hyflo Super Cel (10 g.). The filter-cake was redissolved in a minimum of water, and the filter-aid filtered off. The final solid was washed several times with small amounts of water; the final volume with washings was 400 ml.

(e) *Purification by precipitation of contaminating proteins* (all operations at 5°). The above enzyme concentrate was dialysed in a Visking cellulose casing (size 32/32) against acetate buffer (0·01M; 40 l.). The dialysis bag was placed in a 4·5 × 75 cm. glass tube on a rocking platform, and the buffer allowed to flow through from a reservoir at a flow rate of 500 ml./hr. This treatment resulted in the formation of a copious, coloured precipitate which was centrifuged off on an angle centrifuge, a supernatant fraction (*M*6) (450 ml.) being obtained. More active material was obtained by extracting the remaining precipitate, suspending it with a hand homogenizer in acetate buffer (0·02M; 150 ml.), and re-centrifuging it, giving (*S*2). This was repeated with the same amount of buffer, giving (*S*3). The precipitate from the latter was finally taken up in aqueous sodium chloride (0·2M; 150 ml.), giving fraction (*S*4) (opaque).

(f) *Crystallisation.* Fraction (*M*6) was concentrated by perevaporation (current of air, produced by fan blown at solution in Visking cellulose casing) for 16 hr. at 5° from 450 to 320 ml., to give a 1% solution of protein as measured by the optical density at 280 mμ (higher concentrations up to 2% have been used with advantage in other batches). It was cooled to 0°; 70% v/v ethanol (160 ml.), pre-cooled in a carbon dioxide–acetone bath, was added to it from a burette fitted with a fine capillary, with sufficient stirring to ensure mixing without frothing. The solution in which a precipitate formed was set aside for 16 hr. at −6°. At the same temperature the precipitate was centrifuged off in a refrigerated centrifuge at 1300/g for 40 min. The supernatant liquid (*S*5) was not quite free from suspended precipitate. The main precipitate was dissolved in water (88 ml.), giving a total volume of 100 ml. Phosphate buffer (1M; 1 ml.) and 50% v/v ethanol (10 ml.) were added in the manner described, so that the final alcohol concentration was *ca.* 8% v/v. The solution was placed in a thermostat-controlled bath at −1° for 0·5 hr., the initial precipitate (*S*6) was centrifuged off at −1° and then redissolved in phosphate buffer (1M; 22 ml.). The supernatant liquid was seeded with material obtained from a previous batch and placed in a bath at −1°. Crystals appeared after *ca.* 12 hr. In other cases crystals appeared within 36 hr., without seeding.

After 4 days at −1° the crystals were centrifuged off at −1°, washed twice with phosphate buffer (0·01M; 70 ml. each, containing 7% v/v ethanol). The crystalline centrifugate (see Plate 1) was dissolved in aqueous 0·2M-sodium chloride (*ca.* 45 ml.) and dialysed during 16 hr. against 0·2M-sodium chloride (1 l.) in a 4·5-cm. tube on the rocking platform at 5°. The dialysis bag was emptied and washed out with small amounts of the salt solution and diluted with it to 50 ml. (*M*7).

A second crop was obtained from the centrifugation mother-liquor during 28 days at −1°. The crystals were centrifuged off and dissolved in 0·2M-sodium chloride (15 ml.) (*M*7*a*).

Part of the solution (*M*7) (10 ml.), after 28 days (see footnote ‡ to Table) at 2°, was caused to crystallise again. The enzyme was first precipitated by addition of 70% v/v ethanol (5 ml.),

then centrifuged, and redissolved in water to a final volume of 10 ml. Phosphate buffer (1M; 0·7 ml.) and 50% ethanol (1·5 ml.) were added. The immediate precipitate was centrifuged off after 10 min. at −1° and the supernatant liquid seeded and left at the same temperature for 16 hr. The crystals formed were centrifuged off and dissolved in 0·2M-aqueous sodium chloride (final volume 10 ml.) (*M*8).

Spectrophotometric Measurements and Enzyme Assay.—(a) *Materials* ("AnalaR" unless otherwise stated). (i) Phosphate buffer: Na_2HPO_4 (13·3 g.) and KH_2PO_4 (5·5 g.) dissolved in water (final vol. 2 l.), and chloroform (10 ml.) added as preservative. pH 7·1. (ii) Pyrophosphate buffer: Sodium pyrophosphate decahydrate (71·3 g.) and sodium hydrogen pyrophosphate (B.D.H. Laboratory Reagent grade) (13·3 g.) dissolved in water (final vol. 2 l.), and chloroform (10 ml.) added. pH 8·0. (iii) Xanthine (10—15 mg.; from Genatosan) dissolved in N-sodium hydroxide (2 ml.) and made up to 100 ml. with water. (iv) Buffered xanthine solution: Xanthine solution (3 ml.), N/50-hydrochloric acid (3 ml.), pyrophosphate buffer (12 ml.), and water (7 ml.), prepared weekly and stored in refrigerator.

(b) *Ultra-violet measurements of enzyme solutions at* 280 *and* 450 *mμ.* Samples were used either undiluted or after appropriate dilution with phosphate buffer; E values for the undiluted solution ($E^{1\,cm.}_{undil.}$) were calculated from $(E_{obs.} \times F)/L$ where F is the dilution factor and L the size of cell in cm.

(c) *Activity assays* (see also Kalckar, *loc. cit.*; Morell, *loc. cit.*). The sample was diluted with ice-cold pyrophosphate buffer immediately before being assayed. The dilution was arranged so that the measured rate (see below) was about 0·006—0·03/min.; appropriate dilution factors are 10—25 for buttermilk and digestion filtrates, 100—1000 for concentrates.

Buffered xanthine (2·5 ml.) was placed in a 1-cm. stoppered quartz cell, diluted enzyme solution (0·1 ml.) added, and a stop-watch started. The cell was placed in the thermostat-controlled cell compartment of the spectrophotometer (compartment constructed by Mr. A. L. Stewart; details will be published elsewhere), after its contents had been mixed by inversion. Readings at 295 mμ were taken at 0·5- or 1-min. intervals for 10 min., the first reading being taken as soon as possible after the addition of the enzyme. No comparison cell was used, and the instrument was balanced in the "check" position without any cell in place, before each reading was taken. The temperature of the circulating water was 23·5° ± 0·5°.

E_{295} was plotted against time and the slope $(\Delta E_{295}/\Delta t_{(min.)})$ of the best straight line through the experimental points was calculated. If the rate exceeded about 0·03/min. the reaction tended to slow down towards the end of the 10-min. period. Adequate temperature equilibration appears to occur within about 2 min. of placing the cell in the instrument, and temperature variation should therefore have little effect on the results. The number of units in the sample is equal to $26FV \times (\Delta E_{295}/\Delta t_{(min.)})$, where V is the volume of the sample in litres, F is the dilution factor used in preparing the enzyme solution for assay, and 26 represents the further dilution which takes place in the assay mixture. (N.B.: The final concentrations in the assay solution are as follows: xanthine *ca.* 10^{-4}M, pyrophosphate 0·05M, final pH 8·2.) Q_{O_2} is related to "APR" as described previously (Avis *et al.*, *loc. cit.*), *viz.*, Q_{O_2} = "APR" × 152.

Xanthine Oxidase and Beer's Law.—A solution of xanthine oxidase ("PFR" = 8·1) was dialysed against 0·01M-sodium chloride and centrifuged to give a clear solution, which gave the following readings at 450 mμ:

Solution	Light path (cm.)	Corr. reading	Calc. $E^{1\,cm.}_{undil.}$	Approx. concn. (%)
Undiluted	0·5	1·142	2·28	1·7
Dil. × 3	0·5	0·381	2·29	0·6
Dil. × 25	4	0·362	2·26	0·07

Dilutions were carried out as described above, and the approximate concentrations calculated from the E_{280} by using the $E^{1\%}_{1\,cm.}$ value given previously (Avis *et al.*, *loc. cit.*).

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