# 253. *Cellular Constituents. The Chemistry of Xanthine Oxidase. Part III.* Estimations of the Co-factors and the Catalytic Activities of Enzyme Fractions from Cow's Milk.*

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Analyses for three co-factors of milk xanthine oxidase have been carried out on crystalline and less purified fractions.[1] The purest preparations * contained 8 atoms of iron, 2 molecules of flavin-adenine dinucleotide (FAD), and 1·3—1·5 atoms of molybdenum per molecule of protein. When assayed for enzymic activities these fractions exerted catalytic actions similar to those reported by other workers, except for certain quantitative differences. The latter were found most pronounced when reduced coenzyme I (DPNH) was used as substrate or when cytochrome-*c* was the electron acceptor; particularly in the second case, a very low activity was recorded.

Previous papers of this series [1,2] have described the preparation, from cow's milk, of crystalline and other xanthine oxidase fractions, and their examination for homogeneity. The present paper covers analyses of these fractions for the three co-factors, flavin-adenine dinucleotide (FAD), molybdenum, and iron, and measurements of the catalytic activities of selected samples.

*The Co-factors.*—At the time of writing the following values for these prosthetic groups have been published or can be calculated from data given by the authors: for FAD,† Corran *et al.*,[3] 0·465%, Morell,[4] 0·435%; Richert and Westerfeld,[5] for FAD † 0·49%, for Mo 0·03%, and for Fe 0·14%. Green and Beinert,[6] Totter *et al.*,[7] and Mackler *et al.*[8] have

* Part II, preceding paper.
† Calc. for the anhydrous free acid (*M* 786).

[1] Avis, Bergel, and Bray, *J.*, 1955, 1100.
[2] Avis, Bergel, Bray, James, and Shooter, preceding paper.
[3] Corran, Dewan, Gordon, and Green, *Biochem. J.*, 1939, **33**, 1694.
[4] Morell, *ibid.*, 1952, **51**, 657.
[5] Richert and Westerfield, *J. Biol. Chem.*, 1954, **209**, 179.
[6] Green and Reinert, *Biochem. Biophys. Acta*, 1953, **11**, 599.
[7] Totter, Burnett, Monroe, Whitney, and Comar, *Science*, 1953, **118**, 555.
[8] Mackler, Mahler, and Green, *J. Biol. Chem.*, 1954, **210**, 149.

reported the ratio FAD : Mo as being 2 : 1, and Richert and Westerfeld [5] including their iron values give 2 : 1 : 8.

Our own values are recorded on Table 1. Our determinations of FAD (col. 3*a*) were carried out fluorimetrically and confirmed by microbiological estimations. The values for our crystalline samples (Nos. VI—IX) are of the same order as those published, although some of the latter (Corran *et al.*; [3] Morell [4]) are liable to be high as these workers were unaware, at the time, of the presence of iron which could contribute to the absorption at 450 mμ of their deproteinized solutions (cf. Richert and Westerfeld [5]). Calculating $M$ from the highest FAD content, one obtains 154,000 for one FAD bound to one protein molecule. Doubling this figure for two FAD molecules per unit of protein one arrives at $M$ 308,000, in agreement with the value derived [2] from sedimentation and diffusion data, namely *ca.* 290,000.

The iron content of the crystalline samples (VI—IX) varied little and averages, for the four figures recorded (columns 3*b* and 6), 4·0 atoms of iron per molecule of FAD, in agreement with Richert and Westerfeld's data.[5] This is supported by the constancy of the ratio $E^{450}_{1\,\text{cm.}}$ : Fe(μg./ml.) (0·164 ± 0·007).

While analyses of all preparations described in the literature appear to give a ratio of one atom of molybdenum to two molecules of FAD, our results (columns 3*c* and 7) provide some support for the suggestion by Mahler and Green [9] that native xanthine oxidase might contain one atom of molybdenum per molecule of FAD. In fact, this ratio has been claimed recently by Kielly [10] to exist in her preparations from calf and rat livers. However our ratios are fractional, with an average of 0·70 for our crystalline samples and a probable error for the individual ratios not greater than ±10%. The significance of these fractional ratios in terms of the structure of the enzyme is still obscure.

Unlike Mackler *et al.*,[8] we were unable to remove any substantial amount of molybdenum by dialysis of one of our fractions (LXO59, M5) against ammonia, and the sample retained at the same time almost all its catalytic activity (sample II, column 5). In contrast, another of our fractions (LXO60, ex M7, sample V), which after storage was found to be virtually devoid of xanthine oxidase activity and sedimented [2] similarly to active samples, still gave normal analytical results for the co-factors.*

TABLE 1. *Composition of xanthine oxidase fractions.*

| | 1 | 2 | 3 | | | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|---|---|
| | | | Percentage composition | | | | Activity units [e] per μmole of FAD | Fe (atoms) / FAD (mols.) | Mo (atoms) / FAD (mols.) |
| No. | Batch | Stage | (*a*) FAD | (*b*) Fe [c] | (*c*) Mo [c] | "PFR" [d] | | | |
| I | LXO59 | M5 | 0·19 | 0·06 | 0·020 | 9·9 | 3·7 | 4·6 | 0·87 |
| II | LXO59 | (M5) [a] | Solutions too turbid for accurate | | | | 3·5 | 4·9 | 0·81 |
| III | LXO56 | S4 | absorption measurements at 280 mμ | | | | 3·0 | 6·1 | 0·64 |
| IV | LXO51 | M6 | 0·31 | 0·08 | 0·021 | 8·5 | 2·1 | 3·5 | 0·56 |
| V | LXO60 | Ex M7 [b] | 0·36 | 0·10 | 0·028 | 6·8 | 0·01 | 4·1 | 0·64 |
| VI | LXO55 | M7 | 0·44 | 0·12 | 0·040 | 5·6 | 2·6 | 3·9 | 0·75 |
| VII | LXO56 | M7 | 0·47 | 0·14 | 0·040 | 5·2 | 2·9 | 4·0 | 0·70 |
| VIII | LXO60 | M7 | 0·48 | 0·14 | 0·038 | 5·0 | 2·6 | 4·0 | 0·64 |
| IX | LXO59 | M10 | 0·51 | 0·15 | 0·043 | 5·0 | 1·6 | 4·1 | 0·69 |

[a] After dialysis against ammonia (see Experimental). [b] "Inactive sample" (see Experimental). [c] Metals estimated by colorimetric methods (see Experimental). [d] *I.e.*, $E_{280}/E_{450}$ (see ref. 1). [e] Activity of freshly prepared solutions in X/O assay (see Experimental).

That no other metals formed part of the flavoprotein molecule was borne out by emission spectroscopy of sample LXO55, M7. While iron and molybdenum were present in about the same concentrations as calculated from our colorimetric data, all other metals which could be detected were found at concentrations less than one tenth of that of molybdenum.

The absorption spectrum of our material (LXO60, M7) was very similar to that given

* [*Added, March 20th,* 1956.]—A *crystalline*, inactive sample, similar to the above, has since been obtained.

[9] Mahler and Green, *Science*, 1954, **120**, 7.
[10] Kielly, *Fed. Proc.*, 1955, **14**, 235.

by Mackler *et al.*,[8] though our lower " PFR " indicated higher purity (column 4). The inactive sample (LXO60, ex M7) did not differ substantially from the above.

*Enzymic Activities.*—The main catalytic properties of our samples, in comparison with those in the literature,[11] are given in Table 2.

TABLE 2. *Rates of oxidation of various substrates catalysed by xanthine oxidase.*

Symbols : (*a*) Substrates : X = xanthine, H = hypoxanthine, A = acetaldehyde, D = DPNH. (*b*) Electron acceptors : O = oxygen, Mb = methylene-blue, I = 2 : 6-dichlorophenol-indophenol, Cy = cytochrome-c.

| 1 | 2 | 3 | 4 | 5 | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Absolute rates for reaction | | | Rates as % of corresponding X/O rate | | | | | |
| | | | | *a* | *b* | *c* | *d* | *e* | *f* |
| Sample | X/O | H/Mb[a] | A/Mb[a] | X/O | X/I | A/I | D/I | D/O | H/Cy |
| Corran *et al.*[3] | — | 1·8 | 2·6 | — | — | — | — | — | — |
| Morell[4,b] | 0·52 | — | — | — | — | — | — | — | — |
| Mackler *et al.*[8,c] | (1·25) | — | — | (100) | (106); 49 | (24) | (54) | 6 | 30 |
| LXO56, M7[d] | 1·82 | 1·3; 2·3[e] | — | — | — | — | — | — | — |
| LXO60, M7 (freshly prepared) | 1·68 | 1·1; 1·9[e] | 2·4 | 100 | 41 | 12 | — | 0·7 | — |
| ,, (after 3—4 weeks) | 0·83 | — | — | 100 | 43 | 13 | 1·3 | — | 0·1[f] |
| LXO60, M5 | 0·93 | — | — | 100 | 43 | 18 | 1·5 | 0·7 | 0·1[f] |
| LXO61, M4 | 0·09 | — | — | 100 | 42 | 20 | 1·9 | 0·9 | 0·1[f] |
| LXO61, M1 (buttermilk) | 0·02 | — | — | 100 | 29 | 10 | 3·6 | 1·5[g] | — |
| LXO63, whole milk | — | — | — | 100 | — | — | 6·4 | — | — |
| LXO60; " inactive " sample (ex M7) | 0·005 | — | — | 100 | — | 10 | 400 | 200 | — |

Absolute rates as $\mu$moles of substrate oxidised per mg. of protein per min. For conditions, see Table 3 and Experimental section. Figures in parentheses are extrapolations by the method of Lineweaver and Burk (*J. Amer. Chem. Soc.*, 1934, **56**, 658).

[a] Conditions as given by Corran *et al.*[3] [b] pH 8·3; 19°. [c] pH 7·0; 22°. [d] See ref. 1. [e] Obtained with a reduced hypoxanthine concentration. [f] Identical figures were obtained whether $MoO_3$ was present or absent. [g] Value from Batch LXO58, M1.

While our figures for X/O (column 2; see Table for explanation of symbols), representing observed rates and measured by spectrophotometric methods, were higher than those obtained by other workers, the methylene-blue activities, obtained in Thunberg tubes, with hypoxanthine (H/Mb) or acetaldehyde (A/Mb) (columns 3 and 4 respectively) were of the same order of magnitude as reported by Corran *et al.*[3] Other enzymic activities, expressed in percentages of the corresponding X/O rate on the same sample, such as X/I and A/I (columns 5*b* and 5*c* respectively) showed fair constancy for all fractions examined. Discrepancies from published data have been encountered by us with the reactions involving DPNH-dehydrogenation and cytochrome-*c* reduction, D/I, D/O, and H/Cy (columns 5*d*, *e*, and *f*, respectively). Our freshly prepared crystalline material showed lower activities for the first two than those given by Mackler *et al.*[8] A finding which has not been reported previously was that D/I and D/O decreased rapidly during the early stages (from whole milk to M4) of our purification process. On the other hand, DPNH-activity in more purified samples was observed to be more stable than the activities recorded in the preceding columns. The extreme case was that of sample LXO60, ex M7, mentioned before, which was virtually devoid of X/O and A/I activities (columns 2 and 5*c*), while retaining most of its D/I and D/O potencies. This is reminiscent of the results by Corran *et al.*[12] who described a milk flavoprotein related to xanthine oxidase with DPNH but no X/O activity.

Our figures for H/Cy (column 5*f*) add one more contradiction to those existing in the publications dealing with this enzymic activity.[8,10,13] Our own very low values for the

[11] See review by Singer and Kearny, " The Proteins," ed. Neurath and Bailey, Academic Press, New York, 1954, Vol. IIA, p. 225.

[12] Corran *et al.*, (*a*) *Biochem. J.*, 1938, **32**, 2231; (*b*) also ref. 3. Cf. (*c*) Philpot, *ibid.*, 1938, **32**, 2240; 1939, **33**, 1707; (*d*) and ref. 8.

[13] (*a*) Horecker and Heppel, *J. Biol. Chem.*, 1949, **178**, 683; (*b*) Morell, *Biochem. J.*, 1952, **51**, 666; *Biochem. Biophys. Acta*, 1955, **16**, 258; Mahler, Fairhurst, and Mackler, *J. Amer. Chem. Soc.*, 1955, **77**, 1514.

activities did not increase under anærobic conditions and in absence of salts, nor did they alter whether molybdic oxide was present or not.

*Discussion and Conclusions.*—For the co-factors of our crystalline specimens, the average ratios protein : FAD : Mo : Fe were 1 : 2 : (1·3—1·5) : 8. While FAD and iron appeared to be relatively securely bound, whatever the history of the sample, molybdenum was found in rather variable amounts. The effects of this variability on enzymic activities were not of a simple nature. Mahler and Green [9] postulated that molybdenum is not required for X/O activity, but only for transfer of electrons from specific substrates to single-electron acceptors, such as cytochrome-*c* or ferricyanide. This must be contrasted with the observation by de Renzo *et al.*[14] and by Richert and Westerfeld [5] that loss of molybdenum parallels loss of X/O activity. As to the latter, our results with samples, prior to crystallisation (showing some correlation between molybdenum content and this activity), and with crystalline fractions (showing no such correlation), left us undecided. But there is no doubt that loss of X/O and also X/I and A/I, but not D/O and D/I, activity cannot be caused solely by deficiency of molybdenum. Other modes of damage to the native molecule which still await elucidation must be operative, particularly in the protein moiety. The existence of a milk flavoprotein, having no X/O activity and related in chemical and physicochemical properties to active xanthine oxidase, points in this direction.[2, 12a, 12c, 15] Morell [4] (see his Fig. 2) has determined the amount of X/O inactive molecules in his enzyme preparations by two methods; our own observations [1, 2] on the variability of "AFR" values and of X/O activity per μmole of FAD (see Table 1, column 5) were in agreement with his results. So far, we have not applied his "immediate reduction" method.

Summarising, it can be assumed that our own preparations and those by other workers (which all contained larger amounts of extraneous proteins) still consist of mixtures of two or more of (1*a*) the fully active enzyme, (1*b*) an "inactive" enzyme with a modified protein moiety, possibly capable of catalysing DPNH-dehydrogenation, (2*a*) molybdenum-poor flavoprotein molecules, derived from the native enzyme, and (2*b*) molybdenum-poor molecules with a modified protein moiety; both (1*a*) and (1*b*) have a sufficient molybdenum content; both (2*a*) and (2*b*) are of unknown catalytic potency. While some of our xanthine oxidase preparations (*e.g.*, LXO56, M7) and that described by Corran *et al.*[3] contained a considerable amount of X/O active flavoprotein, the material of Mackler *et al.*[8] contained less and that of Morell [4] still less. Further work must aim at the separation of these various types of closely related molecules.

## EXPERIMENTAL

*Xanthine Oxidase Solutions.*—The preparation of the solutions, batch and stage symbols and conditions of storage, etc., together with "AFR," "APR," and "PFR" values for some of the solutions have been recorded.[1, 2]

*Effect of Ammonia on Xanthine Oxidase.*—A sample of batch LXO59, stage M5 (protein concentration approx. 1%), was dialysed at 0—5° in a Visking cellulose bag against a total of 5 l. of aqueous *ca.* 0·01M-ammonia, in five portions, over a period of 10 days. Then the sample was removed, neutralised, centrifuged to remove precipitated material, assayed, and analysed.

"*Inactive*" *Xanthine Oxidase.*—A sample of washings obtained in the preparation of LXO60, M7, was stored at 0—5° for 8 weeks, the activity of the solution in the X/O assay falling from 35 to 0·3 unit/l. The material was precipitated by the addition of ammonium sulphate and phosphate buffer, centrifuged off, redissolved, and dialysed against *ca.* 9 l. of running 0·2M-sodium chloride at 0—5° on a rocking platform. The solution was centrifuged, and the clear supernatant liquor used for assays and analyses.

*Determination of Absorption Coefficient* $E^{1\%}_{1\,\text{cm.}}$ (280 mμ) *for Xanthine Oxidase.*—The dry weight of a solution of crystallised xanthine oxidase (LXO55, M7) which had $E^{1\,\text{cm.}}_{280}$ 10·0 and $E^{1\,\text{cm.}}_{450}$ 1·80 was determined by three methods: (*a*) Kjeldahl nitrogen. Found: N, 1·35 mg./ml. Taking Ball's value [16] for the nitrogen content of crude xanthine oxidase, *viz.*, 16·3% gives the dry weight as 8·3 mg./ml. (*b*) Gravimetric determination with trichloroacetic acid: [17] 9·3 mg./ml.

[14] de Renzo, Heytler, and Kaleita, *Arch. Biochem.*, 1954, **49**, 242.
[15] See also Table 2, sample LXO 60, ex M7.
[16] Ball, *J. Biol. Chem.*, 1939, **128**, 51.

(*c*) An aliquot of the solution was freeze-dried and weighed, and the weight corrected for loss at 110° and for ash weight : Found, 8·5 mg./ml.

The average $E^{1\%}_{1 \text{cm.}}$ value at 280 mμ is therefore 11·5 ± 0·7. Similar figures have been obtained for a number of preparations, including some as crude as M5. The above value differs slightly from that given previously, *viz.*, 11·0 (Avis *et al.*[18]), while the corresponding figure calculated from Ball's data [16] for cruder material (" PFR " = 12·0) is 13·1.

The value of 11·5 has been used in calculating the dry weight from the 280 mμ optical density for all solutions from stage M5 onwards, when these were required for the calculation of composition, etc. It has also been used for cruder samples, when very approximate values would suffice. As the lowest " PFR " so far obtained is 5·0, the value $E^{1\%}_{1 \text{cm.}}$ (450 mμ) at this purity level is 11·5/5·0, *i.e.*, 2·3.

*Fluorimetric Estimations for FAD* (cf. total riboflavin determinations by Burch *et al.*[19]).—Fluorescence was measured relative to an arbitrary eosin reference solution in a Hilger " Spekker " fluorimeter. This eosin solution was introduced as it was found to be more stable than riboflavin solutions of equivalent fluorescence.[20] The FAD concentration was determined by reference to a riboflavin standard (Roche; dried at 110°).

*Microbiological FAD Estimation.*—A sample of LXO56, M7 was assayed microbiologically for riboflavin, after hydrolysis in 0·1N-hydrochloric acid (determination by Messrs. Galloway and Barton-Wright). Under the conditions of the assay, riboflvin and riboflavin phosphate (Roche) gave identical activities on a molar basis. The riboflavin concentration found corresponded to an FAD content of 0·49% (cf. 0·47% by fluorimetry; Table 1, column 3*a*).

*Colorimetric Iron Analyses.*[21]—Aliquot parts were digested with a mixture of perchloric and sulphuric acid, reduced with aqueous sulphur dioxide, and brought to about pH 4 by the addition of excess of sodium acetate, and the colour devloped by adding 2 : 2′-dipyridyl. Readings were taken at 520 mμ, some hours after making up to constant volume.

*Colorimetric Molybdenum Analyses.*—Aliquot parts were digested with perchloric and sulphuric acid, and the colour was developed and measured according to the method of Dick and Bingley.[22]

*Spectrographic Analysis for Metals* (carried out by J. B. Martin).—A dialysed sample (LXO55, M7) was freeze-dried, and the residue was ashed at 435°; spectrographic analysis of the ash gave the following results, expressed as percentages of metal on the original sample : Mo, 0·04—0·05; Fe, 0·18; Cu, 0·004; Pb, 0·004; Ni, 0·003; Mn, 0·0004.* For the corresponding colorimetric values see Table 1 (sample VI, columns 3*b*, *c*).

*Absorption Spectra.*—These were determined in a phosphate buffer of pH 7·1. Freshly prepared LXO60, M7 had maxima at 275, 325, and 454 mμ, minima at 250, 310, and 401 mμ, and light absorption continued up to at least 800 mμ.

*Methylene-blue Assays* (*Reactions* H/Mb *and* A/Mb).—These were carried out by the Thunberg technique, with hypoxanthine or acetaldehyde as substrate, as described by Corran *et al.*[3] Owing to the considerable effect of hypoxanthine concentration on the activity figures (Table 2, footnote *e*) and to the uncertainty of the final volume of solution in the Thunberg tubes after evacuation and flushing with nitrogen, the methylene-blue figures were somewhat less reliable than spectrophotometric assays. For the acetaldehyde figures, the accuracy was further diminished by loss of substrate during evacuation of the tubes. The activities were calculated in the usual manner.

*Spectrophotometric Enzyme Assays.*—These were carried out with a Unicam S.P. 500 Spectrophotometer, at 23·5° ± 0·5° (cf. ref. 1), generally in 1 cm. stoppered cells, in a total volume of 2·6 ml., reaction being started by adding the enzyme last. Optical-density readings at the wavelength selected were taken at intervals, in most cases without a comparison cell, the instrument being balanced in the " check " position against air (cf. ref. 1). When it was desired to reduce readings of high optical density, due, *e.g.*, to the use of turbid solutions, suitable stable, reference liquids, such as solutions of uric acid, sodium dichromate, or ammoniacal copper sulphate, were used for balancing the instrument. For assays on whole milk (Table 2)

* Zn, 0·004 (by colorimetric analysis of LXO73,M7).

[17] Hoch and Vallee, *Analyt. Chem.*, 1953, **25**, 317.
[18] Avis, Bergel, Bray, and Shooter, *Nature*, 1954, **173**, 1230.
[19] Burch, Bessey, and Lowrey, *J. Biol. Chem.*, 1948, **175**, 457.
[20] Bowen and Wokes, " Fluorescence of Solutions," Longmans, Green & Co., London, 1953.
[21] Koenig and Johnson, *J. Biol. Chem.*, 1942, **143**, 159; Sandell, " Colorimetric Determination of Traces of Metals," Interscience Publ., New York, 1950, p. 362.
[22] Dick and Bingley, *Austral. J. Exp. Biol. Med. Sci.*, 1947, **25**, 193.

the validity of the results was checked by adding known amounts of purified enzyme to the milk and repeating the assays. In one experiment in which the activity added was approximately equal to that originally found in the milk, the recoveries of added activity in the X/O and D/I assays were respectively 84 and 72%.

In no reaction were precautions taken to exclude air, and it is assumed that all reaction solutions were therefore saturated (or very nearly saturated) with air at the start of the reaction.

In all the assay systems described below, it was shown that: (1) no significant change of optical density took place if the substrate or the enzyme or the electron-acceptor (where this was not oxygen) was omitted (in the case of some very turbid assay mixtures, changes of optical density due to changing turbidity did occur in the controls, and appropriate corrections to the activities had to be made); (2) the rate of reaction was proportional to the enzyme concentration (see Table 3 for range of rates used). For calculating enzyme activities a general definition of units, similar to that given previously,[1] was adopted: Units/l. = $(dE/dt) . (f/x)$ (where $dE/dt$ is slope at $t = 0$, except for reaction D/I and D/O for which maximum slopes were taken; $f$ = dilution from stock solution to mixture in cell; and $x$ is cell size in cm.). The relative activity of a given enzyme solution in two different assay systems, A and B, was then obtained by:

$$\text{Relative activity} = 100 \times \frac{\text{Units/l. (reaction A)}}{\text{Units/l. (reaction B)}} \times \frac{\Delta\varepsilon \text{ (reaction B)}}{\Delta\varepsilon \text{ (reaction A)}} \%$$

where $\Delta\varepsilon$ is the difference between the molar extinction coefficients of the oxidised and the reduced form. If the component measured in one of the reactions is undergoing single-electron oxidation or reduction, $\Delta\varepsilon$ is replaced by $2\Delta\varepsilon$ for that reaction in the above expression (*e.g.*, in the cytochrome-*c* reaction). When absolute reaction rates were required for reaction X/O, these were calculated as $\mu$ moles/mg. enzyme/min. from 100 × " APR "$E \times {}^{1\%}_{1\,\text{cm.}}/\Delta\varepsilon$, where $E^{1\%}_{1\,\text{cm.}}$ is the absorption coefficient at 280 m$\mu$ for xanthine oxidase, *i.e.*, 11·5.

*Details of Spectrophotometric Assay Procedures* (see also Table 3).—Buffers: pyrophosphate, pH 8·0, and phosphate, pH 7·1, as used previously [1] for spectrophotometric work.

TABLE 3. *Details of spectrophotometric assay procedures.*

| | | | | Final concentrations, etc. | | | |
|---|---|---|---|---|---|---|---|
| Symbol | $\lambda$ (m$\mu$) | $\Delta\varepsilon$ | pH | Buffer | Substrate concn. ($10^{-4}$M) | Acceptor concn. ($10^{-4}$M) | Range of rates used (1 cm. cell) |
| X/O | 295 | +9,600 | 8·2 | Pyrophosphate (0·05M) | 0·8—1·1 | — | 0·005—0·030/min.[d] |
| X/I | 600 | −21,000 | 8·2 | ,, | 0·8—1·1 | 0·08—0·11 | 0·003—0·050/min. |
| A/I | 600 | −21,000 | 8·2 | ,, | 800 | 0·08—0·11 | 0·003—0·050/min. |
| D/I | 600 | −21,000 | 7·2 | Phosphate (0·03—0·06M) | 0·10—0·14 | 0·08—0·11 | 0·005—0·020/min. |
| D/O | 340 | −6,220[a] | 7·2 | ,, | *ca.* 0·8 | — | 0·002—0·010/min. |
| H/Cy | 550 | +19,600[b] | 7·2 | ,,[c] | 11 | *ca.* 0·4 | 0·002—0·020/min. |

All reactions at 23·5° ± 0·5°, without exclusion of air.

[a] Value from Horecker and Kornberg (*J. Biol. Chem.*, 1948, **175**, 385). [b] Value from Horecker and Heppel.[13a] [c] Reaction mixture contained the following additional components: Ethanol, 0·16M; catalase, *ca.* 7 $\mu$g. of hæmatin/ml.; molybic oxide, 4 × $10^{-4}$M. [d] For reaction X/O, the initial rate remained strictly proportional to enzyme concentration for rates from 0·001—0·20/min., but for routine determination rates were kept within the range specified.

Reaction X/O: Carried out as described previously.[1]

Reaction X/I: Experiments in which the concentration of 2: 6-dichlorophenolindophenol (from B.D.H.) was varied indicated that the quantities given in Table 3 gave maximum activities, with lower activities on increase or decrease of concentration.

Reaction A/I: Acetaldehyde was freshly distilled from paraldehyde and sulphuric acid (older samples which had not deposited crystals were used in some cases); 4% v/v aqueous acetaldehyde was prepared freshly each day and kept cold. (The use of old samples of acetaldehyde appeared to result in slightly low activity figures, and A/I figures are probably less reliable than other assays for this reason.)

Reaction D/I: The stock solution of DPNH (from Sigma material; 0·30—0·35 mg./ml.) deteriorated fairly rapidly at 0—5° (fall in $E_{340}$) and was not kept for more than about 1 week: in this time decomposition was *ca.* 20%. Under the conditions of the assay, the indophenol was decolorised slowly in the absence of enzyme, the rate, $dE/dt$, usually being about −0·0003 per

min. As this rate was always low compared with the enzyme-catalysed rates (see Table 3), no correction was applied. An attempt to use DPNH prepared by reduction of DPN (Light's cozymase) with sodium dithionite [23] was abandoned owing to a very rapid non-enzymic reaction occurring under these conditions. As the unusual features of the D/O reaction (see below) appeared to apply also, to a small extent, to the D/I reaction, maximum d$E$/d$t$ values, usually attained after 1—5 min., rather than initial values, were recorded, and rates higher than 0·02 or lower than 0·005 per min. were not accepted. After complete reduction of the indophenol, a slow re-oxidation usually occurred in this assay, presumably owing to the presence of dissolved oxygen.

Reaction D/O: For DPNH, either Sigma material or reduced Light's cozymase (see above) was used. Unusual features of the reaction were: (1) slow development of maximum rate of reaction; maximum rather than initial d$E$/d$t$ values were therefore recorded; (2) fall of specific activities (or units/l.) at high concentrations of enzyme in the assay mixture (cf. Corran *et al.*[12a]); rates higher than 0·01 or lower than 0·002 per min. were not accepted in routine assays.

Reaction H/Cy: Hypoxanthine was from Genatosan; cytochrome-*c* was from Messrs. Light; horse-liver catalase was kindly supplied by Dr. E. F. Hartree; molybdic oxide ("AnalaR") was dissolved in phosphate buffer. The mixture before addition of the enzyme tended to be somewhat turbid, and was therefore generally centrifuged before use. Owing to the very sharp absorption band of reduced cytochrome-*c* at 550 mμ, it was necessary to use a narrow slit width (*ca.* 0·02 mm.) and to check occasionally the setting of the wavelength scale of the instrument as described by the makers. In some cases, the reading corresponding to complete reduction of the cytochrome was determined with sodium dithionite. These readings were not significantly higher than those at the end of the enzymic reductions. In the few experiments performed in the absence of inorganic salts, solutions were dialysed against water before use (cf. Morell [4]).

An experiment under anærobic conditions in an apparatus similar to that of Lazarow and Cooperstein [24] was carried out, the xanthine oxidase being added to the reaction mixture after dissolved air had been removed by bubbling oxygen-free nitrogen through the solution. Xanthine oxidase, LXO64, M5, gave 70 units/l. in the X/O assay. The anærobic H/Cy reaction rate (without catalase and alcohol) corresponded to 0·8 unit/l., compared with 1·2 units/l. obtained under ærobic conditions. In all "ærobic" H/Cy experiments the amount of enzyme used was large, and would probably be sufficient to exhaust the supply of dissolved oxygen (but not the supply of hypoxanthine) in the first few minutes of the run; it seems likely that all rates measured were therefore effectively "anærobic."

*Determination of* Δε *for Reaction X/O.*—Pure xanthine (Roche Products) was dried at 100°/2 mm. for 2 hr., a solution of known concentration was prepared in the usual buffer mixture, and its absorption measured. The solution (2·6 ml.) was then mixed with a xanthine oxidase concentrate (0·01 ml.), and the absorption was measured again after the rapid reaction had ceased. The molar extinction coefficients for xanthine and uric acid were calculated, after correction for the absorption of the enzyme, for the dilution occurring on addition of the enzyme, and for cell blanks. The values obtained at 295 mμ were: xanthine, ε 2300; uric acid, ε 11,900; giving Δε 9600. The figure for uric acid was confirmed by measurements on a solution prepared from uric acid (from B.D.H.), dried as above: the value obtained differed by less than 1% from that quoted for the enzymically produced uric acid. The Δε value, which corresponds to Δ$E$ (1 μg. of xanthine) = 0·063, is probably more reliable than that given previously.[18] Measurements carried out at the same time on the same solutions, at 290 mμ, gave Δε 7900, *i.e.*, Δ$E$ (1 μg. xanthine) = 0·052, in reasonable agreement with the value in literature.[25]

*Determination of* Δε *for Indophenol Reactions.*—A concentrated solution of indophenol was standardised by titration with pure ascorbic acid and, after suitable dilution with phosphate buffer, the optical density at 600 mμ was determined. A control value obtained after reducing with sodium dithionite (cf. reaction H/Cy) was subtracted, and the Δε value was then calculated directly, giving Δε 21,000.

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[23] Green and Dewan, *Biochem. J.*, 1937, **31**, 1069.
[24] Lazarow and Cooperstein, *Science*, 1954, **120**, 674.
[25] Krebs and Norris, *Arch. Biochem.*, 1949, **24**, 49.

for metals, and Messrs. Galloway and Barton-Wright have performed the microbiological assay. This investigation and that recorded in Part II were supported by grants to this Institute from the British Empire Cancer Campaign, the Jane Coffin Childs Memorial Fund for Medical Research, the Anna Fuller Fund, and the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service.

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