CCXI. XANTHINE OXIDASE AND MILK FLAVOPROTEIN

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Ball [1938; 1939], in a thorough investigation, isolated a flavoprotein from cow's milk and presented evidence which rendered highly probable its identity with the xanthine-aldehyde oxidase. Corran & Green [1938, 1, 2] simultaneously isolated from the same source a flavoprotein which showed no activity as xanthine-aldehyde oxidase but which could catalyse the oxidation of dihydrocoenzyme I. The recorded properties of the two flavoproteins appeared to be identical except with respect to catalytic activity. The aim of the present investigation was to determine by further purification whether there are two flavoproteins, one catalysing the oxidation of hypoxanthine and aldehyde, the other catalysing the oxidation of dihydrocoenzyme; and if there is in fact only one flavoprotein, to explain why Corran & Green were unable to find any xanthine-oxidase activity in their flavoprotein preparation.

We have succeeded by a new method of preparation in isolating a flavoprotein which acts both as xanthine-aldehyde oxidase and as dihydrocoenzyme I oxidase. By appropriate treatment of the flavoprotein, xanthine-aldehyde oxidase activity can be eliminated completely without any loss of dihydrocoenzyme I activity. Thus the two catalytic activities, although associated with the same flavoprotein, are entirely independent functions of this flavoprotein.

Milk flavoprotein is not simply a protein combined with flavin adenine dinucleotide. In addition to flavin there is some other coloured compound of unknown constitution which accounts for the brownish red appearance and the atypical spectrum of milk flavoprotein. The catalytic role, if any, of this additional coloured group has yet to be clarified. In the absence of this information it is unwise to say definitely that flavin is the prosthetic group of milk flavoprotein in the sense that it is the functional group in the oxidation of hypoxanthine, aldehyde and dihydrocoenzyme I.

(1) *Method of testing catalytic activity*

Xanthine oxidase of milk catalyses the direct oxidation of hypoxanthine or xanthine by molecular O_2 [Morgan *et al.* 1922; Dixon & Thurlow, 1924]. With purification of the enzyme the direct reaction with O_2 becomes erratic—the velocity dropping to zero within a few minutes of the initiation. At the highest purity level the reaction at 38° comes to a stop almost at once. Clearly the manometric method is unsuitable for following the activity of the xanthine oxidase in the course of purification. As regards the other substrates, viz. aldehyde and dihydrocoenzyme I, the direct reaction with O_2 is negligible even in the crudest preparations of the enzyme. Addition of methylene blue as carrier increases the

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rate of reaction enormously but even this expedient fails at the higher purity levels to ensure the reliability of the manometric method. Our experience has been that the anaerobic Thunberg method using methylene blue as hydrogen acceptor offers a more satisfactory method of following the activity of the xanthine oxidase.

We have arbitrarily defined as the unit of xanthine oxidase activity an amount of enzyme that in presence of 1 mg. of hypoxanthine will catalyse the reduction of 0·1 ml. of 0·0113*M* methylene blue in 1 min. The experiments were carried out at 38° in a total volume of 2·5 ml. There is a direct proportionality between enzyme concentration and rate of reduction of methylene blue except at low concentrations of enzyme. In practice we have selected for our tests an amount of enzyme which gave a reduction time not greater than 5 min. and not less than 20 sec. Beyond these limits the measurements became unreliable under the conditions of the experiment.

(2) *Method of preparation*

When dealing with a coloured protein having a characteristic absorption band in the visible spectrum the spectrophotometric method offers a quick and reliable measure of the degree of purification attained by a given procedure. The theory of the method is briefly as follows. The light absorption of a flavoprotein at *ca.* 450 mμ is due to the flavin moiety whereas the absorption of 275 mμ is due to both the flavin and the protein. Any colourless protein impurity will increase the absorption at 275 mμ but not at 450 mμ. Thus the ratio $E_{275\,m\mu}:E_{450\,m\mu}$ becomes a measure of the amount of protein impurity in a preparation of flavoprotein. With purification the ratio becomes smaller and approaches a fixed value characteristic of the homogeneous protein. A decrease in the ratio is evidence that purification has been achieved though it is impossible to estimate accurately the decrease in protein impurity from the decrease in the ratio. The light absorption at 275 mμ is a function of the amino-acid content and it does not follow that the protein impurities absorb equally at this wave-length or even absorb at all. In practice we have found the ratio method to be a reliable index to the progress of purification—agreeing reasonably well with the dry weight method.

The following are the details of the method for preparing the flavoprotein which is associated with xanthine-aldehyde-dihydrocoenzyme I activity and which is *ca.* 1000 times more active per mg. dry weight than an average sample of milk. Some 50 l. of milk have to be processed to ensure adequate material in the final stages of purification.

(1) 57 l. of milk containing 11,000 units were warmed to 30°, saturated with NaCl and filtered through fluted papers (unpublished method of M. Dixon & R. Lemberg). Filtration was complete in 24 hr. The clear yellow filtrate (45 l.) was mixed with an equal volume of $(NH_4)_2SO_4$. The precipitate was filtered through fluted papers (time *ca.* 6 hr.). The precipitate was scraped off and dissolved in 750 ml. *M*/10 phosphate buffer *p*H 7·2—final vol. 1270 ml. containing 2780 units. Allowing for the $(NH_4)_2SO_4$ introduced by the precipitate the solution was made 40% saturated with respect to $(NH_4)_2SO_4$. The precipitate was centrifuged off and redissolved in water—final vol. 820 ml. containing 2300 units. The enzyme solution was deep brown at this stage and somewhat opalescent.

(2) The above solution was dialysed for 2 hr. against running tap water and then submitted to the first $(NH_4)_2SO_4$ fractionation. Enough *M*/2 Na_2HPO_4 was added to bring the *p*H to 7·5 and the enzyme solution was then made 35% saturated with respect to $(NH_4)_2SO_4$ by slow addition of a saturated solution.

The precipitate was discarded. The supernatant fluid was brought to 45% saturation: the precipitate was centrifuged and dissolved in water, final vol. 223 ml. containing 1270 units.

(3) The above solution was dialysed for 2 hr. against running tap water, cooled to 0° and then mixed with 1/5 vol. $M/2$ acetate buffer pH 4·6. Ethyl alcohol was added sufficient to bring the concentration up to 13%. The solution was cooled to −5° and centrifuged at the same temperature. The precipitate was dissolved in $M/100$ phosphate buffer, final vol. 117 ml. containing 1000 units.

(4) The above solution was acidified to pH 5·8 with 10% acetic acid. Alumina Cγ gel containing 15 mg. per ml. was added in five successive lots (25 ml.) each of which was centrifuged separately. The first two lightly coloured lots were discarded and the final more deeply coloured lots were combined, washed with water and eluted with $M/2$ phosphate buffer pH 7·2 until almost colourless. The combined eluates (240 ml.) contained 600 units. The flavoprotein was concentrated by precipitating with 42% saturated $(NH_4)_2SO_4$ and redissolving in 50 ml. water (550 units). To remove the last traces of alumina the solution was spun hard for 30 min. The supernatant fluid was water-clear and deep brownish red.

(5) The above solution was dialysed against running distilled water for 36 hr. at 3°. The slightly coloured precipitate which was formed was discarded. The clear centrifuged solution was brought to pH 7·5 by the addition of $M/10$ Na_2HPO_4 solution and subjected to the second $(NH_4)_2SO_4$ fractionation. Saturated $(NH_4)_2SO_4$ was added dropwise until a definite turbidity formed. After 15 min. the precipitate was centrifuged. In this way four successive fractions were obtained between the limits 35–45% saturation of $(NH_4)_2SO_4$. The first and fourth fractions were the least coloured and were discarded. The second and third fractions were dissolved in water and combined, final vol. 25 ml. containing 300 units. The procedure of dialysis and removal of insoluble material followed by $(NH_4)_2SO_4$ fractionation was repeated twice again before the highest purity level was reached. The third and fourth $(NH_4)_2SO_4$ fractionations were carried out between the narrower limits of 38 and 45% saturation. We have found no rule governing which of the fractions will have the highest purity level. In practice the most deeply coloured fractions were selected and the correctness of the selection checked by determining the ratio $E_{275\,m\mu}:E_{450\,m\mu}$. The final solution (20 ml.) containing 200 units had an extinction ratio of 6·2. The overall yield from the original milk to this stage was $\frac{200}{11{,}000}\times 100$ or 1·8%.

At the extinction ratio 6·2 stage 0·60 mg. of the flavoprotein is equivalent to one enzyme unit. In an average sample of cows' milk 5 ml. containing 624 mg. dry weight are equivalent to one enzyme unit. The degree of concentration relative to milk is therefore $\frac{624}{0{\cdot}60}=1040$. One l. of milk of average activity would contain 120 mg. of the flavoprotein at the 6·2 extinction ratio stage.

Table I summarizes the various procedures used in the method and the degree of purification obtained by each stage. There is considerable variation from one lot of milk to another in the efficiency of the various purification procedures. The values recorded in the table represent the averages of many experiments.

At the purity level represented by extinction ratio 6·2 the flavoprotein is still not homogeneous. Mr J. Philpot discusses the ultracentrifugal data in the Addendum to this communication. The flavoprotein component accounts for 80 to 83% of the total sedimenting material in the preparation. Repetition of $(NH_4)_2SO_4$ fractionation failed to separate the flavoprotein from the persistent

Table I. *Summary of purification procedures*

Stage	Volume ml.	Enzyme units	$E_{275\,m\mu}:E_{450\,m\mu}$
Milk	57,000	11,000	—
$(NH_4)_2SO_4$ precipitate of NaCl filtrate	1,270	2,780	—
After 1st $(NH_4)_2SO_4$ fractionation	223	1,270	50
After alcohol precipitation	117	1,000	25
After alumina adsorption	50	550	15
After 2nd $(NH_4)_2SO_4$ fractionation	25	300	10
After 4th $(NH_4)_2SO_4$ fractionation	20	200	6·2

impurity referred to as the γ component in the photograph of the ultracentrifuge run. A selective reagent has yet to be found for the final stages of the purification.

In general the xanthine-aldehyde oxidase activity is strictly proportional to the flavoprotein concentration. The ratio (xanthine-aldehyde activity : flavoprotein concentration) remains constant from the first clear extract which can be assayed to the final stage. However, we have observed particularly in the summer months that the ratio falls off in the early stages of the purification. For example, a neutral dilute salt solution of the flavoprotein at the end of stage 1 of the purification lost 50 % of its xanthine-aldehyde activity after storage for 12 hr. at 0° although the concentration of the flavoprotein remained unaltered. High salt concentrations and low temperatures (−10°) retard this inactivation but we have failed to find any method of stopping it apart from rapid manipulation. The phenomenon was not observed during the autumn and winter months. The inactivating agent appears to be removed after the alumina stage. Flavoprotein preparations of ratio $E_{275\,m\mu}:E_{450\,m\mu}=12$ or less are stable at room temperature in presence of 5–10 % $(NH_4)_2SO_4$ or at 0° in absence of salts. Capryl alcohol can be used as a preservative without any effect on the enzyme.

(3) *Absorption spectrum*

Fig. 1*a* shows the absorption spectrum of a solution of milk flavoprotein at the extinction ratio 6·2 stage. There are maxima at 275, 350 and at 450–453 mμ. The same solution and its control were treated with hydrosulphite. The spectrum recorded after reduction is shown on Fig. 1*b*.

The dry weight[1] of the solution was 2 mg. per ml. On the assumptions (1) that all the absorption at 450 mμ is due to the flavin group, and (2) that the absorption coefficient $\beta=2{\cdot}4\times10^7$ as for other flavoproteins then the lactoflavinphosphate content of this preparation should be 0·77 %. If the first of the above assumptions were correct we should expect that addition of hydrosulphite would completely eliminate absorption at this wave-length. In fact the hydrosulphite reduced solutions of flavoprotein show about 40 % of the absorption of the oxidized form at 450 mμ. It would seem, then, that not more than 60 % of the absorption at 450 mμ can be due to flavin.

The flavin moiety of milk flavoprotein can replace the flavin adenine dinucleotide coenzyme of the *d*-amino-acid oxidase [Ball, 1939; Corran & Green, 1938, 2]. The method of estimating the dinucleotide by the *d*-amino-acid oxidase test has already been described by Corran *et al.* [1939]. By this method the

[1] The dry weight estimations were carried out as follows. Samples of the test solution which had been dialysed for 5 days against distilled water were frozen and dried in high vacuum over H_2SO_4 for 48 hr. The dry weight was determined in triplicate after moisture equilibrium had been reached. Drying at 100° *in vacuo* led to an 8 % loss in weight. There was also an ash residue of 4·5 % after combustion (Weiler, Oxford). The dry weight was corrected for residue ash and moisture.

amount of dinucleotide present in any given solution of flavoprotein can be determined. The flavin group was split off from the protein by exposing the solution to $N/6$ HCl for 5 min. at room temperature. The solution was then rapidly neutralized by the addition of solid Na_2HPO_4. The flavin concentration as

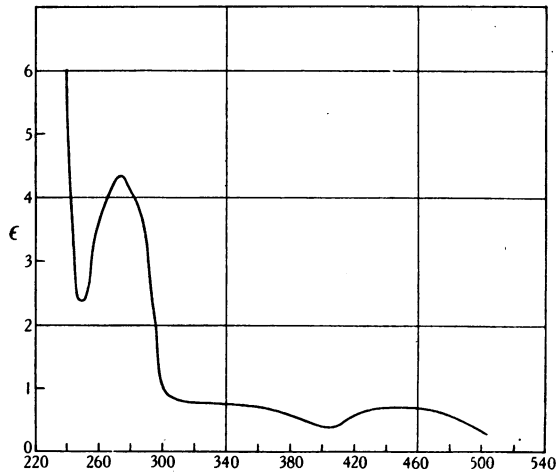

Fig. 1 *a*.

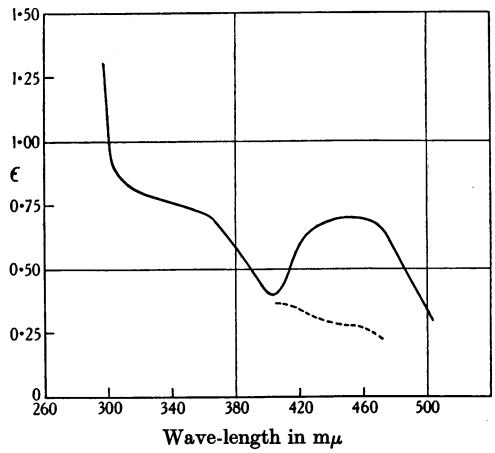

Fig. 1 *b*.

Fig. 1. (*a*) Absorption spectrum of 0·2% milk flavoprotein solution (0·77% apparent flavin-phosphate). (*b*) Comparison of oxidized (——) and hydrosulphite-reduced (- - - -) flavoprotein (0·2%).

determined by the O_2 uptake in the *d*-amino-acid oxidase test system was compared with that calculated on the basis that all the absorption at 450 mμ was due to flavin. The results of several experiments are shown in Table II. A maximum figure of 35% was obtained for the ratio

$$\frac{\text{flavin determined as flavindinucleotide}}{\text{flavin on basis of total absorption at 450 m}\mu}.$$

Table II

Stage	μg. apparent flavinphosphate/ml. as determined by height of 450 mμ band	μg. flavinphosphate/ml. as determined in *d*-amino-acid test	$\frac{\text{actual flavin}}{\text{apparent flavin}} \times 100$
(i) After 1st $(NH_4)_2SO_4$ fractionation	0·53	0·14	26%
(ii) After alcohol precipitation	0·55	0·18	33%
(iii) After alumina adsorption	0·66	0·23	35%
(iv) After 2nd $(NH_4)_2SO_4$ fractionation	0·56	0·17	31%
(v) Final preparation	0·61	0·21	35%

By varying the conditions for liberating the flavin group the yields were for the most part lower but never higher than 35%.

The amount of flavin split off from the denatured flavoprotein can also be estimated spectrophotometrically. This requires rather more concentrated solutions, and again the conditions under which denaturation takes place determine the yield of flavin. Splitting in dilute HCl under the conditions described above gave the highest recovery of flavin. For example, 3 ml. of a solution of flavoprotein containing 51·6 μg. apparent flavinphosphate per ml. were mixed with 1·2 ml. saturated $(NH_4)_2SO_4$ and 0·6 ml. *N* HCl. After 5 min. the precipitate was centrifuged off and the supernatant fluid neutralized with solid Na_2HPO_4. The slight colourless precipitate which formed was centrifuged off and a clear yellow solution was thus obtained. After estimating spectrophotometrically the amount of flavinphosphate present from the height of the 450 mμ band and allowing for dilution, the flavinphosphate content of the original solution was calculated to be 18·3 μg. per ml. or 35% of that expected on the basis of the 450 mμ absorption of the flavoprotein. Thus the two independent methods of estimating flavin are in good agreement that flavin accounts for about 35% of the total light absorption of flavoprotein at 450 mμ. On the other hand, 60% of the light absorption is abolished by addition of hydrosulphite indicating that another 25% of the total absorption, while not apparently due to flavin, is eliminated by reduction with hydrosulphite. In addition the hydrosulphite-reduced flavoprotein solution still contains some group absorbing 40% as much as the oxidized compound.

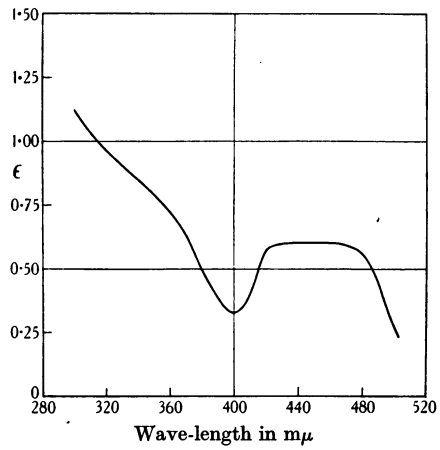

Fig. 2. Calculated absorption spectrum of non-flavin coloured group of milk flavoprotein (0·264%).

Previously Corran & Green [1938, 2] estimated that flavin accounted for 80% of the total light absorption of the flavoprotein at 450 mμ. This estimate has now been shown to be incorrect.

The available data allow the calculation of the approximate visible spectrum of the non-flavin group in milk flavoprotein (cf. Fig. 2). This was arrived at by subtracting the absorption of the flavin moiety from that of the flavoprotein. The band in the visible region shows a broad peak between 440 and 460 mμ.

(4) *Properties of the flavin group*

We have used two methods for the isolation and study of the flavin group; the first involves splitting off the flavin from a highly purified sample of the flavoprotein; the second involves splitting the flavoprotein at a very crude stage. By the first method a few procedures suffice to reach the practically pure flavin but then the scarcity of starting material makes it difficult to obtain sufficient material for chemical investigations. The second method is more elaborate but it has the virtue of making the isolation less expensive.

(1) A sample of milk flavoprotein at the stage of extinction ratio 15 was heated at 70° for 5 min. in presence of 15% $(NH_4)_2SO_4$ and dilute acetic acid (*p*H 3·8). After filtration the protein-free solution was saturated with $(NH_4)_2SO_4$ and extracted at *ca.* 70° with molten phenol. Water was added and the phenol was extracted with ether. The spectrum of the flavin thus obtained is shown in Fig. 3. A comparison of the relative heights of the three absorption bands at

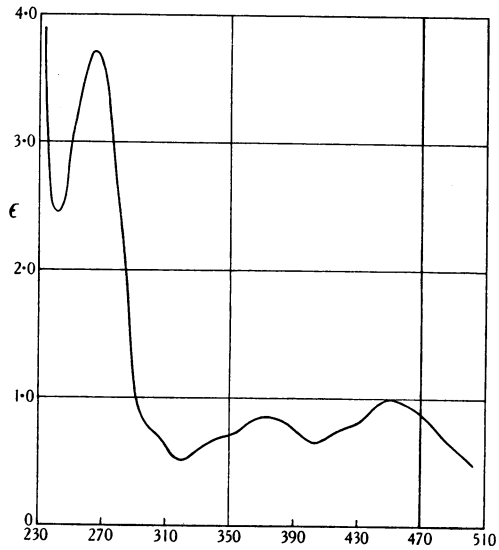

Fig. 3. Absorption spectrum of liberated flavin group of milk flavoprotein.

265, 370 and 450 mμ respectively with those for flavin adenine dinucleotide of yeast [Warburg & Christian, 1938] is given below.

λ in mμ	Milk flavin	Yeast flavin
265	3·7	3·2
370	0·85	0·8
450	1·0	1·0

Ball [1939] found even closer agreement between the two respective spectra.

We have shown previously that the flavin can act as the coenzyme of the *d*-amino-acid oxidase. Comparison of the milk and yeast flavins shows that they have the same activity within the limits of experimental error.

	0·5 μg. flavinphosphate	
	Milk	Yeast
μl. O_2 per 10 min. in test system	68	76
	75	70

(2) Whey powder (cf. Corran & Green [1938, 2] for preparation) was shaken mechanically for 2 hr. with 75 % methanol. The insoluble protein was filtered off and the methanol removed by distillation *in vacuo*. The aqueous solution was saturated with $(NH_4)_2SO_4$ and heated to 80°. The heavy protein precipitate was filtered off. The filtrate was extracted with molten phenol at 70°. Water was added to the separated phenol phase and the phenol extracted with ether. The aqueous solution was acidified to *p*H 3 with nitric acid and a large excess of $AgNO_3$ added. The silver salt was decomposed with H_2S and the AgS filtered off. The flavin solution was then frozen and thawed. This procedure aggregated colloidal AgS which defied the first filtration.

The following are typical results carried out on the above flavin solution:

	Found	Theory for dinucleotide on the basis of flavin
Flavin	$0{\cdot}82 \times 10^{-7}$ *M*	—
Phosphorus	$1{\cdot}78 \times 10^{-7}$ *M*	$1{\cdot}64 \times 10^{-7}$ *M*
Nitrogen	$7{\cdot}4 \times 10^{-7}$ *M*	$7{\cdot}4 \times 10^{-7}$ *M*

Flavin was estimated spectrophotometrically assuming that the absorption coefficient $\beta = 2{\cdot}6 \times 10^7$ cm.^{2} per mole; phosphorus was estimated by the method of Kuttner & Lichtenstein [1932]. Miss V. Rogers kindly carried out the N estimations by the ultra-micro-Kjeldahl method of Needham & Boell [1939].

The orcin colorimetric method for the estimation of sugar [cf. Pirie, 1936] was used to demonstrate that carbohydrate was present in milk flavin in approximately the correct order of magnitude (80 %). Adenine was tested for as uric acid by conversion into hypoxanthine with nitrous acid, and then oxidation to uric acid by molecular oxygen in presence of the xanthine oxidase (unpublished method of Mr R. Markham). About 80 % of the theory for adenine was found.

Although the chemical analyses by themselves are not conclusive, yet, taken in conjunction with the close similarity of milk and yeast flavins with respect to spectrum and catalytic activity they leave little doubt of the identity of the two compounds.

Various attempts were made to split off flavin reversibly from the flavoprotein. Dialysis of the flavoprotein solution for long periods against dilute acetic acid and dilute HCl at 0° led to the removal of the flavin without apparent denaturation of the protein. But it was not found possible to produce an active enzyme by adding milk dinucleotide to the flavin-free protein. Negative results were also obtained by the application at low temperatures of the HCl-$(NH_4)_2SO_4$ method of Warburg & Christian [1938] used with such striking success in the splitting of the *d*-amino-acid oxidase and yeast flavoprotein. Ball [1939] has succeeded in the reversible resolution of milk flavoprotein by prolonged dialysis against distilled water at 0°. This method has failed in our hands. In fact we have consistently used the dialysis procedure as a successful means of purifying milk flavoprotein in the final stages.

(5) *Catalytic properties*

Milk flavoprotein catalyses the oxidation of hypoxanthine, aldehyde and dihydrocoenzyme I. A summary of the catalytic constants at the stage of extinction ratio 6·2 is given in Table III. The three catalytic activities are of the same order of magnitude. The concentrations of hypoxanthine and aldehyde used were sufficient to saturate the enzyme, thus ensuring maximal activity in each case. The problem of saturating the enzyme in respect to dihydrocoenzyme I is somewhat complicated and the reader is referred to Corran & Green [1938, 2] for the conditions under which maximal activity is reached. Crude preparations of the

Table III. *Summary of catalytic properties*

	Hypoxanthine	Acetaldehyde	Dihydrocoenzyme I
Methylene blue reduction time	1·12 min.(1)	0·60 min.(2)	1·33 min.(3)
Relative activities (hypoxanthine =100)	100	186	85
Turnover number	306	570	260
Q_{MB}* per mg. flavinphosphate	9×10^5	$1{\cdot}67 \times 10^6$	$7{\cdot}6 \times 10^5$
Q_{MB} per mg. protein	2420	3500	2060

(1) 1 ml. buffer (7·2), 0·1 ml. 0·0113*M* methylene blue, 0·2 ml. 0·5% hypoxanthine and 0·3 ml. of a flavoprotein containing 5·35 μg. actual flavinphosphate/ml. (15·3 μg. apparent flavinphosphate).
(2) Details as for (1) except 0·2 ml. *M* acetaldehyde in place of hypoxanthine.
(3) 1 ml. lactic enzyme, 1 ml. 2% coenzyme I, 0·2 ml. 2*M* HCN, 0·1 ml. 0·0113*M* methylene blue, 0·2 ml. *M* lactate and 0·3 ml. of flavoprotein solution as in (1).
* Q_{MB} = μl H_2 transferred to methylene blue per hr. per mg. dry weight.

xanthine oxidase in presence of hypoxanthine react at the same speed with methylene blue as with O_2 [Green & Dixon, 1934]. The Q_{MB} (μl H_2 transferred to methylene blue per hour per mg. dry weight) can therefore be considered to be roughly equivalent to the Q_{O_2}. The difficulties of making aerobic measurements with highly purified flavoprotein preparations were explained in Section 1.

(6) *The association of the three catalytic activities with the flavoprotein*

All the available evidence has been consistent with the view that our milk preparations contain only one flavoprotein at all stages of purification. The possibility, however, cannot be excluded that our flavoprotein preparations are mixtures of two or three flavoproteins of similar molecular weight, spectrum and chemical properties and that this mixture cannot be resolved by the procedures used. The possibility is indeed remote but it cannot be dismissed until more precise and varied criteria of the homogeneity of proteins become available. For ease of presentation, however, we shall assume that we are dealing with one flavoprotein.

Table IV. *Proportionality between hypoxanthine activity and apparent flavinphosphate content at different stages of purity*

Stage	1 Hypoxanthine activity (units/ml.)	2 Apparent flavin-phosphate (μg./ml.)	2/1
1st $(NH_4)_2SO_4$ fractionation	5·2	26·4	5·1
After alcohol precipitation	5·8	27·4	4·7
After 1st alumina adsorption	4·0	16·4	4·1
After 2nd alumina adsorption	3·2	14·2	4·4
Ratio 8·3	4·3	22	5·1
Ratio 7·4	8·8	41·6	4·7
Ratio 6·4	5·0	22	4·4
Ratio 6·2	3·3	15·3	4·6
		Average	4·6

Table IV shows the proportionality between the enzyme units (hypoxanthine) and the apparent flavinphosphate content of various flavoprotein preparations. A strict proportionality exists at all stages of purification. If the three activities are associated with the same flavoprotein we should expect the activity ratio, hypoxanthine : aldehyde : dihydrocoenzyme I to be identical at all stages. Such has been found to be the case. Dixon [1939] and Booth [1935] have already established that xanthine oxidase of milk is a xanthine-aldehyde oxidase. Ball

[1939] in turn has identified the xanthine-aldehyde oxidase with milk flavoprotein. Finally our results identify the xanthine-aldehyde and the dihydrocoenzyme oxidase with the same flavoprotein.

The three catalytic activities, although apparently associated with the same flavoprotein, are not invariant functions of the flavoprotein. Preparations which have been dried or treated with cyanide lose activity towards hypoxanthine and aldehyde but are still active towards dihydrocoenzyme I. It is significant that hypoxanthine and aldehyde activities always run parallel. No procedure has yet been devised which will eliminate dihydrocoenzyme I activity without affecting either xanthine or aldehyde activity. The hypoxanthine-aldehyde function is fragile compared with that of dihydrocoenzyme, and the two functions clearly must involve different active groups in the protein part of the molecule.

Inactivation by drying. A sample of milk flavoprotein at the stage of extinction ratio 7·0 was frozen and dried in high vacuum over H_2SO_4. Tests of the redissolved flavoprotein showed that 75 % of the activity towards hypoxanthine was lost. No apparent difference was observed in the spectrum of the flavoprotein or in its solubility. The inactivation was not, therefore, the result of any gross alteration in the flavoprotein molecule.

At lower stages of purity, inactivation was a slower process. For example, at the stage of extinction ratio 50, drying for 3 days under the above conditions brought about only a 50 % loss in hypoxanthine activity although dihydrocoenzyme I activity was unaffected. Dried whey powder retained xanthine-aldehyde activity for a period of months. But here again deterioration was taking place though at a slower rate. For example, a whey powder which had stood for 4 months yielded a flavoprotein which was completely inactive towards hypoxanthine and aldehyde though still active towards dihydrocoenzyme I. This inactivation by drying took place equally well in presence or in absence of salts.

We have repeated the isolation of milk flavoprotein by the original method of Corran & Green [1938, 2] with a view to determining the exact stages at which xanthine-aldehyde activity was lost. The following results were obtained:

Stage	Enzyme units (hypoxanthine)
1st dried preparation (whey powder)	620
2nd dried preparation	90
After lead treatment	0

There was about a 30 % loss of flavoprotein in proceeding from the first to the second dried preparations whereas the loss in hypoxanthine activity was 85 %. After the lead treatment the flavoprotein present was completely inactive towards hypoxanthine.

Inactivation by cyanide. Dixon & Keilin [1936] have shown that xanthine oxidase when incubated with cyanide becomes irreversibly inactivated. We have utilized this effect as a tool for the rapid elimination of xanthine-aldehyde activity without affecting dihydrocoenzyme I activity.

A preparation of the flavoprotein at the stage of extinction ratio 15 was made $M/10$ with respect to HCN. After 10 min. the flavoprotein was precipitated with half-saturated $(NH_4)_2SO_4$. The precipitate was washed with a large volume of half-saturated $(NH_4)_2SO_4$ solution and then dissolved in phosphate buffer. The ratio, hypoxanthine activity : dihydrocoenzyme I activity was found to be 1·0 : 2·6 instead of the usual ratio of 1·0 : 0·85. The above procedure was then repeated. After this treatment no more hypoxanthine-aldehyde activity was present although dihydrocoenzyme I activity was unimpaired and the spectrum and chemical properties of the flavoprotein were not noticeably altered.

(7) *Mechanism of reaction*

The flavoproteins hitherto described in the literature are known to catalyse the oxidation of their respective substrates by undergoing a cycle of reduction by the substrate and then oxidation by O_2 or some other oxidizing agent. This cycle concerns only the flavin part of the molecule and hence flavin is regarded as the prosthetic group. By analogy we should expect a similar mechanism to apply to milk flavoprotein. The analogy however is not complete. Whereas the other flavoproteins contain only one coloured prosthetic group, viz. flavin, milk flavoprotein contains some other coloured grouping in addition to flavin which may be concerned in the catalytic role of the enzyme. Furthermore, milk flavoprotein contains 2–3 mol. of flavin per mol. of protein whereas the ratio is 1 : 1 for the other flavoproteins. Finally, milk flavoprotein catalyses three distinct oxidations in contrast to the one specific reaction catalysed by the other flavoproteins. These differences in properties do not exclude a mechanism involving cyclical reduction and oxidation of the flavin groups but they weaken the force of analogy and call for caution in the interpretation of results.

A strong solution of milk flavoprotein is orange-red in colour. On addition of hydrosulphite the colour is practically bleached except for a residual pale brown colour. It is interesting to note that although to the eye the colour has largely disappeared yet spectrophotometric estimation discloses 40 % of the original light absorption at 450 mμ. On addition of hypoxanthine or aldehyde to the flavoprotein under anaerobic conditions this colour change does not take place at once. After incubation at 38° for 15 min. there is a slight but definite diminution of colour compared with the untreated control. The paling becomes more accentuated with time. After some hours' incubation the flavoprotein solution takes on the appearance of the hydrosulphite-reduced control. Admission of air restores the original colour in the tubes containing either hypoxanthine or hydrosulphite as reductant for the flavoprotein. These experiments show that the flavin groups are slowly reduced by hypoxanthine and aldehyde, and that reduced flavin is autoxidizable in air. The rate, however, is some thousand times slower than would be expected if the flavin groups were reduced and oxidized in the course of catalytic activity. The following experiment clearly demonstrated this discrepancy in velocity: 1 ml. of a solution of the flavoprotein containing 35 μg. apparent flavinphosphate per mol., i.e. 12 μg. actual flavinphosphate was mixed with 1 mg. hypoxanthine under anaerobic conditions. About 2 hr. were required before the complete bleaching of the flavin colour was pronounced. A parallel test showed that 1 ml. of the flavoprotein solution in presence of hypoxanthine catalysed the reduction of 0·1 ml. of 0·0113*M* methylene blue in 7 sec. Assuming that the reduction of methylene blue by hypoxanthine involves a cycle of the flavin groups the ratio (μl. H_2 transferred to methylene blue : μl. H_2 equivalent of flavoprotein) should give the number of times each flavin group would have to be reduced and oxidized in 1 min. The ratio turns out to be 306. It follows, therefore, that under anaerobic conditions the flavin groups should have been reduced in *ca.* 0·2 sec. The observed time was more like 2 hr.

The possibility arises that the substrate reduces a small part of the flavin groups at once and that the slow reduction of the bulk of the flavin groups has nothing to do with the catalysis. To test this hypothesis it would be necessary to make rapid spectrophotometric measurements of the flavoprotein immediately after mixing with the substrate under anaerobic conditions. Unfortunately we have been unable to carry out such an experiment. Measurements made within 10 min. of mixing invariably show partial reduction of the flavo-

protein though the degree of reduction observed varies from one preparation to another.

To summarize we may say that none of the three substrates can rapidly bleach the flavoprotein to completion. No decision has been reached as to whether there is an instantaneous partial reduction of the flavoprotein by the three substrates. Furthermore, there does not appear to be summation of the substrates with respect either to the degree or velocity of reduction. The interpretation of these results must await more knowledge of the nature of the additional coloured grouping in the molecule. The hypothesis of a flavin cycle is neither excluded nor supported by our experiments.

Ball [1939] has reported that both hypoxanthine and aldehyde reduce milk flavoprotein rapidly and almost as completely as hydrosulphite. This observation is difficult to explain except on the basis that the flavoprotein isolated by our method is a mixture of flavoproteins only a small proportion of which is represented by the xanthine-oxidase flavoprotein whereas that of Ball's consists principally or entirely of xanthine-oxidase flavoprotein. However, comparison of the catalytic activities of the flavoproteins isolated by Ball's and our methods does not support this possibility.

(8) *Liver xanthine oxidase*

The isolation of the xanthine oxidase offered an independent method of confirming the conclusions reached with the milk enzyme. We have succeeded in purifying the liver enzyme to a point where the flavoprotein colour in the preparation can be seen directly, but as yet coloured impurities have not been sufficiently removed to permit satisfactory spectroscopic measurements.

The following is a brief résumé of the method of isolation. Minced pig liver was mixed with 3 vol. water and 0·4 vol. saturated $(NH_4)_2SO_4$ at *p*H 4·6. The insoluble material was discarded by centrifuging; the supernatant fluid was brought to 60% saturation of $(NH_4)_2SO_4$. The precipitate was filtered and resuspended in water—insoluble material being discarded by centrifuging. The dialysed solution was acidified to *p*H 4·6 at 0° and in presence of 16% alcohol. The precipitate was dissolved in buffer at *p*H 7·2 and some inactive protein removed by heat-coagulation at 56°. The solution was finally fractionated between the limits of 45 and 60% saturation of $(NH_4)_2SO_4$.

The final solution was strong yellow in colour. Only part of the colour was bleached by hydrosulphite. Shaking with air restored the original depth of colour. The presence of a flavoprotein in the preparation containing bound flavin adenine dinucleotide was also demonstrated by splitting off the flavin group and observing a significant O_2 uptake in the *d*-amino-acid oxidase test system.

The above preparation of liver flavoprotein catalysed the oxidation of hypoxanthine, aldehyde and dihydrocoenzyme I:

	Relative activities	
	Liver	Milk
Hypoxanthine	100	100
Aldehyde	28	186
Dihydrocoenzyme I	56	85

The ratio of the three activities was not the same as for milk. Aldehyde had 0·28 the activity of hypoxanthine in the case of the liver enzyme and 1·86 the activity of hypoxanthine in the case of the milk enzyme. The significant fact, however, is that all three catalytic activities are associated with both the liver and the milk flavoproteins.

The highest Q_{MB} reached with the xanthine oxidase of liver was 400 as compared with 2400 for the best preparation of milk enzyme. The degree of concentration relative to the original liver was *ca.* 200. Work on the purification is still in progress.

Pig liver contains an aldehyde oxidase in addition to the hypoxanthine-aldehyde oxidase. This enzyme, however, was removed early in the purification and could not account for the aldehyde activity observed in the purified flavoprotein preparation.

The xanthine oxidase of liver was extremely unstable at all stages of purification. Only by rapid manipulation was it possible to reach the higher stages of purity without excessive loss in activity.

Summary

A method is described for preparing a flavoprotein which catalyses the oxidation of hypoxanthine, aldehydes and dihydrocoenzyme I, and is *ca.* 1000 times more active per mg. dry weight than milk. The three catalytic activities although associated with the same flavoprotein can be differentially inactivated. Drying and incubation with cyanide abolish hypoxanthine-aldehyde activity without affecting either dihydrocoenzyme I activity or the spectrum and chemical properties of the flavoprotein.

The flavin moiety has been shown to be very similar to if not identical with flavinadenine dinucleotide. Flavin accounts for only 35 % of the total absorption at 450 mμ. Evidence is presented for the existence of a non-flavin coloured group in the molecule. The catalytic role of this additional group has yet to be clarified. No direct evidence has been obtained that the flavin groups undergo a cycle first of reduction by the three substrates and then of oxidation by some oxidizing agents.

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REFERENCES

Ball (1938). *Science*, **88**, 131.
—— (1939). *J. biol. Chem.* **128**, 51.
Booth (1935). *Biochem. J.* **29**, 1732.
Corran & Green (1938, 1). *Nature, Lond.*, **142**, 149.
—— —— (1938, 2). *Biochem. J.* **32**, 2231.
—— —— & Straub (1939). *Biochem. J.* **33**, 793.
Dixon (1939). *Enzymologia*, **5**, 198.
—— & Thurlow (1924). *Biochem. J.* **18**, 976.
—— & Keilin (1936). *Proc. roy. Soc.* B, **119**, 159.
Green & Dixon (1934). *Biochem. J.* **28**, 237.
Kuttner & Lichtenstein (1932). *J. biol. Chem.* **95**, 661.
Morgan, Stewart & Hopkins (1922). *Proc. roy. Soc.* B, **94**, 109.
Needham & Boell (1939). *Biochem. J.* **33**, 149.
Pirie (1936). *Brit. J. exp. Path.* **16**, 497.
Warburg & Christian (1938). *Biochem. Z.* **298**, 368.

ADDENDUM: EXAMINATION IN THE ULTRACENTRIFUGE

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The new specimen of milk flavoprotein described above was ultracentrifuged at 54,000 r.p.m. in a 12 mm. cell at a concentration of approximately 0·5% (as judged from the refractive increment 0·00097).

Fig. 4 shows a photograph by the "diagonal schlieren" method. Three components are present, with $S_{20} \times 10^{13}$ equal to 6·8, 12·6 and 19·2. Comparison with the previous results [Philpot, 1938, 1] shows that the first two components correspond with the ones formerly labelled "γ" and "δ", while the third component "ϵ" is a new one peculiar to the latest specimen. The "δ" component, which is the flavoprotein itself, is now for the first time the most prominent. Integration shows that it accounts for 68% of the total refractive increment, or 81% of the observably sedimenting material. The "γ" and "ϵ" components account for 11 and 5%, respectively, of the total refractive increment, while the remaining 16% is due to uncentrifugable or highly polydisperse material. These figures are all ± about 5%.

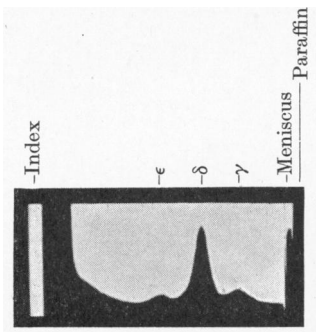

Fig. 4.

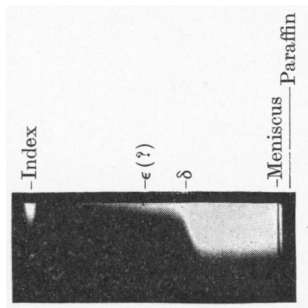

Fig. 5.

Fig. 5 shows a photograph taken during the same run by a self-plotting absorption method. The optical system is identical with that of the diagonal schlieren method [Philpot, 1938, 2]; but the diagonal edge is set vertical, to let all the light through, and an Ilford carbon-gelatin wedge, with horizontal gradient, is placed in front of the photographic plate. With monochromatic light of suitable wave-length any line of constant intensity plots concentration against distance from the meniscus. With imperfectly monochromatic light a wedge cell containing the protein is preferable to the carbon wedge, but this requires a lot of material.

The prominent S-shaped step in Fig. 5 shows that most of the light absorption at 475 mμ, the wave-length used, is due to a single component, whose sedimentation constant is $12{\cdot}6 \times 10^{-13}$, and which is therefore the "δ" component. On close inspection it is possible to see that 5–10% of the light absorption is caused by faster-moving material with sedimentation constant about 17×10^{-13}. This

may mean that the "ϵ" component of Fig. 4 is coloured; but the accuracy of the absorption method is insufficient to give any certainty. If the "ϵ" component were coloured it could be a twofold aggregate of the main "δ" component. An attempt to dissociate it with 0·01*M* hexametaphosphate was, however, unsuccessful. None of the coloured material is uncentrifugable.

If the "δ" component forms 60–80 % of the material and contains 90–100 % of the flavin, if the overall percentage of flavinphosphate is 0·27 % as found above, and if the molecular weight is 220,000–320,000, then there must be 1·4–3·1 flavin groups per molecule. The previous conclusion that there were eight groups per molecule was based on a higher flavin percentage which has since been revised.

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REFERENCES

Philpot (1938, 1). *Biochem. J.* **32**, 2240.
—— (1938, 2). *Nature, Lond.*, **141**, 283.