CCLXXXVII. THE ISOLATION OF A FLAVOPROTEIN FROM COWS' MILK

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(*Received 1 November 1938*)

The starting-point of this investigation was the observation that concentrated solutions of the xanthine oxidase of milk were characterized by a pronounced orange coloration. We have been able to isolate a flavoprotein compound which accounts for some though not all of this colour. There are, very likely, other flavoprotein compounds in milk besides the one to be described.

I. *Isolation*

(1) Fresh untreated cows' milk is warmed to 35° and treated with rennet as described by Dixon & Kodama [1926]. The curd is broken up and filtered through muslin. The filtrate is mixed with 1·1 vol. of sat. $(NH_4)_2SO_4$. The precipitate rises to form a sharp layer, and the fluid underneath is sucked off and discarded. The layer of precipitate is filtered with suction on Büchner funnels. The well-packed cake of precipitate is dried *in vacuo* over H_2SO_4 and the fat is extracted with ether. The yield is *ca.* 700 g. from 100 l. of milk. The powder keeps indefinitely when stored dry.

(2) A 10 % solution of the powder is cooled to 0° and mixed with 0·56 vol. of sat. $(NH_4)_2SO_4$. Glacial acetic acid is added with vigorous stirring until the *p*H is 4·0 (just yellow to bromocresol green). The precipitate is centrifuged, and mixed with water equal in volume to the discarded supernatant fluid. After neutralizing with NaOH, the total volume is measured and the degree of saturation of the solution with respect to $(NH_4)_2SO_4$ is calculated on the assumption that the cake of precipitate is 36 % saturated. 0·1 vol. of ethyl alcohol is added with vigorous stirring followed by sufficient sat. $(NH_4)_2SO_4$ to bring the degree of saturation to 25 %. After acidification with glacial acetic to *p*H 4·0 the precipitate is centrifuged and discarded. The supernatant fluid is made 50 % saturated with $(NH_4)_2SO_4$ and the precipitate filtered with suction and dried *in vacuo*. The yield is *ca.* 200 g. from 100 l. of milk.

(3) A 5 % solution of the above powder is dialysed against running tap water for 12 hr. and mixed with 0·05 vol. of 25 % basic lead acetate solution. The bulky precipitate is rapidly centrifuged and discarded. The relatively clear supernatant fluid is mixed with an equal volume of sat. $(NH_4)_2SO_4$. The centrifuged precipitate, which contains $PbSO_4$ and protein, is resuspended in 5 vol. of water. After neutralizing with NaOH, the suspension is centrifuged. The supernatant fluid which is retained has a pronounced orange colour. After dialysis against running tap water until free of $(NH_4)_2SO_4$, the solution is mixed with an equal volume of ethyleneglycol monoethyl ether and the *p*H adjusted to 3·8 by addition of glacial acetic acid. The precipitate is centrifuged and redissolved in the minimum quantity of *N*/100 NaOH. The solution is dialysed against running tap water and finally

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against distilled water until free of the reagent. The yield at this stage is about 2 g. from 100 l. of milk.

(4) At 38 % saturation of $(NH_4)_2SO_4$ and *p*H 7·0 only part of the flavoprotein is precipitated together with a large amount of colourless material. The precipitate is discarded. The supernatant fluid is made 50 % saturated with respect to $(NH_4)_2SO_4$ and centrifuged. The above procedure is repeated twice. The orange-red precipitate is dissolved in the minimum volume of *N*/100 NaOH and the solution dialysed against distilled water until salt-free. The yield is *ca.* 0·25 g. from 100 l. of milk.

(5) Milk flavoprotein is readily adsorbed on alumina Cγ at *p*H 6–7 and can be eluted from the adsorbing agent with a mixture of $(NH_4)_2HPO_4$ and NH_3 according to the method of Weygand & Stocker [1937]. This method of purification is only successful after extensive concentration of milk flavoprotein. The minimum quantity of alumina necessary to adsorb flavoprotein completely from a 0·5 % solution is used and this quantity must be determined experimentally for each preparation. Two elutions are sufficient for extraction of at least 90 % of the adsorbed flavoprotein. The combined eluates are dialysed against distilled water. After two adsorptions the yield is *ca.* 0·12 g. from 100 l. of milk.

Table I contains a summary of the scheme of purification and of the degree of concentration at the various stages. Assuming a 95 % loss in the purification

Table I. *Preparation of milk flavoprotein*

	Flavoprotein present in
Milk	
↓	
Rennin treatment, 35°	Filtrate
↓	
50 % sat. $(NH_4)_2SO_4$	Precipitate
↓	
Dried and extracted with ether	Precipitate
↓	
38 % sat. $(NH_4)_2SO_4$ *p*H 4·0 and 0°	Precipitate
↓	
25 % sat. $(NH_4)_2SO_4$ *p*H 4·0 and in 10 % alcohol	Filtrate
↓	
50 % sat. $(NH_4)_2SO_4$	Precipitate
↓	
Precipitate dried and dialysed (0·01 % flavinphosphate)	—
↓	
5 % solution treated with 0·05 vol. 25 % basic lead acetate	Filtrate
↓	
50 % sat. $(NH_4)_2SO_4$	Precipitate
↓	
Precipitate redissolved and dialysed after centrifuging off $PbSO_4$	Filtrate
↓	
Treated with equal volume ethyleneglycol monoethyl ether (0·1 % flavinphosphate)	Precipitate
↓	
Redissolved precipitate dialysed and 38 % sat. $(NH_4)_2SO_4$ at *p*H 7·0	Filtrate
↓	
50 % sat. $(NH_4)_2SO_4$ (0·2 % flavinphosphate)	Precipitate
↓	
Repeat twice the 38 and 50 % sat. $(NH_4)_2SO_4$ procedures (0·35 % flavinphosphate)	—
↓	
After dialysis, adsorb on alumina Cγ and elute twice with $(NH_4)_2HPO_4$ and NH_3. Dialyse. Repeat process (0·53 % flavinphosphate)	—

there should be 2·4 g. of flavoprotein in 100 l. of milk. Since each litre contains 120 g. dry weight, the degree of concentration at the 0·53 % flavinphosphate stage should be 12,000/2·4 = 5000. It is difficult for various reasons to determine directly the initial concentration of flavoprotein in milk. Most important is the fact that fresh milk and the whey powder contain an enzyme which inactivates coenzyme I. Since the presence of flavoprotein at these stages can only be detected by a catalytic test, involving the use of coenzyme I, obviously no accurate estimation of concentration is possible. In addition to this complication, there is the fact that the concentration of flavoprotein in milk lies within the limits of error of the method of detection. The estimation of the loss in the purification process, particularly in the early stages, is approximate and merely gives order of magnitude. Stages 1 and 2 together involve about a 30 % loss mainly due to the bulkiness of the precipitate at 25 % saturation of $(NH_4)_2SO_4$ and *p*H 4·0. Stages 3, 4 and 5 involve losses of approximately 75, 60 and 30 % respectively.

A preparation of flavoprotein at the 0·53 % flavinphosphate stage is not homogeneous in an ultracentrifugal field. The amount of the impurity and the theoretical % of flavinphosphate which a homogeneous preparation should contain are discussed in the appendix by J. St L. Philpot. The low initial concentration of flavoprotein in milk and the great losses in the purification procedure render attempts at further purification extremely expensive. Starting with 50 l. of milk, one practically reaches the vanishing point at the 0·53 % flavinphosphate stage. In the final stages, scarcity of material is the limiting factor in the purification.

II. *Physical and chemical properties*

A concentrated solution of purified milk flavoprotein (> 10 mg./ml.) is orange-red in colour. Dilute solutions (< 2 mg./ml.) appear brownish yellow. On addition of hyposulphite to a neutral solution of the protein, the colour is bleached and restored by shaking with air. Re-oxidation is practically instantaneous as soon as all the hyposulphite is oxidized to sulphite.

Milk flavoprotein is rapidly denatured at room temperature in solutions of *p*H 3·8 or less, likewise in concentrated aqueous solutions (> 40 %) of methyl alcohol and acetone. Temperatures higher than 50° denature the protein within a few min. Denaturation is always accompanied by the liberation of the prosthetic flavin. Indeed the appearance of greenish fluorescence serves as a delicate indicator of denaturation. Loss of catalytic activity can be used as a quantitative measure of denaturation of flavoprotein. No success has yet been attained in the reversible resolution of flavoprotein into prosthetic flavin and undenatured protein.

The absorption spectrum of our purest flavoprotein preparation (0·53 % flavinphosphate) is given in Fig. 1. There are three main absorption bands with maxima at 279, 350 and 450 mμ respectively. The spectrum was recorded by a Hilger "Spekker" photometer. The positions of the band peaks are identical in all preparations examined although with purification the ratio

$$\log \frac{I_0}{I}\,(279\ \mathrm{m}\mu) \Big/ \log \frac{I_0}{I}\,(450\ \mathrm{m}\mu)$$

increased.

The spectrophotometric method was used to estimate the flavin content of preparations of known dry weight. The assumption was made that the absorption coefficient β at 450 mμ was $2{\cdot}4 \times 10^7$. The justification for this assumption is that all known flavin-protein compounds have the same β value for their main

absorption band in the visible range of the spectrum. Since $\beta=\frac{1}{c}.\frac{1}{d}.\ln\frac{I_0}{I}$ then by determining the value of $\ln\frac{I_0}{I}$ at 450 mμ in a 2 cm. cell, the concentration of riboflavin, c, in mol. per ml. can be calculated from the formula. The concentration was always expressed in terms of g. % of flavinphosphate.

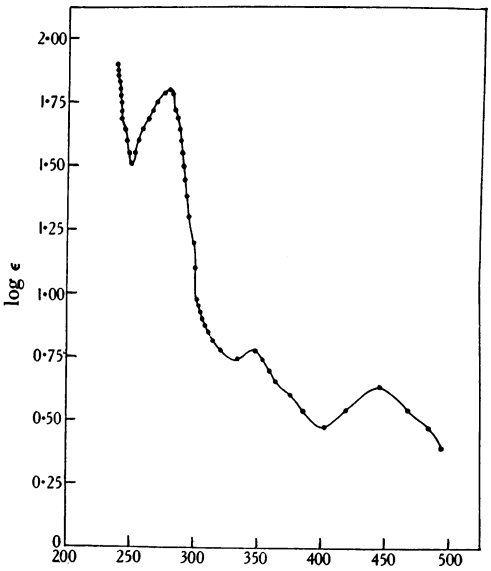

Fig. 1.

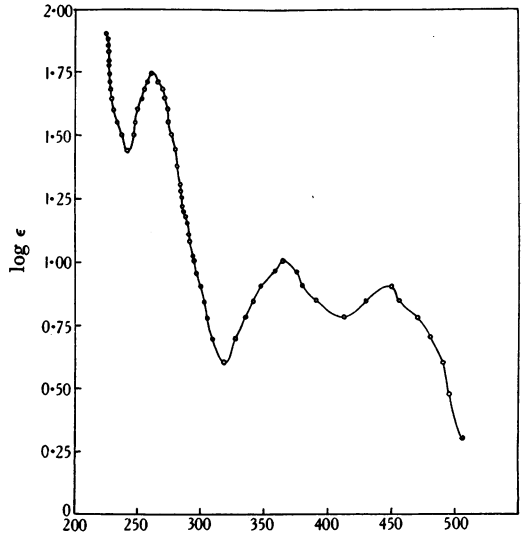

Fig. 2.

Fig. 1. Absorption spectrum of 1·76 % flavoprotein solution at the 0·53 % flavinphosphate stage; *p*H 7.

Fig. 2. Absorption spectrum of the prosthetic flavin after precipitation as the Ag and Ba salts; *p*H 7.

Estimation of the flavin content both directly in the combined form and indirectly after extraction with 75 % methyl alcohol from the protein shows that the yield of free flavin is about 80 %. We are satisfied that this small discrepancy is referable (1) to the inefficiency of extraction, and (2) to the marked tendency of the prosthetic flavin to become adsorbed on the protein precipitate. Fig. 2 shows the absorption spectrum of a purified sample of the prosthetic flavin. There are three main absorption bands with peaks at 260, 365 and 450 mμ. The spectrum is similar to, but not identical with, that of riboflavinphosphate.

III. *Prosthetic flavin*

By extracting a dry preparation of flavoprotein with 75 % methanol, the prosthetic flavin is obtained in solution and the protein is denatured. After removing methyl alchohol by distillation *in vacuo*, and precipitating residual denatured protein with $(NH_4)_2SO_4$, a clear yellow highly fluorescent aqueous solution is obtained. The prosthetic flavin is insoluble in benzyl alcohol; hence it cannot be riboflavin [cf. Emmerie, 1938] and is more probably a phosphorylated derivative. Cataphoretic measurements kindly carried out by Dr R. A. Kekwick of the Lister Institute show that the prosthetic flavin migrates anodically in a

cataphoretic field more rapidly than lactoflavinphosphate. At *p*H 7 in phosphate buffer, at 0° and $\mu=0{\cdot}02$ the mobilities of lactoflavinphosphate and the prosthetic group were, respectively, $1{\cdot}8\times10^{-5}$ and $2{\cdot}4\times10^{-5}$ cm.^{2}/volt^{-1}/sec.$^{-1}$. To quote Dr Kekwick: "these measurements are not very accurate owing to the large diffusion constants of the material, but it is safe to say that the unknown flavin migrated the faster". It was therefore clear that the prosthetic group was a phosphorylated derivative not identical with lactoflavinphosphate.

Straub [1938] and Warburg & Christian [1938] have demonstrated that the coenzyme of the amino-acid oxidase is a flavin-adenine dinucleotide. With the assistance of Dr Straub, we have been able to show that the prosthetic group of milk flavoprotein can act as the coenzyme of the amino-acid oxidase (cf. Table II).

Table II

The quantities used were: 0·7 ml. flavin-free amino-acid oxidase prepared by an unpublished method of Dr Straub; 1 ml. phosphate buffer, *p*H 7·2; 0·2 ml. *M* *d*-alanine. Total vol. 2·9 ml. Air in gas space.

Flavin added as μg. flavinphosphate per ml.	0	0·16	0·4	0·8	2
μl. O_2/10 min.	0	36	69	108	108

The high specificity of oxidation coenzymes in general argues the close chemical similarity of the prosthetic flavin and the coenzyme of the amino-acid oxidase, but biological interchangeability is by no means conclusive evidence of identity. In collaboration with A. H. Gordon and S. Williamson we are pursuing further the question of its relation to the amino-acid oxidase coenzyme. The physical and chemical properties of the two compounds do not seem to be identical.

IV. *Catalytic properties*

Reduced coenzyme I (diphosphopyridinenucleotide) is oxidized extremely slowly by methylene blue and other oxidation-reduction indicators. In presence of milk flavoprotein these reactions are enormously accelerated. The catalysis has been quantitatively measured in the following three test systems:

(1) reduced coenzyme + methylene blue;
(2) lactic dehydrogenase system + oxidized coenzyme + methylene blue;
(3) lactic dehydrogenase system + oxidized coenzyme + methylene blue (or pyocyanine) + O_2.

In systems (1) and (2) the velocity of the catalytic reaction is determined by measuring the rate of decoloration of methylene blue under anaerobic conditions. The difference between the two systems lies in the method of reducing the coenzyme. In (1) reduced coenzyme prepared by treating oxidized coenzyme with hyposulphite is used. In (2) the lactic dehydrogenase system is used to reduce oxidized coenzyme *in situ*. Since methylene blue is autoxidizable it is possible to study the reaction manometrically by measuring the rate of oxygen absorption as in (3). Apart from the different methods of measuring the reactions in (2) and (3) respectively, we are dealing in (2) with a steadily falling concentration of methylene blue and in (3) with a constant concentration of methylene blue. There should, therefore, be differences in the respective rates of reaction.

Table III shows the catalytic effect of flavoprotein on the reaction between reduced coenzyme I and methylene blue. The coenzyme was reduced by hyposulphite in *M*/2 $NaHCO_3$ solution according to the method of Green & Dewan [1937]. For the details of the Thunberg technique, cf. Green & Dixon [1934]. The

Table III

The quantities used were: 0·8 ml. of 1% coenzyme solution and 0·2 ml. 0·0125 *M* methylene blue. Total vol. 2·5 ml.

Flavoprotein as μg. flavinphosphate	0	10	2	1	0·2
Reduction time in min.	15	0·5	0·9	1·5	2·1

rate of reduction of methylene blue by reduced coenzyme is within limits proportional to the concentration of flavoprotein. Increase of the concentration of flavoprotein beyond 4 μg./ml. of flavinphosphate has no influence on the catalytic rate under the conditions of the above experiment. It is also interesting that the efficiency of flavoprotein as a catalyst increases with dilution. In other words, the greatest efficiency is reached when the ratio

$$\frac{\text{concentration of reduced coenzyme}}{\text{concentration of flavoprotein}}$$

becomes very large.

In test system (2), the lactic dehydrogenase+lactate is used as the reducing agent of the coenzyme. The enzyme is prepared from rabbit skeletal muscle by the method of Green *et al.* [1937]. The product of oxidation, pyruvic acid, inhibits the reduction process and must be fixed with cyanide. Fig. 3 shows the dependence of the rate of methylene blue reduction on the concentration of flavoprotein. Above 4 μg. flavinphosphate per ml. increase of flavoprotein concentration effects no further increase in the rate of decoloration of methylene blue. As little as 0·04 μg. flavinphosphate per ml. is sufficient to demonstrate a positive catalytic test. No reduction occurs in absence of flavoprotein.

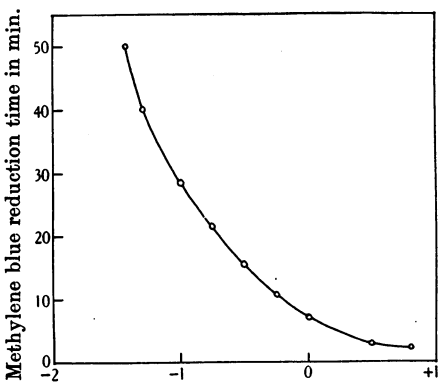

Fig. 3.

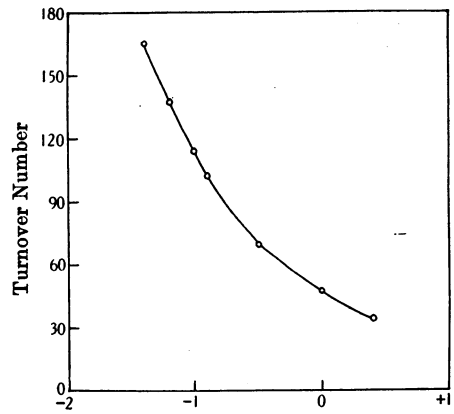

Fig. 4.

Fig. 3. The effect of the concentration of flavoprotein (expressed as μg. flavinphosphate) on the rate of reduction of methylene blue by reduced coenzyme.

Fig. 4. The dependence of the Turnover Number on the concentration of flavoprotein (expressed as μg. flavinphosphate).

Assuming that flavoprotein undergoes a cycle of oxidation and reduction, thereby catalysing the reaction between reduced coenzyme I and methylene blue, one can easily calculate from the above data the number of times flavoprotein would have to be reduced or oxidized in 1 min. (Turnover Number) in

order to account for the overall reaction. The exact molarity of the methylene blue solution was 0·0125. Therefore 0·2 ml. of the methylene blue solution is equivalent to 56 μl. H_2. Also 1 μg. flavinphosphate is equivalent to 0·049 μl. H_2. The Turnover Number (T.N.) is equal to the hydrogen equivalent of the methylene blue reduced per min., divided by the hydrogen equivalent of the flavoprotein present. Fig. 4 shows the dependence of the T.N. on the concentration of flavoprotein. Under the conditions of the experiment a limiting value of about 165 per min. was reached.

In the above experiment, the concentration of coenzyme I was 0·38 mg./ml. The T.N. increases with the concentration of coenzyme. For example with 1 mg. coenzyme, 0·42 μg. flavinphosphate and 0·2 ml. 0·0125 *M* methylene blue in a total volume of 3·6 ml. the T.N. was 105. Repeating the experiment with 3·3 mg. coenzyme the T.N. was 294. Fig. 5 shows the dependence of the T.N. on the concentration of flavoprotein using 2·6 mg. coenzyme per ml. Under optimum conditions, i.e. high coenzyme and low flavoprotein concentrations, the limiting T.N. is about 550.

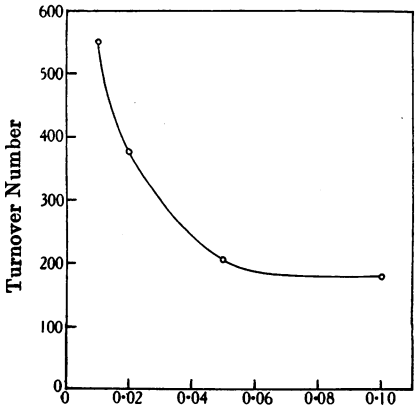

Fig. 5.

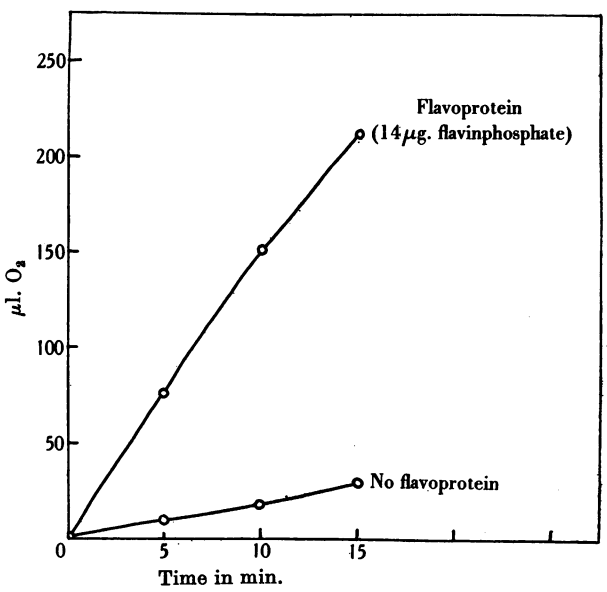

Fig. 6.

Fig. 5. The dependence of the Turnover Number on the concentration of flavoprotein using excess coenzyme. The quantities used were: 1 ml. enzyme; 1 ml. of 0·7% coenzyme I; 0·2 ml. 2*M* HCN; 0·2 ml. 0·0125*M* methylene blue and 0·2 ml. *M* lactate.

Fig. 6. The O_2 uptake of the lactic dehydrogenase system + pyocyanine + coenzyme I ± flavoprotein. The quantities used were: 1·5 ml. enzyme solution; 1 ml. 0·1% coenzyme I; 0·2 ml. 2*M* HCN; 0·2 ml. 0·1% pyocyanine and 0·1 ml. 2*M* lactate.

Table IV

The quantities used were: 1·5 ml. lactic dehydrogenase; 1 ml. 0·1% coenzyme I; 0·2 ml. 2*M* HCN; 0·2 ml. 2*M* lactate; 0·2 ml. 0·1% pyocyanine. Total vol. 4·1 ml.

ml. flavoprotein solution (14 μg. flavinphosphate per ml.)	1·0	0·3	0·1	0·03	0	1·0 (No pyocyanine)
μl. O_2/10 min.	152	94	43	31	22	28

Warburg & Christian [1932] have isolated a flavoprotein compound from yeast which also catalyses the oxidation of reduced coenzyme I by methylene blue. We compared the relative catalytic efficiencies of the two flavoproteins under identical experimental conditions. The ratio of activities milk flavoprotein : yeast flavoprotein was 7. This is in rough agreement with the limiting value of 50 for the T.N. of yeast flavoprotein, as compared with 500 for the milk compound.

The lactic dehydrogenase system + oxidized coenzyme + pyocyanine hardly absorbs oxygen. On addition of flavoprotein a vigorous uptake ensues (cf. Fig. 6). The dependence of the rate of O_2 uptake on the concentration of flavoprotein is shown in Table IV. The blank without flavoprotein is not inappreciable, a fact which rules out experiments with low concentrations of flavoprotein.

There is an unexpected result in Table IV, viz. that in absence of pyocyanine, flavoprotein shows no catalytic activity. In the previous section it was pointed out that reduced flavoprotein is autoxidizable. Two possibilities were open: either that reduced flavoprotein was not autoxidizable under the experimental conditions or that the assumption of flavoprotein undergoing a cycle of reduction and oxidation was incorrect. If the rate of oxidation of reduced flavoprotein by molecular O_2 was the limiting factor we should expect a higher rate of uptake in an atmosphere of O_2 than in air. Experiment showed that flavoprotein in absence of pyocyanine had no catalytic activity, regardless of whether an atmosphere of air or pure O_2 was used. The autoxidation of flavoprotein was therefore not the factor in question. If flavoprotein does not undergo a cycle of reduction and oxidation, it should be possible to demonstrate that flavoprotein is not reduced under the conditions of the experiment. The following experiments show that this is indeed the case. 8 mg. reduced coenzyme and flavoprotein in a concentration of 20 μg. flavinphosphate per ml. were mixed anaerobically in a Thunberg tube at 38°. No decoloration of flavoprotein was observed in the course of 1 hr. In another tube, 0·2 ml. of 0·0125 M methylene blue was added to the above mixture. Reduction of the methylene blue was complete in 30 sec. whereas the colour of oxidized flavoprotein persisted for more than 1 hr. Simple calculation shows that if flavoprotein were undergoing a cycle of reduction and oxidation, it would have to be reduced and oxidized $56/0{\cdot}98 \times 60$ or 3400 times in 1 hr. in order to account for the observed rate of reduction of methylene blue. Yet even in 1 hr. there was no evidence of complete reduction. The same result was obtained when using the lactic dehydrogenase system + oxidized coenzyme instead of hyposulphite-reduced coenzyme. The colour of flavoprotein in these experiments was sufficiently intense to allow visual observation of changes in colour. A control tube with flavoprotein reduced by hyposulphite was used for comparison with the experimental tubes.

The above facts suggest the following picture of flavoprotein catalysis. Reduced coenzyme combines with flavoprotein. The pyridine ring in this complex is easily dehydrogenated by oxidation-reduction indicators such as methylene blue, pyocyanine etc. When oxidation of the pyridine ring takes place, the complex dissociates into oxidized coenzyme and the original flavoprotein. That is to say, flavoprotein remains in the oxidized state during the cycle of its catalysis. The cataphoretic method should easily decide whether such complex formation does in fact take place.

We have carried out similar experiments with the Warburg and Christian flavoprotein of yeast. Although this compound definitely undergoes a cycle of oxidation and reduction our calculations indicate that the cycle is not sufficiently rapid to account for the catalytic effect on the oxidation of reduced coenzyme I

by methylene blue. This may be due to the presence in our yeast preparations of the flavoprotein described by Haas [1938] which does not react directly with O_2.

It is possible to use many variations of the three test systems for demonstrating the catalytic action of milk flavoprotein. For example, the lactic dehydrogenase system can be replaced by other systems which require coenzyme I such as the malic, triose, triosephosphoric and α-glycerophosphoric systems. Similarly methylene blue or pyocyanine can be replaced by flavinphosphate or cytochrome *c*. There were indications that coenzyme I (diphosphopyridinenucleotide) could be replaced by coenzyme II (triphosphopyridinenucleotide), but the experiments were not conclusive.

Preparations of flavoprotein at and beyond the 0·1 % flavinphosphate stage of purity have no xanthine oxidase activity. There is no question, therefore, of the identity of flavoprotein with the xanthine oxidase.

V. *Catalysts for the oxidation of reduced phosphopyridinenucleotides*

There are four catalysts known to catalyse the oxidation of phosphopyridinenucleotides. Table V summarizes essential information about their properties. Coenzyme factor is by far the most active catalyst of this group and is the only

Table V

	Reference	Source	Prosthetic group	Absorption bands mμ	Colour
Coenzyme factor	Dewan & Green [1938], Adler *et al.* [1937]	Animal tissues and yeast	Unknown	—	—
Warburg-Christian flavoprotein	Warburg & Christian [1932], Theorell [1934]	Yeast	Riboflavinphosphate	275, 380, 465	Yellow
Haas flavoprotein	Haas [1938]	Yeast	Dinucleotide of adenine and riboflavin	275, 377, 455	Greenish yellow
Milk flavoprotein	—	Milk	Unidentified flavin	279, 350, 450	Orange-red

one which has a wide distribution in animal tissues and micro-organisms. Nothing is known of its prosthetic group. All three flavoproteins differ in their physical and chemical properties. The differences are referable not only to the flavin but also to the protein portion.

SUMMARY

The isolation and properties of a flavoprotein and its prosthetic group from milk are described.

We are grateful to Messrs Boots, Ltd., and to Mr Slater of this Department for co-operating in the processing of large quantities of milk. One of us (D. E. G.) is indebted to the Ella Sachs Plotz Foundation for a research grant.

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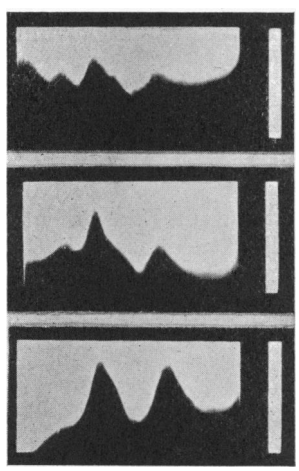
ADDENDUM: EXAMINATION IN THE ULTRACENTRIFUGE

By J. St L. Philpot, *Department of Biochemistry, Oxford* (*with* E. Dodwell, *Technical Assistant*)

Three specimens of milk flavoprotein at different stages of purification were sent by Messrs Corran and Green. The ultracentrifuge results are given in Table I. In every case the flavin appeared to belong wholly to a component having $S_{20} = 11\text{–}12 \times 10^{-13}$. Table I shows that in the purest preparation, containing

Table I

Photograph	Flavin-phosphate %	Sedimentation constants $\times 10^{13}$: Refraction method α	β	γ	δ	Absorption method δ	Concentration of δ component % of total protein
	0·06	1·1	3·9	6·6	11·5	*ca.* 11·0	—
	0·26	—	3·8	6·9	11·7	11·3	15–25
	0·53	—	—	6·2	12·1	12·5	39–46

0·53% flavin (expressed as lactoflavinphosphate) the flavoprotein forms 39–46% of the total protein. Hence the pure flavoprotein must contain 1·15–1·36% flavin, and its mol. wt. divided by the number of flavin groups per mol. must be 34,000–40,000. In this it differs from yeast flavoprotein, which has one flavin group per mol. of weight 78,000 [Kekwick & Pedersen, 1936].

From the sedimentation constant 12×10^{-13} and, assuming the usual partial specific volume 0·75, the minimum mol. wt. (i.e. that of a spherical particle) is about 220,000. Hence there must be at least $220/40 = 5{\cdot}4$ flavin groups per mol. Actually all known proteins with sedimentation constant about 12×10^{-13} have mol. wt. about 280,000 [cf. Svedberg, 1937], which is sixteen times the approximate "Svedberg unit" 17,600. The figure 34,000–40,000 given above suggests that milk flavoprotein may contain one flavin group to every two Svedberg units. If it were composed in all of sixteen Svedberg units it would therefore have eight flavin groups in a molecule of weight 270,000–320,000. Until the homogeneous protein is available this seems the most reasonable assumption.

I am grateful to the Medical Research Council and the Nuffield Trust for financial assistance.

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