Biochem. J. (1975) **147**, 417–423
Printed in Great Britain

Association of Xanthine Oxidase with the Bovine Milk-Fat-Globule Membrane

NATURE OF THE ENZYME–MEMBRANE ASSOCIATION

By MICHAEL S. BRILEY* and ROBERT EISENTHAL
Biochemistry Group, University of Bath, Bath BA2 7AY, U.K.

(*Received* 15 *October* 1974)

1. Xanthine oxidase (EC 1.2.3.2) was found to represent more than 8% of the intrinsic protein of the bovine milk-fat-globule membrane. 2. Less than 25% of the xanthine oxidase activity of the fat-globule membrane was solubilized with 0.1M-sodium pyrophosphate buffer or 2M-NaCl. Of the particulate activity remaining, 56% was solubilized with Triton X-100. 3. The xanthine oxidase activity solubilized with buffer, 2M-NaCl or Triton X-100 was not liberated as the free enzyme. Only tryptic digestion was found to release the free enzyme from the fat-globule membrane. Tryptic digestion also liberated free xanthine oxidase from those fractions solubilized by buffer or NaCl, but not from those fractions solubilized with Triton X-100 or by sonication. 4. The effect of membrane association on the catalytic properties of the enzyme could be mimicked by low pH or by the presence in the assay mixture of certain concentrations of 2-methylpropan-2-ol, but not 1,4-dioxan, suggesting that hydrogen-bonding rather than low dielectric constant may be involved. 5. The origin of the milk-fat-globule membrane is discussed with reference to the intrinsic nature of the associated xanthine oxidase activity.

Much of the xanthine oxidase (EC 1.2.3.2) present in milk is found associated with the membrane surrounding the milk fat-globule (Morton, 1954; Zittle *et al.*, 1956). These membranes arise from membranes of the secretory cell (Keenan *et al.*, 1971; Wooding, 1971*a*,*b*) and are concentrated in the aqueous phase (buttermilk) resulting from phase inversion (churning) of cream.

Using gel filtration Briley & Eisenthal (1974) isolated three fractions containing xanthine oxidase from buttermilk; (i) xanthine oxidase bound to the fat-globule membrane (fraction BM_1), which was excluded from Sepharose 2B; (ii) xanthine oxidase in a soluble lipoprotein-containing fraction (fraction BM_2), which was retained by Sepharose 2B but excluded from Sephadex G-200; and (iii) free xanthine oxidase, which was retained by Sephadex G-200. Under certain experimental conditions the ratio of xanthine oxidase activity to NADH oxidase activity (X/N) is characteristic of the state of the enzyme. Membrane-bound enzyme (e.g. fraction BM_1) has an X/N value of about 50 and the free enzyme an X/N value of about 110.

When fraction BM_1 was heated to remove associated enzyme activities, and increments were then added to free xanthine oxidase, the NADH oxidase activity was progressively enhanced to a value where its X/N value was characteristic of the membrane-bound enzyme. This was shown to be due to the binding of free enzyme to heated fraction BM_1.

In the present study we have used the X/N value as a parameter of enzyme–membrane association to investigate the nature of the association of xanthine oxidase with the milk-fat-globule membrane.

* Present address: Departement de Biologie Moléculaire, Institut Pasteur, 25, Rue du Docteur Roux, Paris, France.

Experimental

Methods

Preparation of milk-fat-globule membrane. Milk-fat-globule membrane and a soluble lipoprotein fraction containing xanthine oxidase activity were prepared from buttermilk as described previously (Briley & Eisenthal, 1974), where they were designated fractions BM_1 and BM_2 respectively. This nomenclature is also used here.

Centrifugation. Centrifugations were carried out at 4°C in an MSE Superspeed 50 centrifuge in either a 3×25ml or 3×10ml 'swing-out' rotor. Pelleted material was resuspended at 0°C by gentle homogenization in an all-glass hand-homogenizer. Discontinuous sucrose density gradients were constructed in polycarbonate tubes by using the method described by Briley (1973*a*). Samples were applied to the top of the gradient and brought to isopycnic equilibrium by centrifugation at 100000*g* for at least 14h at 4°C. Tubes were punctured at the bottom, the contents pumped out and fractions (0.5ml) collected.

Concentration of samples. Where necessary samples of protein or membrane fractions were concentrated

by dialysis against a saturated solution of polyethylene glycol (mol.wt. >6000) in 0.1M-sodium pyrophosphate buffer, pH7.0. Xanthine oxidase and NADH oxidase activities were not affected by this treatment unless concentration was continued to a stage where the protein solubility was exceeded; in such cases samples were discarded.

Enzyme assays. Xanthine oxidase and NADH oxidase activities were determined spectrophotometrically as described previously (Briley & Eisenthal, 1974). One xanthine oxidase unit is defined as the amount of enzyme causing a change in E_{290} of 1.0/min under the conditions used. One unit of NADH oxidase activity is defined similarly in terms of a change in E_{340} of 1.0/min with NADH as substrate.

Chemical analyses. Protein was measured by the method of Lowry *et al.* (1951), with dry bovine serum albumin as standard.

FAD was estimated fluorimetrically (Burch, 1957).

Materials

Purified xanthine oxidase (from milk), lipase (from wheat germ), phospholipase A (from *Vipera russelli*) and phospholipase C (from *Clostridium perfringens*) were obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K., NADH (grade II) and FAD were from Boehringer Corp. (London) Ltd., London W5 2TZ, U.K. and all other reagents were from BDH Chemicals Ltd., Poole, Dorset, U.K.

Results

Solubilization of xanthine oxidase activity associated with the fat-globule membrane

A sample of fat-globule membrane (fraction BM_1) in 0.1M-sodium pyrophosphate buffer, pH7.0, was centrifuged (all centrifugations in this section were at 100000*g* for 1h) and the resulting pellet was resuspended in the same buffer and centrifuged again. This was repeated twice more. The thrice-washed pellet was resuspended in 2M-NaCl and again centrifuged. The resulting pellet was suspended in 0.1% (v/v) Triton X-100 and left at 0°C for 1h before centrifuging. The pellet resulting from this was again resuspended in 0.1% Triton X-100 and this time left for 20h at 4°C before centrifuging.

After each separation the supernatant and resuspended pellet were assayed for xanthine oxidase and NADH oxidase activities and the protein content was determined. The FAD content of certain fractions was also measured. Table 1 shows that less than 25% of the total xanthine oxidase activity was solubilized by extraction with buffer. (Some of this activity was associated with a slight cream layer of very small fat-globules separated in the first centrifugation and therefore not solubilized.) Further extraction with 2M-NaCl released very little xanthine oxidase activity. Of the remaining particulate activity 56% was solubilized by 0.1% Triton X-100.

Table 1. *Solubilization of membrane-bound xanthine oxidase from milk-fat-globule membrane*

Details of sequential treatments and centrifugations are given in the text. Recoveries in all cases were approx. 90% and were taken into consideration in calculations.

Treatment	Activity in pellet (% of original xanthine oxidase activity)	Activity solubilized (% of original xanthine oxidase activity)
Fraction BM_1	100	0
Thrice-washed with buffer*	76.0	24.0
Washed with 2M-NaCl	74.5	1.5
Extracted with 0.1% Triton X-100 (1h)	44.5	30.0
Extracted with 0.1% Triton X-100 (20h)	29.8	14.7

* The first supernatant had a slight cream layer of very small fat-globules, which artificially increased the xanthine oxidase activity.

In Table 2 the amount of xanthine oxidase present in each fraction is expressed as a percentage of the total protein content of the fraction. These values were calculated by two methods: (i) by comparison of the FAD/protein ratio with that of pure xanthine oxidase (5.75μg of FAD/mg of protein), calculated on the basis of 2mol of FAD/mol of enzyme (Bray & Swann, 1972) and a 'protein molecular weight' of 272800 [from a molecular weight of 275000 (Andrews *et al.*, 1964), minus the contribution of the non-protein components, i.e. metals and flavins]; and (ii) by comparison of the specific activities of the various fractions with that of purified xanthine oxidase whose xanthine oxidase content is known by method (i).

On the basis of FAD content, commercially purified xanthine oxidase preparations were found to contain only 15.3% of the enzyme. This is consistent with its quoted specific activity of 0.5unit/mg of protein (1unit = 1μmol/min), in comparison with the highest specific activity obtained for milk xanthine oxidase, 3.5units/mg of protein, under similar conditions (Hart *et al.*, 1970). Assuming the latter to be 100% enzyme, the commercial preparation would be expected to contain 14.3% xanthine oxidase.

Table 2 shows that xanthine oxidase comprises approx. 5.5% of the total protein of the fat-globule membrane as represented by fraction BM_1 and approx. 7.5% of the fraction remaining after

Table 2. *Amount of xanthine oxidase present in fractions derived from fraction BM_1*

Experimental details are as in Table 1. Methods (i) and (ii) for calculating the amount of enzyme protein present as a percentage of total membrane protein, based on FAD content and specific activity respectively, are explained in the text.

Fraction	FAD/protein (μg/mg)	Specific activity (xanthine oxidase units/mg of protein)	% enzyme	
			Method (i)	Method (ii)
Commercially purified xanthine oxidase	0.88	4.00	15.3	—
(1) Fraction BM_1	0.34	1.37	5.9	5.2
(2) Second buffer extract of (1)	0.35	1.15	6.1	4.4
(3) Pellet after third buffer extract of (1)	—	1.51	—	5.8
(4) 2M-NaCl extract of (3)	—	1.29	—	4.9
(5) Pellet from (4)	0.47	1.72	8.2	6.6
(6) Triton X-100 extract (1h) of (5)	0.56	1.86	9.7	7.1
(7) Pellet from (6)	0.52	1.48	9.0	5.7
(8) Triton X-100 extract (20h) of (7)	0.58	2.32	10.1	8.9
(9) Pellet from (8)	0.46	1.31	8.0	5.0

extraction with buffer and 2M-NaCl. The xanthine oxidase content of these extracts is approx. 4.5%, whereas the fractions solubilized by Triton X-100 contain approx. 9.0% xanthine oxidase. In all cases the proportion of enzyme present in the various extracts is similar to that of the fraction from which they were solubilized.

The release of xanthine oxidase from the fat-globule membrane by sonication was also investigated. A sample of fraction BM_1, thrice washed with 0.1M-sodium pyrophosphate buffer, pH7.0, was sonicated by using a 200W sonicator with a titanium probe oscillating at 20kHz for four bursts of 30s each, separated by 30s of cooling in an ice–salt bath. This treatment resulted in a clarification of the suspension and a 17% loss of total xanthine oxidase activity. On centrifugation of the sonicated material 27% of the xanthine oxidase activity was found in the supernatant fraction.

Characterization of the solubilized fractions

The fractions solubilized from fraction BM_1 with buffer, 2M-NaCl, or Triton X-100 or by sonication all had X/N values of about 50, characteristic of membrane-bound xanthine oxidase. After heating in boiling water for 10min, the addition of increments of any of these fractions to free xanthine oxidase had no effect on the NADH oxidase activity, in contrast with the effect of the addition of heat-treated fraction BM_1.

The soluble, lipoprotein fraction, BM_2, also had an X/N value of about 50, and when heated it also had no effect on the NADH oxidase activity of free xanthine oxidase (Briley & Eisenthal, 1974). To investigate the possibility that fraction BM_2 and the solubilized material were the same, their behaviour in a sucrose density gradient was compared.

Material eluted in the second xanthine-containing peak on separation of buttermilk through Sepharose 2B [known from Briley & Eisenthal (1974) to contain a mixture of fraction BM_2 and free xanthine oxidase] was concentrated and centrifuged in a sucrose density gradient (Fig. 1*a*). The xanthine oxidase activity was partially resolved into two fractions. A denser shoulder (fraction BM_2) of the main peak was isopycnic with a density in the region of 1.11. This shoulder contained 10% of the xanthine oxidase activity and had an X/N ratio characteristic of membrane-bound xanthine oxidase. Most of the remaining xanthine oxidase activity (86% of that applied) was found in the main peak, which was isopycnic with a density of 1.07 and had an X/N value of approx. 110.

Centrifugation of fraction BM_1 in a sucrose density gradient (Fig. 1*b*) separated the xanthine oxidase activity into two fractions. The denser fraction, which was turbid, was isopycnic with a density of 1.18. The less dense fraction, which was clear, was isopycnic with a density of 1.11. Both fractions had X/N values characteristic of membrane-bound xanthine oxidase. The density of the less dense fraction in Fig. 1(*b*), i.e. material solubilized from fraction BM_1, was identical with that of the denser shoulder in Fig. 1(*a*), fraction BM_2.

Release of xanthine oxidase by hydrolytic enzymes

Pancreatin is known to liberate xanthine oxidase from cream (Ball, 1939) and is now an integral part of most procedures for the isolation of the enzyme. The release of free xanthine oxidase from the fat-globule membrane by digestion with trypsin, a major component of pancreatin, has been shown previously (Briley & Eisenthal, 1974). To investigate this release further, a once-washed pellet of fraction

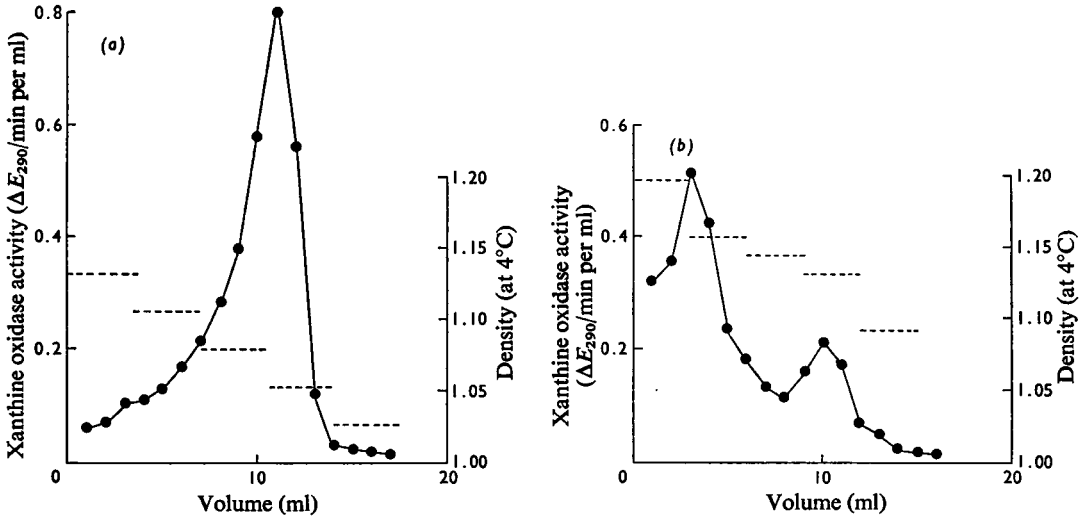

Fig. 1. *Comparison of the behaviour of fraction BM_2 and buffer-solubilized membrane material on sucrose density gradients*

The gradients, which comprised five layers (3.6ml each) of sucrose solution in 0.1M-sodium pyrophosphate buffer, pH7.0, with the densities as indicated (----), were constructed as described in the Experimental section. The samples, (*a*) 2.5ml of material concentrated from the second peak of xanthine oxidase activity on the separation of buttermilk through Sepharose 2B, and (*b*) 5ml of fraction BM_1 were applied to the top of the gradient and equilibrated as described in the Experimental section. In each case, fractions (0.5ml) were collected and xanthine oxidase activity (●) was assayed.

Table 3. *Tryptic digestion of various soluble fractions containing xanthine oxidase activity*

All reactions were carried out in 0.1M-sodium pyrophosphate buffer, pH7.0. Fractions containing 0.38–0.78 xanthine oxidase unit were incubated at 23–26°C with 15mg of trypsin/ml for 60min. Samples were taken at intervals and the NADH oxidase activity was assayed. Xanthine oxidase activity was also assayed at the beginning and end of each experiment.

	X/N before	Decrease in NADH oxidase activity (%)	X/N after
Fraction BM_2	50	60.0	96
Buffer extract of fraction BM_1	48	51.7	96.4
Triton X-100 extract (1h) of fraction BM_1	65.5	1.0	66.0
Material released from fraction BM_1 by sonication	53.2	5.0	55.8

BM_1 (14.52 xanthine oxidase units) was resuspended in 0.1M-sodium pyrophosphate buffer, pH7.0, and incubated with 8mg of trypsin at 28°C for 2h. During this time the X/N value increased from 46.5 to 78.5. The mixture was then centrifuged at 100000*g* for 1h. The yellow supernatant contained 82% of the xanthine oxidase activity with an X/N value of 85, whereas the white pelleted material contained 18% of the xanthine oxidase activity with an X/N value of 48. Incremental additions of pelleted trypsin-digested material had no effect on the NADH oxidase activity of free xanthine oxidase. However, after heating the same material for 10min in boiling water, the addition of increments to free enzyme increased the NADH activity to a plateau value (X/N = 52) in a similar manner to that caused by the addition of heat-treated fraction BM_1.

The possibility that the lipolytic activities of pancreatin may also contribute to the release of xanthine oxidase was examined. Samples of fraction BM_1 were incubated with purified lipase (370μg of lipase/xanthine oxidase unit in 2ml of 50mM-sodium pyrophosphate buffer, pH7.0, containing 0.5mM-$CaCl_2$, at 27°C for 30min), purified phospholipase A (115μg of phospholipase A/xanthine oxidase unit in 1ml of 0.1M-sodium pyrophosphate buffer, pH7.0, at 25°C for 1h) or purified phospholipase C (175μg of phospholipase C/xanthine oxidase unit in 0.1M-sodium pyrophosphate buffer, pH7.0, at 27°C for 1h). In all cases the xanthine oxidase and NADH oxidase activities remained unchanged.

Fraction BM_2 and those fractions solubilized from fraction BM_1 with buffer, Triton X-100 or by sonication were each incubated with trypsin to determine whether free xanthine oxidase could be liberated from these fractions. The experimental

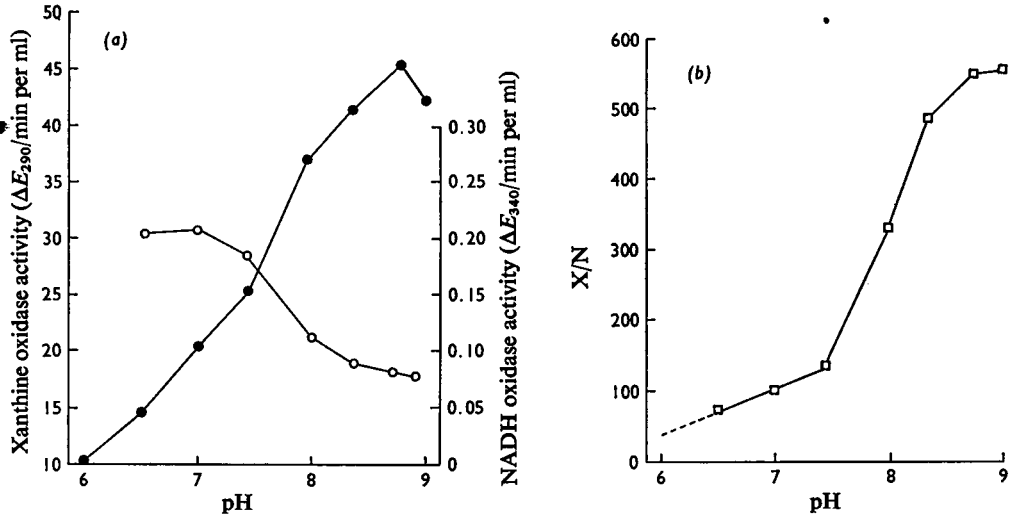

Fig. 2. *pH-dependence of xanthine oxidase and NADH oxidase activities of purified xanthine oxidase*

(*a*) Xanthine oxidase (●) and NADH oxidase (○) activities of purified xanthine oxidase were assayed as described in the Experimental section at pH values between 6.0 and 9.5 (nominal values). The pH values plotted are those measured in the reaction immediately after each assay. E_{290} for xanthine oxidation varies with pH owing to a shift in the absorption maximum for xanthine. Xanthine oxidase activities are corrected to E_{290} at pH 7.0 by using the data of Krebs & Norris (1949). Values are the means of duplicates. (*b*) X/N values (□) were calculated from the xanthine oxidase and NADH oxidase activities at the same pH (within 0.1 pH unit).

conditions and results are presented in Table 3. The NADH oxidase activities of fraction BM_2 and the fraction solubilized with buffer both decreased progressively during tryptic digestion, increasing their X/N values to 96.0 and 96.4 respectively. Tryptic digestion did not, however, affect the NADH oxidase activity of fractions solubilized with Triton X-100 or by sonication.

Effect of environment on the xanthine oxidase and NADH oxidase activities of purified xanthine oxidase

The effect of pH on the xanthine oxidase and NADH oxidase activities of purified xanthine oxidase was studied over the pH range 6.0–9.0. The maximal xanthine oxidase activity (at pH 8.5) was 2.8 times greater than that at pH 6.0 (Fig. 2*a*). This agrees closely with the earlier results of Krebs & Norris (1949). NADH oxidase activity reached a maximum value at pH 6.5–7.0, which was 2.3 times higher than the activity at pH 8.7 (Fig. 2*a*). A similar increase in NADH oxidation at low pH has also been reported (Nakamura & Yamazaki, 1973). The X/N values varied from 74.3 at pH 6.5 to 373 at pH 8.7 (Fig. 2*b*). Acid hydrolysis of NADH made it impossible to measure NADH oxidase activity at pH values lower than 6.5. However, when the plateau value for NADH oxidase activity at pH 6.5–7.0 is used with the xanthine oxidase activity at pH 6.0 an X/N value of 52.4 is obtained.

The activities of free xanthine oxidase were examined in various mixtures of buffer and 1,4-dioxan or buffer and 2-methylpropan-2-ol. Xanthine oxidase activity decreased proportionally with decreasing dielectric constant (Fig. 3*a*) irrespective of the solvent used. NADH oxidase activity varied differently with the two solvents used. In the presence of 2-methylpropan-2-ol the activity increased with decreasing dielectric constant (Fig. 3*b*), whereas with 1,4-dioxan the activity decreased with decreasing dielectric constant (Fig. 3*b*). As a result, the presence of either solvent caused a decrease in the X/N value of the free enzyme (Fig. 3*c*). E_{290} for xanthine and E_{340} for NADH were not affected by dielectric constant in the range used.

Discussion

Intrinsic nature of membrane-bound xanthine oxidase

Green and co-workers have defined two types of membrane proteins (Capaldi & Green, 1972; Vanderkooi, 1972). Extrinsic proteins are released by reagents that disrupt electrostatic interactions, whereas intrinsic proteins are only released by

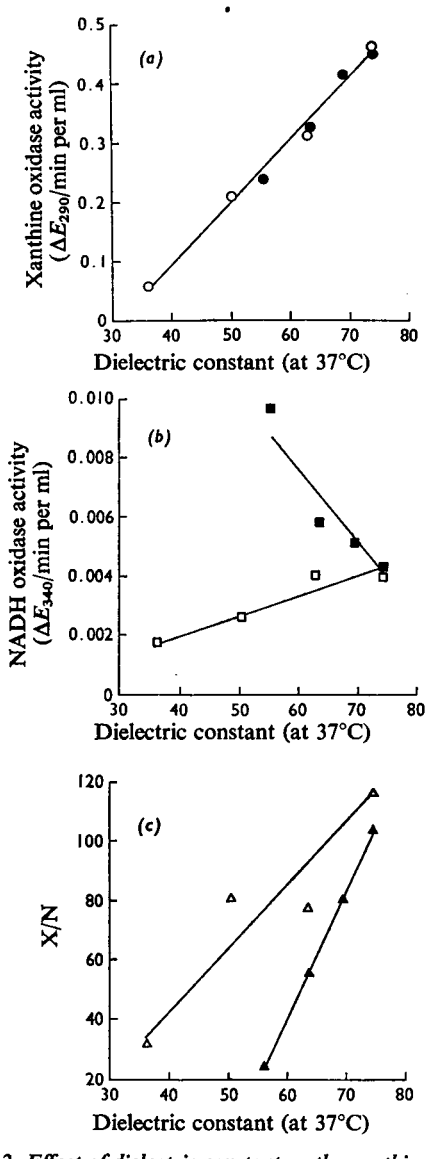

Fig. 3. *Effect of dielectric constant on the xanthine oxidase and NADH oxidase activities of free xanthine oxidase*

(*a*) Xanthine oxidase and (*b*) NADH oxidase activities of purified xanthine oxidase were assayed as described in the Experimental section with the addition of 4.2–21.5% (v/v) 2-methylpropan-2-ol (closed symbols) or 5.0–26.0% (v/v) 1,4-dioxan (open symbols) to the standard assay mixture (the pH was not adjusted). Values are the means of duplicates. The dielectric constant D' of the assay mixture was calculated from the equation:

$$\log D' = \phi_1 \log D_1 = \phi_2 \log D_2$$

where ϕ = the volume fraction and D = dielectric constant (Von Hippel, 1954). X/N values in (*c*) were calculated from the values in (*a*) and (*b*). Correlation coefficient for (*a*) >0.99.

reagents that disrupt hydrophobic interactions. Less than 25% of the xanthine oxidase activity of the fat-globule membrane was solubilized by buffer or 2M-NaCl. Most of the xanthine oxidase present in the fat-globule membrane can therefore be classified as intrinsic protein, suggesting that at least part of the molecule is deeply embedded in the hydrophobic core of the membrane.

Xanthine oxidase represents more than 8% of the intrinsic protein of the membrane. The proportion of xanthine oxidase calculated on the basis of specific activity was slightly lower than that determined by FAD content. This agreement may be fortuitous since the amount of 'inactivated' and demolybdo forms of the enzyme (Bray & Swann, 1972) and/or the amount of FAD in the membrane, not associated with xanthine oxidase may vary substantially from one preparation to another.

The enzyme solubilized from the membrane with buffer or Triton X-100 or released by sonication appears not to be free xanthine oxidase. Unlike free xanthine oxidase, these fractions all have X/N values of about 50, although, after heating, unlike fraction BM_1, they do not bind free xanthine oxidase. These properties are similar to those of the soluble lipoprotein fraction, BM_2 (Briley & Eisenthal, 1974). Material solubilized from the membrane by buffer has a similar density in a sucrose density gradient to fraction BM_2, suggesting that they may be the same material. Indeed, fraction BM_2 may represent material solubilized from the fat-globule membrane during preparation.

Free xanthine oxidase can only be released from the membrane by tryptic digestion. Trypsin releases the free enzyme from fraction BM_1, fraction BM_2 and from material solubilized from the membrane with buffer. The trypsin resistance of Triton X-100-solubilized material may be explained by the presence of bound Triton shielding the trypsin-susceptible parts of the complex. Similarly, vesiculation of the membrane material during sonication may also result in the non-availability of the trypsin-susceptible areas. The particulate material remaining after tryptic digestion of fraction BM_1 is incapable of binding free xanthine oxidase (as shown by a change in the X/N value) until after heat treatment. This suggests that heat treatment, possibly involving some membrane modification, is required before reconstitution of the enzyme–membrane complex is possible.

Effect of enzyme–membrane association

The X/N value of free xanthine oxidase appears to approach 50 at about pH 6.0, although this is not possible to measure directly. The differences seen in the catalytic properties of free and membrane-bound

xanthine oxidase may therefore result from the enzyme binding to the membrane in a micro-environment of low pH.

Xanthine oxidase activity of the free enzyme is decreased in the presence of 2-methylpropan-2-ol or 1,4-dioxan. The decrease in activity is proportional to the dielectric constant of the assay mixture and independent of the solvent used. The presence of 1,4-dioxan caused a decrease in the NADH oxidase activity of the free enzyme which was proportional to dielectric constant. With 2-methylpropan-2-ol, however, there appears to be a more specific activation, overcoming the general inhibitory effect of low dielectric constant. The addition of 2-methylpropan-2-ol therefore results in a decrease in the X/N value which is due, in part, to an increase in NADH oxidase activity, suggesting that 2-methylpropan-2-ol may be, at least partially, mimicking the effect of membrane association.

Unlike 1,4-dioxan, 2-methylpropan-2-ol is capable of acting as a donor in hydrogen-bond formation. Thus the enhanced NADH oxidase activity of the free enzyme in the presence of 2-methylpropan-2-ol may result from hydrogen-bonding with the solvent. By analogy the enhancement of NADH oxidase activity of the membrane-bound enzyme may result from a similar hydrogen-bonding in the enzyme–membrane complex. At present it is not possible to state whether the effects of 2-methylpropan-2-ol and low pH are due to the same general phenomenon. The answer to this and to the exact nature of the micro-environment of the binding site must await further investigation.

Origin of the fat-globule membrane

Much of the xanthine oxidase in milk thus appears to be present as an integral part of the fat-globule membrane and it is therefore unlikely that it is simply adsorbed on to the membrane surface, as has been suggested (Ball, 1939).

Xanthine oxidase has not been detected in the plasma membrane of any tissue, although it has been found in mammary-gland microsomal fractions of the lactating cow (Bailie & Morton, 1958). This fraction would, however, have contained membrane material from plasma membrane, Golgi vesicles, endoplasmic reticulum and probably also from the membranes surrounding the fat-globules within the cell, as observed by Wooding (1971*b*). Thus suggestions that the milk-fat-globule membrane is wholly derived from the plasma membrane (Dowben *et al.*, 1967; Keenan *et al.*, 1970, 1971; Patton & Trams, 1971) appears to be an over-simplification.

There is now considerable evidence to suggest that the milk-fat-globule membrane consists of two layers (reviewed by Briley, 1973*b*), one surrounding the fat-globule within the cell (Wooding, 1971*b*) and the other acquired from the plasma membrane as the fat-globule is secreted from the cell (Bargmann & Knoop, 1959). Thus one might imagine xanthine oxidase to be an integral part of the intracellular fat-globule membrane. More evidence, however, is required before the relationship between the plasma membrane and the fat-globule membrane is clear. Xanthine oxidase may prove to be a useful tool for further investigation into the origin of the milk-fat-globule membrane.

We thank Unigate Ltd. and the Science Research Council of Great Britain for financial support under the C.A.P.S. scheme, and for a studentship for M.S.B.

References

Andrews, P., Bray, R. C., Edwards, P. & Shooter, K. V. (1964) *Biochem. J.* **93**, 627–632
Bailie, M. J. & Morton, R. K. (1958) *Biochem. J.* **69**, 35–44
Ball, E. G. (1939) *J. Biol. Chem.* **128**, 51–67
Bargmann, W. & Knoop, A. (1959) *Z. Zellforsch. Bd.* **49**, 344–388
Bray, R. C. & Swann, J. C. (1972) *Struct. Bonding (Berlin)* **11**, 107–144
Briley, M. S. (1973*a*) *Lab. Practice* **22**, 679
Briley, M. S. (1973*b*) Ph.D. Thesis, University of Bath, U.K.
Briley, M. S. & Eisenthal, R. (1974) *Biochem. J.* **143**, 149–157
Burch, H. B. (1957) *Methods Enzymol.* **3**, 960–962
Capaldi, R. A. & Green, D. E. (1972) *FEBS Lett.* **25**, 205–209
Dowben, R. M., Brunner, J. R. & Philpott, D. E. (1967) *Biochim. Biophys. Acta* **135**, 1–10
Hart, L. I., McGartoll, M. A., Chapman, H. R. & Bray, R. C. (1970) *Biochem. J.* **116**, 851–864
Keenan, T. W., Morre, D. J., Olsen, D. E., Yunhans, W. N. & Patton, S. (1970) *J. Cell Biol.* **44**, 80–93
Keenan, T. W., Olsen, D. E. & Mollenhauer, H. H. (1971) *J. Dairy Sci.* **54**, 295–299
Krebs, E. G. & Norris, E. R. (1949) *Arch. Biochem.* **24**, 49–54
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
Morton, R. K. (1954) *Biochem. J.* **57**, 231–237
Nakamura, M. & Yamazaki, I. (1973) *Biochim. Biophys. Acta* **327**, 247–256
Patton, S. & Trams, E. G. (1971) *FEBS Lett.* **14**, 230–232
Vanderkooi, G. (1972) *Ann. N.Y. Acad. Sci.* **195**, 6–15
Von Hippel, A. R. (1954) *Dielectrics and Waves*, p. 231, John Wiley and Sons, New York
Wooding, F. B. P. (1971*a*) *J. Cell Sci.* **9**, 805–821
Wooding, F. B. P. (1971*b*) *J. Ultrastruct. Res.* **37**, 388–400
Zittle, C. A., Dellamonica, E. S., Custer, J. H. & Rudd, R. K. (1956) *J. Dairy Sci.* **39**, 528–535