ACTIVATION AND INACTIVATION OF MILK XANTHINEOXIDASE BY PHYSICOCHEMICAL MEANS

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Xanthineoxidase (X.O.) is found in fresh milk in a particular physicochemical state, attached to the surface of fat globules as lipoprotein cenapses. These cenapses may be broken by a variety of physicochemical means: heat, pressure, ultrasonic waves and detergents, which, before denaturing the enzyme, cause a steep rise in xanthineoxidase activity. This activation is a characteristic feature of milk X.O.

We studied the kinetics and the mechanism of activation and of inactivation of X.O. at different stages of purity, by means of heat, high pressures and ultrasonic waves, and

calculated the activation energy of its heat denaturation. The inactivation of X.O. by ultrasonic waves has been compared with the destruction of riboflavine in the same conditions.

These studies throw some light on the mechanism of the action of the enzyme and on the nature of its lipoprotein complex in milk.

Milk X.O. is one of the most intensively studied enzymes, its structure and mechanism of action being now relatively well understood.[10, 12, 24, 25] Recent progress in cell fractionation techniques has furthered studies on the variation of enzyme activity in relation to changes of structural elements. A good understanding of such phenomena is essential for elucidating the mechanism of multienzyme action, e.g. in mitochondria or in microsomes.[5, 14, 29]

Milk X.O. is integrated into morphological elements in the fat globules, its activity being largely conditioned by the state of a lipoprotein complex. A characteristic feature of milk X.O. is that its activity is low in very fresh milk and increases considerably the longer the milk stands. This phenomenon, observed by Dixon,[4] described by Wieland [30] and studied by Polonovski, Neuzil, Baudu [15-19] and by us,[26-27] seems to be a consequence of a change in the physicochemical state of X.O. produced by a variety of chemical and physical factors. In the present paper we give a brief description of our experiments on this topic and its interpretation in terms of structural organization.

EXPERIMENTAL

MATERIALS.—Fresh milk was directly milked into a Dewar and brought to the laboratory; its temperature varied slightly around 35° C. Purification of X.O. was achieved by different methods. For the high pressure and ultrasonic (U.S.) studies we used partially purified preparations obtained by a method described previously,[26, 27] using organic solvents (ether + propanol) for the delipidation. In other experiments preparations according to Dixon and Kodama,[4] to Ball,[2] and to Green *et al.*[12] were used, as indicated. All chemicals used were of analytical grade. Aldehydes were redistilled before use. Purines were supplied by Roche and riboflavine came from the Lederle laboratories.

METHODS.—(i) Enzyme activity was measured by the Thunberg method using conventional tubes which had been evacuated for 10 min at the water pump and filled with nitrogen. Methylene Blue was placed in the stopcock, and the decoloration time (d.t.) measured at 37° C. For the experiments where rapid successive activity determinations were needed (e.g. kinetics of the thermoactivation), narrow (8 mm diam.) haemolysis tubes, sealed with a paraffin layer, were used. Using whole milk as the enzyme source this method gives reproducible results for decoloration times between 0·5 and 15 min.[19, *]

(ii) In other experiments, spectrophotometric assays were used (Kalckar [7]) with O_2, Methylene Blue or triphenyl-tetrazolium chloride (TPT) as electron acceptors (Unicam quartz spectrophotometer). With the first, uric acid formation was followed at 290 or 300 mμ; with the second, decoloration was measured at 675 mμ. With TPT, formazan formation was followed at 500 mμ.

It seemed interesting to compare reduction kinetics of 2 acceptors reduced simultaneously by the X.O. + hypoxanthine (H.X.) system.

Fig. 1 gives the results of a typical experiment. The 300 mμ plot shows that production of uric acid with O_2 as acceptor is of zero order, at least over the first 10 min. If Methylene Blue is added, its rate of reduction measured at 675 mμ is not constant but gets faster as the O_2 is exhausted. The same is true for TPT, where reduction is preceded by a rather long time-lag followed by a linear rise of optical density at 500 μm. The affinity of this dye for the enzyme system is much smaller than that of Methylene Blue. If the X.O. + H.X. system contains both dyes, Methylene Blue is preferentially reduced and the time lag for TPT reduction is prolonged (see fig. 2).

* Enzyme activity is expressed as the reciprocal of decoloration time (10^2/d.t. min) in minutes per unit enzyme (ml of milk, mg of purified enzyme). In activation experiments the activation coefficient, (a.c.) gives the ratio: enzyme activity after activation/enzyme activity before activation.

Thus we used TPT only occasionally for qualitative demonstration of enzyme activity and Methylene Blue or O_2 for quantitative studies. With the substrate/acceptor ratios used (0·05-0·25 μM Methylene Blue for 5 μM of H.X.), the reciprocal of the decoloration time is proportional to the initial rate of oxidation of H.X. Reproducibility of results is within $\pm$ 10 % with Methylene Blue in presence of air: parallel determination of decoloration time of 0·25 μM of Methylene Blue by 2 ml of milk (final volume 3 ml, pH 8·2) yielded [19] a mean of 4·6 min with a standard deviation of 0·43.

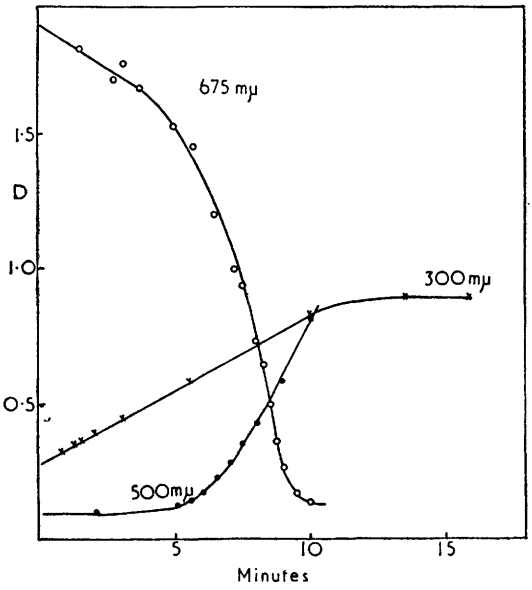

FIG. 1.—Kinetics of oxidation of hypoxanthine (5 μM) by purified X.O. (according to Green *et al.*[12]) and different electron acceptors. Abscisses: time in minutes; ordinates: optical density. 1 ml of enzyme is mixed with 0·5 ml 0·1 M phosphate buffer. pH 7·2, 5 μM hypoxanthine, the acceptor, and 2·8 ml dist. water. Acceptors: 675 mμ plot 0·05 μM Methylene Blue; 500 mμ plot: 7 μM TPT; 300 mμ plot: oxygen, 1 cm quartz cell, at approx. 20° C. Reduction of Methylene Blue and of TPT in the presence of air.

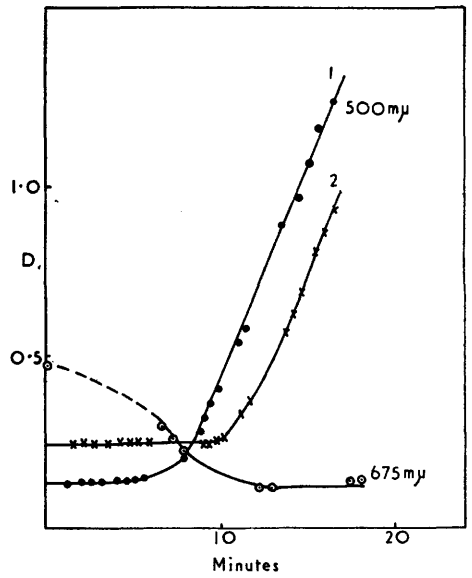

FIG. 2.—Effect of Methylene Blue on the reduction of TPT by X.O. The same condition as in fig. 1. 675 mμ plot: reduction of Methylene Blue in the TPT + Methylene Blue mixture; 500 mμ plot no. 1: reduction of 7 μM TPT in presence of air; 500 mμ plot no. 2: reduction of TPT in presence of air and 0·05 μM Methylene Blue.

(iii) In the Warburg experiments the enzyme and buffer were placed in the main compartment, and 0·2 ml 5 % KOH in the centre well; the substrate is added after a 5-min equilibration period. As acetaldehyde distils into the KOH, its final concentration is uncertain.

PHYSICAL TREATMENTS.—(i) Experiments with U.S. waves were performed with a S.C.A.M. type of instrument [15] having a quartz plate of 6-cm diam. connected to a 2 kW generator. The frequencies used varied between 176 and 960 kc/s, and the power emitted was approximately 2·9-3·9 W/cm^2 (82-102 acoustic watts). The solution was placed in a glass cylinder having a Cellophane membrane at the bottom, and this was cooled during irradiation by tap water at about 15° C.

(ii) The high pressure work was done using the presses constructed by J. and J. Basset.[27] The enzyme was submitted at constant temperature (37° C) to pressures varying from 5,000 to 12,000 kg/cm^2. Compressions and expansions were sufficiently slow as to avoid changing the temperature of the sample. The enzyme solution was placed in a cylindrical glass container, closed by non-vulcanized rubber tubing by which the pressure of the oil chamber was transmitted.

(iii) High speed centrifugations were performed on a Spinco preparative ultracentrifuge * at 0°.

RESULTS

TEMPERATURE-ACTIVATION AND INACTIVATION OF MILK X.O.

As was emphasized by Wieland and the late M. Polonovski and colleagues,[15-17] the dehydrogenase activity of fresh milk is very low and is strongly activated by a brief exposure to low temperatures (< 15° C). If immediately after milking, fresh milk is centrifuged at 37° C, enzyme activity is entirely confined to the globules. If milk is refrigerated before separation of the fat globules, the separated liquid is rich in X.O.

Fig. 3 shows manometric experiments with refrigerated and fresh whey and with refrigerated cream suspension separated by centrifugation at 5,000 rev/30 min. The low residual activity of fresh (non-refrigerated) whey is due to small fat globules and liposomes,[13] it disappears after centrifugation at 25,000 rev/min (45,000 g).

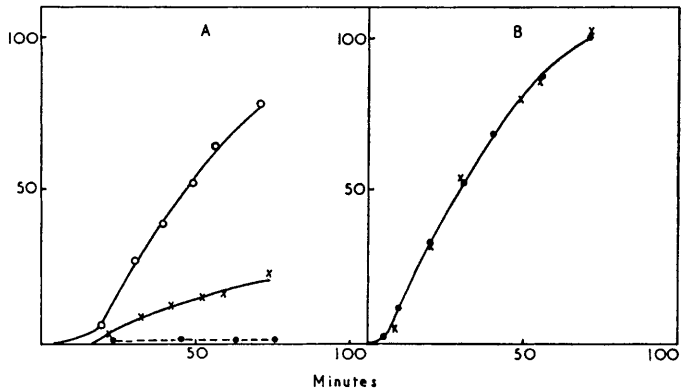

FIG. 3.—X.O. activity of different fractions of fresh and refrigerated milk, as determined by the manometric technique at 37° C. Abscisses: time in minutes, ordinates: μl. oxygen absorbed per mg dry weight. (A) O—O whey of milk centrifuged after cooling 2 h to — 5° C. ×—× whey of fresh milk; centrifuged at 37° C.·— —· the same after centrifugation 45 min at 45,000 g, 0° C. (B) ·—· cream of fresh milk refrigerated in phosphate buffer, ×—× cream of refrigerated milk. 5 μM hypoxanthine and 0·1 mM phosphate buffer, pH 7·2 in a total volume of 3·2 ml.

We have studied the kinetics of this activation to find out whether it is of a physical or a chemical nature. Fig. 4 gives the results of such experiments as a three-dimensional plot, showing the activation coefficient as a function of time and temperature in the first 10 min.

Between 0 and 20° C, the activity (10^2/d.t. min per ml milk) rises to 3-5 times its initial value and remains at this level. Between 20 and 40° C, fresh milk retains its original low activity (A.C. = 1) (though not indefinitely) but a slow activation appears after a few hours or days, probably due to the action of bacterial or milk enzymes (lipases 15).

* Thanks are due to Dr. Rebeyrotte for the ultracentrifugations.

At high temperatures, activation and thermal inactivation of X.O. proceed simultaneously, giving rise to a curve with a maximum. Both degree and rate of activation and rate of inactivation depend on temperature. At 0° C and above 80° C activation seems to be nearly linear with time, but the exact kinetics cannot be obtained by this method. Between 0° and 20° C, and 50° and 80° C, activation curves are S-shaped.

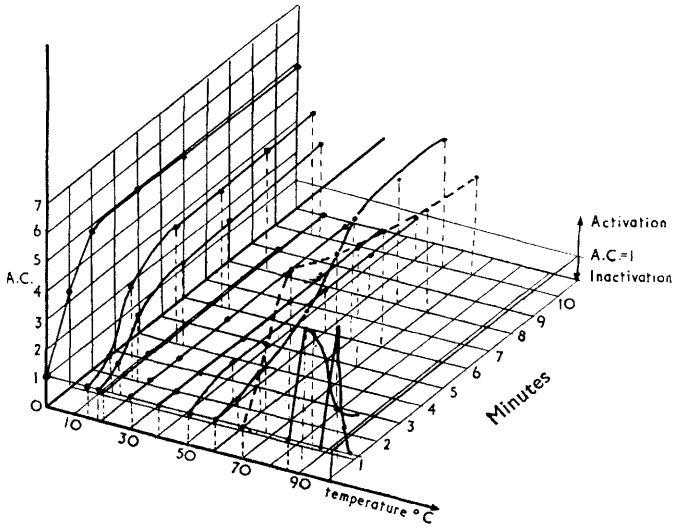

FIG. 4.—Kinetics of thermal activation and inactivation of milk X.O. 5 μM hypoxanthine, 0·25 μM Methylene Blue, 2 ml milk, final volume 3 ml. Methylene Blue decoloration time determined in haemolysis-tubes in presence of air at 37° C.

Table 1 gives the times of attainment of half maximal activity and maximal activity, as a function of temperature (half times are given because they are proportional to zero order and to first order rate constants). These data enabled us to estimate apparent activation energies of the thermal activation process.

TABLE 1.—MAXIMAL ACTIVATION COEFFICIENT $(d.t.)_0/(d.t.)_t$ AND HALF-TIMES AND TIMES OF ITS ATTAINMENT ($t_{\frac{1}{2}}$ AND t_{max}) AS A FUNCTION OF TEMPERATURE

(determined by Methylene Blue decoloration in haemolysis tubes with 2 ml milk, 5 μM hypoxanthine, 0·25 μM Methylene Blue; final volume 3 ml, pH 8·2 at 37° C)

temp. °C	min.		activation coefficient max
	$t_{\frac{1}{2}}$	t_{max}	
0	1	4	5
14	2	22	4·9
17	3·5	—	3·4
50	6·5	16	2
60	4·4	6	4·5
70	1	2	5·1
78	0·5	1	4·5
86	0·25	0·5	4·4
97	0·10	0·7	4·6

Fig. 5 shows the Arrhenius plots (log $1/t_{\frac{1}{2}}$ or log $1/t_{max}$ against reciprocal of absolute temperature, where $t_{\frac{1}{2}}$ and t_{max} are the times required to attain half of the maximal activity and maximal activity respectively. Satisfactory straight lines are obtained with both $t_{\frac{1}{2}}$ and t_{max} between 50 and 100° C. For the high-temperature activation, the activation energy is about 21 kcal/mole. The activation of the enzyme is followed by its heat denaturation above 60° C.

Table 2 gives the graphically computed first-order constants of the heat inactivation. Fig. 5B shows the corresponding Arrhenius plot with a slope corresponding to an activation energy of about 40 kcal and a frequency factor, log $PZ \simeq 22$. The enthalpy,

TABLE 2.—FIRST-ORDER CONSTANTS FOR THERMAL INACTIVATION OF MILK X.O. AS A FUNCTION OF TEMPERATURE (same method as for table 1)

t °C	k_m sec^{-1} × 10^4
70	11·65
78	38·8
86	85·5
97	332

entropy and free energy of activation of the heat denaturation are: $\Delta H^* = 39{\cdot}4$ kcal/mole; $\Delta S^* = +40{\cdot}5$ cal/mole deg.; $\Delta F^* = 26{\cdot}7$ kcal/mole.

The degree of activation of X.O. by chilling or heating varies with the substrate. It is more important with hypoxanthine than with the aldehydes.

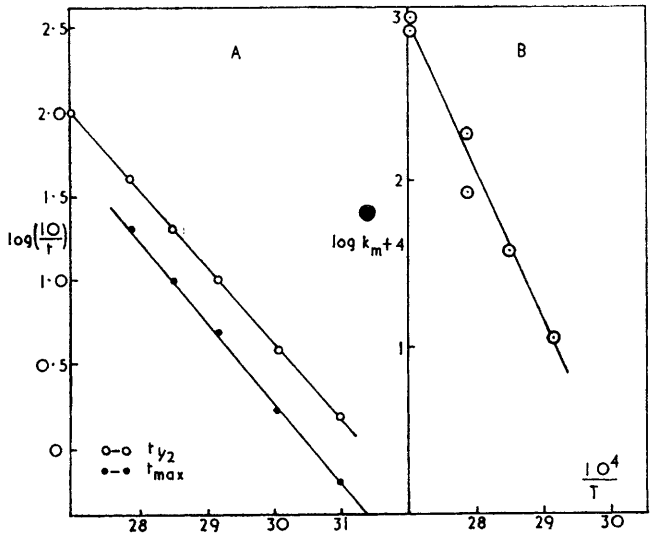

FIG. 5.—Arrhenius diagrams for the thermal activation and inactivation of milk X.O. Abscisses: reciprocal of absolute temperature; ordinates: (A) log of reciprocal of the time necessary to attain the maximal and half-maximal activity. (B) log of the first-order rate constant of thermal inactivation of X.O. Rates determined as indicated in fig. 4.

Table 3 gives rates of oxidation of H.X. and different aldehydes by fresh and activated milk-enzyme (1 h at — 10° C). Measurements were performed in the Warburg apparatus, using 1 ml milk, 1 ml phosphate buffer in a final volume of 3·2 ml at 37° C. It is seen that the thermal activation coefficient is near unity for the lower aldehydes and increases with molecular length and volume.

TABLE 3.—RELATIVE RATES OF OXIDATION OF ALDEHYDES BY FRESH AND COLD-ACTIVATED MILK; MANOMETRIC METHOD, 37° C, IN PRESENCE OF 0·1 mM PHOSPHATE BUFFER, FINAL VOLUME 3·2 ml

substrate mM		rate of oxidation, µl. O_2/60 min per ml of milk		activation coefficient	pH
		fresh milk	cold activated milk		
acetaldehyde	0·5	86	71	0·83	7·1
propylaldehyde	0·5	105	94	0·89	7·1
butylaldehyde	0·5	87	146	1·68	6·3
heptylaldehyde	1·0	75	115	1·5	7·4
salicylaldehyde	0·5	18	31	1·72	6·9
hypoxanthine	0·005	46	152	3·3	8
	0·010	42	102	2·4	7·8

HIGH PRESSURES

As the thermal activation experiments suggested a physical mechanism, it seemed interesting to submit milk X.O. to other physicochemical treatments. Activity against time curves with a maximum would substantiate this hypothesis. Mechanical agitation was shown to provoke a similar activation to that of chilling.[15, 30] So we submitted milk to hydrostatic pressures varying from 5 to 12,000 kg/cm^2. Variation of physicochemical properties of proteins and enzymes submitted to high pressures (5-20,000 kg/cm^2) has been intensively investigated by the Macheboeuf school. Barbu and Joly [3] found evidence for modification in the physical state of proteins above 4000 kg/cm^2.

Fig. 6 gives activation coefficients as a function of time at different pressures for fresh milk. As the pressure-chamber was thermostatted at 37° C, thermal activation was avoided. These curves are similar to those of fig. 4, with the exception that inactivation does not proceed by a first-order mechanism. At 7000 kg/cm^2 this rise, if taking place, is followed so rapidly by inactivation that we could not measure it (after 2 min the

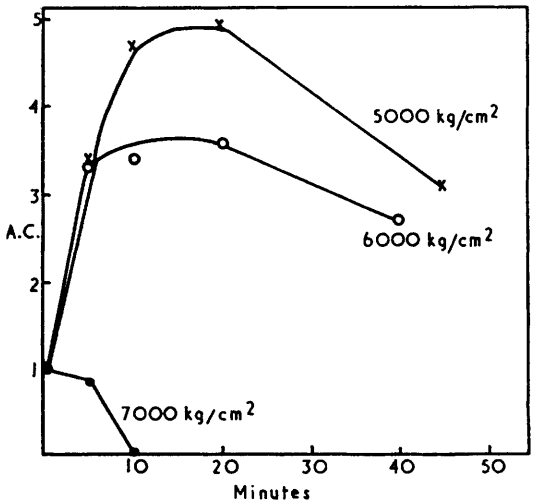

FIG. 6.—High pressure activation and inactivation of milk X.O. Abscisses: time of application of pressure; ordinates: activation coefficient. Rate of hypoxanthine dehydrogenation determined by the Thunberg method, 2 ml of milk, 0·1 mM phosphate buffer, pH 7·4, 5 μM, hypoxanthine 0·1 μM; Methylene Blue, final volume 3·6 ml; decoloration time determined at 37° C.

enzyme is practically inactivated). Similar results can be obtained with acetaldehyde as substrate,[27] but the ratio of activities with both substrates

$$\left[\frac{\text{t.d. min. (acetaldehyde)}}{\text{t.d. min. (hypoxanthine)}}\right]$$

varies with time and pressure (from 1·0 to 2·2).

With washed fat globules and with purified enzyme (by the organic solvent method [26]) there is no activation, enzyme activity disappearing at 7000 kg/cm^2 in 30 min. Purified enzyme and fat globules show about the same sensitivity to pressure (table 4).

TABLE 4.—INACTIVATION OF X.O. IN WASHED FAT GLOBULES AND IN THE PURIFIED ENZYME AFTER 30 MIN AT DIFFERENT PRESSURES (ENZYME ACTIVITY ESTIMATED BY THE THUNBERG METHOD: 2 ml ENZYME SOLN., 2 ml PHOSPHATE BUFFER (0·1 M, pH 7·4) HYPOXANTHINE 5μM, METHYLENE BLUE, 0·1 μM, FINAL VOLUME 4·6 ml)

pressure kg/cm^2	% activity remaining after 30 min	
	fat globules	purified enzyme
5000	100	88
6000	25	28
7000	—	0
8000	0	—

ULTRASONIC WAVES

Effect of U.S. waves on fresh milk is shown in fig. 7, with both H.X. and acetaldehyde as substrate.

Activation and inactivation follow a similar course as that at high pressure, and are more important with hypoxanthine as substrate, than with acetaldehyde. Activation by U.S. waves was sometimes observed even with washed fat globules (d.t. of Methylene Blue diminished in the ratio 3 : 1), or with partially purified enzymes (organic solvent method). The preparations obtained by the use of propanol or butanol were only partially soluble in the phosphate buffer media. U.S. waves by their depolymerizing and dispersing

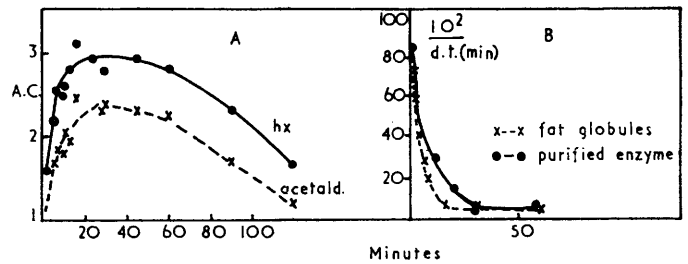

FIG. 7.—Activation and inactivation of milk X.O. by ultrasonic waves. Abscisses: time of irradiation; ordinates: (A) activation coefficient, (B) reciprocal of decoloration time. Dehydrogenase activity determined in Thunberg tubes at 37° C with 2 ml milk or enzyme solution and 0·2 mM phosphate buffer, pH 7·4; 5 μM hypoxanthine acetaldehyde 0·68 mM, 0·1 μM Methylene Blue; final volume 4·6 ml. (A) fresh milk; (B) fat globule suspension (about 10 % w/v) and partially purified enzyme (organic solvent method [26]); 8·9 mg/ml); final pH 7·1. U.S. waves of 960 kc/s, 100 W, 20° C.

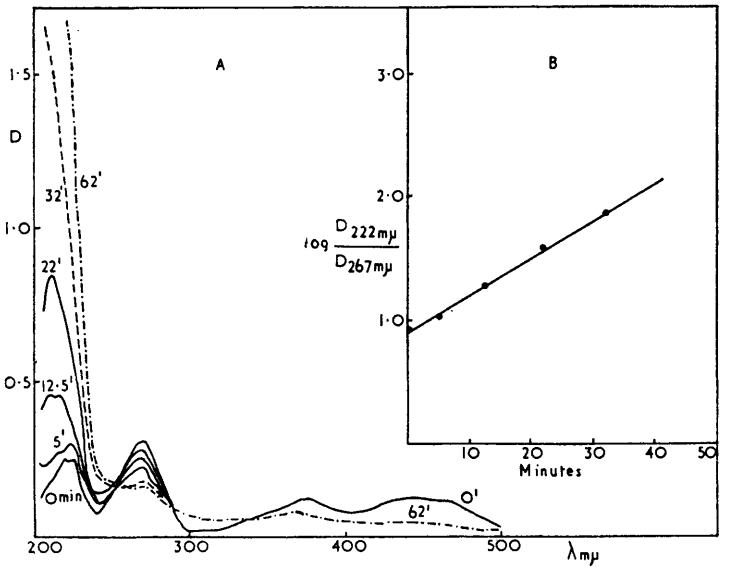

FIG. 8.—Oxidation of riboflavine by ultrasonic waves.

9·7 × 10^{-6} M solution in 0·075 M borate buffer, pH 8·42. U.S. waves of 960 kc/s, 130 W, refrigerated in ice. (A) U.-v. and visible spectrum of riboflavine as a function of time of irradiation. (B) log of ratio of the 222 and 267 mμ bands against time.

effect greatly solubilized the preparation.[6] This caused the apparent activation, which was followed by a rapid inactivation of the fat globule suspension and the purified preparation.[26] This inactivation of enzyme is approximately first-order, in the first 10 to 20 min. K_m varies between 7·7 and 19·1 × 10^{-4} sec^{-1} for the purified preparation and is about 24 to 42 × 10^{-4} for fat globules.

Prudhomme [23] showed that the effect of U.S. waves is diminished by increasing the protein concentration and ionic strength. The mechanism of U.S. inactivation may be due to at least two factors: mechanical degradation and free-radical reactions induced by cavitation. It is difficult to decide upon the exact role played by these two factors in X.O. inactivation. An approximate idea about free radical participation in the inactivation mechanism may be obtained by comparing enzyme inactivation to coenzyme destruction. We carried out some model experiments with riboflavine, part of the X.O. coenzyme. U.V. and visible spectra of riboflavine solutions were obtained as a function of irradiation time.

Fig. 8 gives the results of a typical experiment. The intensity of all but one band decreases with time of irradiation. The intensity of the 222 mμ band increases and the log of the ratio of the two absorption coefficients at 222 and 267 mμ ($\log \epsilon_{222}/\epsilon_{267}$) increases with time according to a first-order mechanism, yielding a rate constant $k_m = 9{\cdot}5 \times 10^{-4}$ sec^{-1} (fig. 8).

Comparison of these data suggests that the rate-determining step in enzyme inactivation is not the same as in riboflavine oxidation. The low rate constant for the milk enzyme, may be explained by the protective effect of proteins. This is substantiated by the much higher rate of inactivation of X.O. in fat globule suspensions than in milk, and by protection of riboflavine by casein against U.S. oxidation: irradiation of riboflavine, $1{\cdot}3 \times 10^{-5}$ M in a 120 mg casein solution at pH 6·5, does not eliminate the fluorescence nor the yellow colour; both disappear in the control solution without casein after 20 min (960 kc/s, 80 W). [A new substance appears in the irradiated riboflavine solution, which migrates very slowly ($R_f \sim 0{\cdot}01$) in butanol + acetic acid + water (4 : 1 : 5) on Whatman no. 1 paper (ascending chromatography); it has a blue fluorescence on the paper and is not identical with lumiflavine or lumichrome.[28]]

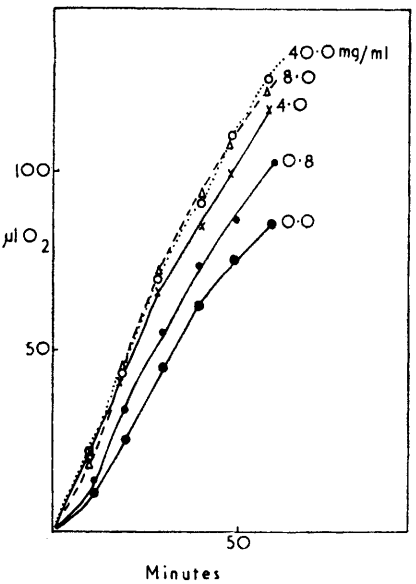

FIG. 9.—Activation of milk X.O. by lauryl sulphate. Manometric method, 1 ml milk (after 8 h at 15° C), 5 μM hypoxanthine, 0·1 mM phosphate buffer, pH 7·2, final volume 3·2 ml, final pH 7·0. Lauryl sulphate as indicated on the figure, 37° C.

After having been left a few hours, milk contains reductases of bacterial origin. These enzymes (as measured by the d.t. of Methylene Blue without any substrate added) are much more sensitive to U.S. waves and to high pressure than X.O.[26, 27] and may be selectively eliminated from contaminated milk.

ACTIVATION AND INACTIVATION OF MILK X.O. BY DETERGENTS

Detergents activate the X.O. of fresh milk.[15, 17, 18] Fig. 9 gives the results of a typical experiment. Fresh milk was partially activated by standing 8 h at 15° C, then mixed in Warburg vessels with increasing amounts of lauryl sulphate, and the rate of H.X. oxidation determined. Activation increases with concentration up to 8 mg/ml of milk; even 40 mg/ml did not inhibit the enzyme. This experiment shows that detergents are more effective as activators than is refrigeration. If 20 ml of fresh milk (at 37° C) are mixed with 8 mg of lauryl sulphate, oxygen consumption is doubled (with 5 μM H.X. as substrate at pH 7·4). Non-ionic detergents such as Tweens have a similar effect.

Fig. 10 gives activation coefficients as a function of time with Tween 60 (as determined by the haemolysis tube method). Similar results are obtained with cationic detergents.[22] The resistance of milk enzyme is relatively great even to this class of substances which have very strong denaturing action. 50 mg of alkyltrimethylammonium chloride (zephirol) per ml of a 5-times washed fat globule suspension—this avoids activation by zephirol—equivalent to 270 mg of detergent per gram of dry weight, effects only 43 % inhibition; 1 mg/ml cream suspension (5·4 mg/g dry weight) gives 10 % inhibition.

DISTRIBUTION OF X.O. IN MILK, WHEY AND FAT GLOBULE SUSPENSION BETWEEN THE PARTICULATE FRACTION AND THE SOLUTION

Morton's interesting work [13] suggested the following experiments, the aim being to decide whether all the X.O. obtained from the fat globules in the activation process is contained in the microsomal or liposomal fraction or if it goes immediately in " true " solution. Earlier work on X.O. purification [12] and its recent work on crystallization [1] leaves little doubt that X.O. can be obtained in true solution free of microsomal particles. On the other hand, even 5-times washed fat globules (0·182 g/ml suspension of cream,

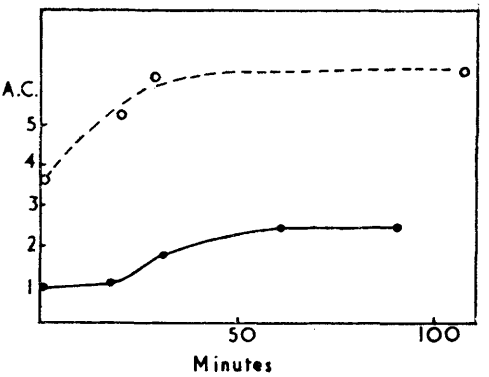

FIG. 10.—Activation of fresh milk X.O. by Tween 60 (monostearate of polyoxymethylene-sorbitane). Abscisses: time of incubation with the detergent at 37° C; ordinates: activation coefficient. Enzyme activity determined by the haemolysis tube method, as in fig. 4. Final concentration of Tween, 5 mg/ml.

washed at 0° with 0·1 M phospate buffer; pH 7·3, centrifuged 30 min at —7°C at 5000 rev/min) retain a considerable activity (175 μl. O_2 consumed in 60 min per g. of dry weight, corresponding to 4550 μl. O_2 per hour per g of non-lipid dry weight. Lipids are extracted by butanol + light petroleum ether + chloroform and methanol.) Table 5 shows the distribution of enzyme activity after centrifugation at 45,000 g (45 min at 0° C) between the different milk fractions.

TABLE 5.—DISTRIBUTION OF ENZYME ACTIVITY AFTER 45,000 g CENTRIFUGATION (45 min at 0° C)

(Enzyme activity measured by the manometric technique with 5 μM hypoxanthine, 0·1 mM of phosphate buffer at 37° C, pH 7·3 in final vol. of 3 ml).

	activity in μl. O_2 absorbed per hour per mg dry weight				
	refrigerated milk	milk and lauryl-sulphate	separated milk	cream* suspension in H_2O	cream† suspension in phosphate
top layer (fat globules)	1·2	1·5	—	0	0·7
liquid layer	0·44	0·6	1·1	2·8	8·2
precipitate	0·16	0·56	1·2	24·0	17·0
precipitate in presence of 0·05 μM Methylene Blue 100 γ-cytochrome C, 1 μM MoO_3	0·55	—	0·9	—	—

* Refrigerated milk (2 h at — 5° C) centrifuged at 5000 rev/min for 30 min to separate the cream layer.

† Fresh cream suspension, twice washed in water, suspended in water, refrigerated at 0° overnight, churned by shaking 15 min, centrifuged at 5000 rev/min at —7° C for 30 min, and the liquid separated; the cream suspended in 0·1 M phosphate buffer, pH 7·3, left at — 2° C for 2 h, centrifuged and separated as before; the top layer activity, in the 5th column refers to this 4-times extracted cream suspension.

It can be seen that the distribution of enzyme activity is different for milk and fat globule suspension. A non-negligible fraction of total activity appears in solution (at a degree of dispersity not sedimented by the gravitational field used). Only in refrigerated cream suspension does the greatest part of activity in the liposomes appear. Lauryl sulphate increases enzyme activity in all 3 fractions. The relatively low figures in table 5 are due to the high lipid and protein content of milk. If this is related to protein

concentration, we find 720-655 μl. O_2 per hour per mg protein for the typical liposomal fractions, and 0·6 μl. O_2 for the "casein" particles of separated milk (see Morton.[13]) So we can confirm all but one of Morton's statements, viz., X.O. seems not to be wholly present in liposomes. There is a non-negligible fraction firmly attached to the fat globules and another fraction which passes directly into solution following activation by cold or other physicochemical means.

DISCUSSION

The related experiments together with earlier work from this laboratory [15] enable us to formulate an hypothesis about the mechanism of the activation process. X.O., in its natural state in milk, seems to be wholly attached to the fat globules by lipoprotein cenapses of varying strength (see scheme). The globule is protected by a peripheral structure, permeable to short-chain molecules (no activation for aldehydes of 2-3 carbon units).

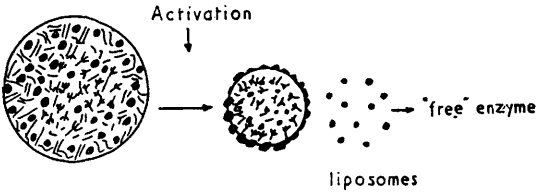

FIG. 11.

On cooling, this protective layer is disrupted, a fraction of the low-melting lipids goes into solution ("oiling off") and the enzyme is partially liberated as liposome complexes which dissociate to yield soluble enzyme. The relatively high energy barrier (20 kcal) of the activation process may be explained by the fission of the lipoprotein complex. The fraction of enzyme which cannot be detached from the fat globule proves the solid character of these cenapses, which could not be due only to adsorption. Activation by detergents substantiates the analogy between these cenapses and serum lipoproteins (Macheboeuf [11]).

We may assign a functional role to the lipoid constituent of the enzyme complex: during Thunberg experiments the reduced Methylene Blue penetrates the fat globules, the leucobasis being much more lipo-soluble than the oxidized dye. This lipo-penetration of the reduced acceptor may displace the oxidation-reduction equilibrium in favour of complete reduction.[20, 21] Whether liposomes are preformed elements existing before activation in the fat globule or adsorbed on it cannot be answered yet. The dependence of the activation on the substrate may be explained by two alternative hypotheses:

(i) a steric hindrance limited to molecules greater than C_3-C_4 aldehyde:
(ii) a change in the structure of the active site during the activation process.

In favour of the second hypothesis we note the experiments of Knobloch [9] on the change in the relative rates of oxidation of acetaldehyde and hypoxanthine [26, 27] during pressure and U.S. activation. Knobloch finds evidence for a different mechanism of oxidation of aldehydes than of purines by milk X.O. Though Knobloch claims the existence of two enzymes, these experiments do not prove this. The probability of the existence of a different mechanism of oxidation for aldehydes may be taken into consideration as an alternative hypothesis.

The reorganization of the structure of fat globules after refrigeration and compression is shown by the studies of King.[8] This author demonstrated the transformation of fat globules into spherical shell-crystals after such treatment. On cooling and compressing fat molecules previously distributed at random undergo a radial orientation. This may be considered as the last stage of structural change before the rupture of the globule, being moreover accompanied by

the separation of " butter oil " (low melting lipids). The thermal activation process may interfere with calculations of yield in enzyme purification [4, 19] and should not be neglected, in such experiments.

It is quite reasonable to assume that milk phosphatase and lipase have physico-chemical structures similar to X.O. and that similar activation of this enzyme might also be expected.[13, 15] Comparative studies on the physical state of X.O. in the mammary gland may throw light on the mechanism of synthesis of this particular enzyme — lipoprotein complex.

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