Comparative Properties of Microsomes from Cow's Milk and from Mammary Gland

1. ENZYMIC ACTIVITIES

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It has been well established that the occurrence of cancer of the mammary gland of certain strains of mice is greatly influenced by a factor which is transmitted to the young via the mother's milk (Bittner, 1936; see also Dmochowski, 1953; Greenstein, 1954). The nature of this 'milk factor' is still uncertain. Huseby, Barnum & Bittner (1950) found that a large proportion of the milk-factor activity of dispersions of mouse mammary gland was sedimented by centrifuging at 23 000 g for 1 hr. Particles of the size-range of viruses and of microsomes were observed in electron micrographs of the precipitate from the mammary gland and also in a precipitate obtained under similar conditions from mouse milk containing the milk factor. Huseby *et al.* (1950) therefore suggested that the milk factor might be associated with microsomes. However, they did not report any examination of milk known to be free of the factor, and they were apparently unaware of the particulate nature of casein in milk.

Morton (1953, 1954*a*) showed that in addition to casein, which represents the major particulate component, normal cow's milk also contains enzymically active lipoprotein particles which he called 'milk microsomes'. Preliminary observations of the chemical and enzymic properties of microsomes from cow's milk and from mammary gland indicated that the milk particles probably originated directly from the secretory cells of the gland (Morton, 1954*a*). It was therefore suggested that the milk factor could be associated with microsomes and thus pass into mouse milk from the mammary gland (Morton, 1953).

This and the following paper report investigations related to this hypothesis. The cow was chosen as the experimental animal because material could be obtained in relatively large quantities. It has been found that, although microsomes from milk and mammary gland are not identical, milk microsomes are probably derived from gland microsomes.

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MATERIALS AND METHODS

Enzyme substrates. Cytochrome *c* was prepared from ox hearts according to Keilin & Hartree (1945). Reduced diphosphopyridine nucleotide (DPNH) was obtained from Boehringer und Soehne, Mannheim, Germany. All other substrates were commercial products.

Inorganic phosphate. This was determined according to Weil-Malherbe & Green (1951).

Phenol. This was estimated as previously described (Morton, 1955*a*).

Nitrogen. This was determined by a micro-Kjeldahl method essentially as already described (Morton, 1955*b*), except that optical densities were measured with a colorimeter (Evans Electroselenium Ltd., with filter no. 627).

Dry weights. The particles in 0.25 M-sucrose were sedimented by centrifuging at 60 000 g for 90 min., suspended in 0.15 M-NaCl (at pH 7.4), and resedimented at 60 000 g for 60 min. The final pellet was dried to constant weight over conc. H₂SO₄ *in vacuo* at room temperature.

Determination of enzymic activities

Alkaline phosphatase. Hydrolase activity was estimated as follows. The enzyme preparation was incubated for 10 min. at 38° with 5 mM-disodium phenyl phosphate, mM-MgCl₂ and 40 mM-ethanolamine-HCl buffer, pH 10.1 at 38°. The total volume was 2 ml., and the amount of enzyme was adjusted so that between 5 and 30 μg. of phenol was liberated in 10 min. Activities are expressed as μmoles of inorganic phosphate liberated/mg. dry wt./hr. at 38°.

Phosphotransferase activity was estimated by measuring phosphorylation of glycerol and of glucose essentially as described by Morton (1955*c*, 1958). With glycerol as acceptor, the reaction mixture (final vol. 0.5 ml.) contained 4 M-glycerol, 0.01 M-phenyl phosphate, 0.02 M-MgCl₂ and 0.05 M-ethanolamine-HCl buffer, pH 10.1 at 38°. The reaction was stopped by addition of 0.1 ml. of 30% (v/v) HClO₄, then a predetermined amount of N-KOH was added to adjust the reaction to pH 7.4; the precipitate of potassium perchlorate was removed by centrifuging, the precipitate washed and the supernatant and washings were made up to 2 ml. The amount of glycerol esterified was calculated as the difference between phenol and inorganic phosphate liberated. With glucose as acceptor, 2 M-glucose replaced glycerol but otherwise the reaction mixture was similar. Glucose 6-phosphate was estimated enzymically by Slater's (1953) procedure.

The percentage transfer was calculated according to Axelrod (1948) as

100 × moles of ester synthesized / moles of phenol liberated.

Dehydrogenases. These were estimated as described by Morton (1954a) by measuring the rate of reduction of 2,6-dichlorophenolindophenol (final concn. approx. 10 mM) at 600 m μ with a Beckman spectrophotometer, model DU. All measurements were made with a final volume of reactants of 3 ml. in cuvettes of 1 cm. light path and at room temperature (approx. 19°). The reaction was started by the addition of substrate, which was omitted from the control cuvette. For anaerobic studies, the measurements were carried out with cuvettes sealed to a Thunberg tube (see Martin & Morton, 1956). The substrate was placed in the hollow stopper, and the assembly evacuated and filled with oxygen-free nitrogen three times and then sealed. The reaction was begun by tipping the substrate from the stopper. As particulate enzyme preparations were used, the contents of the cuvettes were mixed thoroughly between readings. With a volume of 3 ml., the amount of dye reduced (in μ moles) is given by $E_{600} \times 0.218$, where E_{600} is the change in optical density at 600 m μ due specifically to the dehydrogenase activity. The enzyme was diluted to give a change in optical density/min. of about 0.10. Activities are expressed as μ moles of dye reduced/mg. dry wt./hr. at room temperature.

For xanthine dehydrogenase, the reaction mixture contained 13.2 mM-xanthine, 0.03 M-sodium pyrophosphate-HCl buffer, pH 8.1 at 20°, and dye. The stock xanthine was dissolved in 0.05 N-NaOH and an appropriate amount of 0.1 N-HCl was added to the cuvette to neutralize the alkali.

For succinic dehydrogenase, the reaction mixture contained 0.066 M-sodium succinate, 0.05 M-sodium phosphate buffer, pH 7.5 at 20°, and dye.

For DPNH-diaphorase, the reaction mixture contained 30 mM-DPNH, approx. 0.1 M-KCN, 0.05 M-veronal (sodium diethylbarbiturate)-sodium acetate-HCl buffer (Michaelis, 1931), pH 7.5 at 20°, and dye.

Reduced diphosphopyridine nucleotide-cytochrome c reductase. Activity was estimated similarly to dehydrogenase activity, except that cytochrome c replaced the dye and optical densities were measured at 550 m μ . The concentrations of reactants, in a final volume of 3 ml., were as follows: 30 mM-DPNH, 50 μ M-ferri-cytochrome c, approx. 0.01 M-KCN and 0.05 M-veronal-sodium acetate-HCl buffer, pH 7.5 at 20°. With a volume of 3 ml., the amount of cytochrome c reduced (in μ moles) is given by $E_{550} \times 0.154$ (see Morton, 1955c). Activities are expressed as μ moles of cytochrome c reduced/mg. dry wt./hr. at room temperature.

Xanthine oxidase. The activity was measured spectrophotometrically by the method of Kalckar (1947) as modified by Morell (1952). The reaction mixture was the same as used for measurement of xanthine dehydrogenase activity except that the dye was omitted and optical densities at 270 and 290 m μ were measured. Under the experimental conditions, the oxidation of 1 μ g./ml. of xanthine to uric acid gave an increase in optical density of 0.048 at 290 m μ . Results are expressed as μ moles of xanthine oxidized/mg. dry wt./hr. at room temperature.

γ -Glutamyl transferase. Measurement of activity was based on the observations of Hanes, Hird & Isherwood (1952). The appearance of new compounds on paper chromatograms was taken as evidence of enzymic activity. The following reactions were studied.

(a) Hydrolysis of γ -glutamyltyrosine. The reaction

mixture comprised 0.1 ml. of 0.1 M- γ -glutamyltyrosine, pH 7.8, 0.1 ml. of 0.2 M-sodium phosphate buffer, pH 7.8, and 0.1 ml. of microsome preparation.

(b) Transfer to glycine. The reaction mixture comprised 0.1 ml. of 0.1 M-reduced glutathione, 0.1 ml. of 0.5 M-glycine in 0.2 M-sodium phosphate buffer, pH 7.8, and 0.1 ml. of microsome preparation.

(c) Transfer to leucine. The reaction mixture was similar to that for (b) except that 0.1 ml. of 0.15 M-L-leucine in 0.2 M-sodium phosphate buffer, pH 7.8, replaced the glycine solution.

In each case the reaction was carried out under nitrogen in small stoppered glass tubes at 38° for 1 hr. Samples of about 1.5 μ l. were removed at the beginning and after 1 hr. and applied to Whatman no. 1 paper and immediately dried. Appropriate amino acids and peptides were also applied to the paper as markers. The papers, with pads of filter paper clipped to the bottom, were run in propan-1-ol-water (80:20, v/v) at 25° for 40-45 hr., after which they were removed, dried and sprayed with ninhydrin reagent (Hanes *et al.* 1952).

Isolation of microsomes from lactating mammary gland

Preliminary studies. As it was desired to compare the properties of cytoplasmic particles from secretory cells with those of particles from milk, glands from cows in lactation were used. This ensured that there was a high proportion of secretory tissue as compared with connective and muscle tissue. As a result, however, the ducts and collecting vessels of the gland contained a considerable amount of milk, which had to be removed to prevent contamination of the cytoplasmic particles with casein and other particles from the milk. 'Milking-out' the udder before the cow was killed, and perfusion of the excised udder with water or 0.15 M-NaCl soln. removed only part of the milk. It was necessary to wash the minced tissue several times in order to remove extracellular milk.

Several preliminary experiments were carried out to determine a satisfactory means of disruption of the secretory cells with minimum disintegration of connective and muscular tissue, and without disruption of the mitochondria. Disintegrated lipoprotein membranes from mitochondria are in the size range of microsomes and may contaminate this fraction (see Martin & Morton, 1956). Since succinic dehydrogenase activity is localized in the mitochondria in liver (Hogeboom, Schneider & Palade, 1948), it was assumed that it was also localized in the mitochondria of mammary gland. Succinic dehydrogenase activity was therefore used as a guide to the localization of mitochondria (or fragments thereof) in the several fractions separated from dispersions of gland by differential centrifuging. It was found that grinding of the tissue with sand in a pestle and mortar caused considerable disintegration of the mitochondria, since the specific activity of the 'large-particle' fraction was low as compared with that of the 'small-particle' (microsome) fraction. Disintegration in a Waring Blendor for a short period appeared to disrupt sufficient of the secretory cells without disintegrating many mitochondria and this method was therefore used for preparation of the tissue dispersions. Preliminary observations with 0.88 and 0.25 M-sucrose media for preparation of the tissue dispersion showed that enzymic

The washed pieces were minced, and the mince suspended in about ten times its weight of 0.25M-sucrose and collected by filtration through coarse muslin. This was repeated. About 100 g. of mince was then dispersed into about 250 ml. of 0.25M-sucrose with a Waring Blendor at full speed for 30 sec. The dispersion was filtered through muslin, and the retained tissue was returned to the Blendor and retreated as before. The filtrates through muslin were combined and fractionated by differential centrifuging as shown in Fig. 1. The various fractions were washed by suspending them in about 30 ml. of 0.25M-sucrose and re-centrifuging. Owing to a mechanical breakdown in the high-speed centrifuge usually used, the differential centrifuging for cow no. 1 differed somewhat from that for cows nos. 2 and 3, as indicated in Fig. 1. An International Model PR1 centrifuge was used for sedimenting at 1500 g, a Sorvall Model SS2 centrifuge for 19 000 g and a Spinco Model L centrifuge for 50 000, 60 000 and 72 000 g.

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graph TD
    A["Dispersion in sucrose  
(about 600 ml.)"] --> B["Centrifuged  
at 1500 g for 20 min."]
    B --> C["Precipitate of unbroken  
cells, cell debris and  
nuclei. Discarded  
(cow no. 1), or washed by  
suspending in sucrose  
and recentrifuging  
(cow no. 2)"]
    B --> D["Supernatant"]
    D --> E["Centrifuged at  
19 000 g. for 5 min."]
    E --> F["Mitochondria  
Washed by suspending in  
sucrose and recentrifuging  
(cows nos. 1 and 2)"]
    E --> G["Supernatant"]
    G --> H["(Cow no. 1)  
Divided into  
two portions  
and centrifuged"]
    G --> I["(Cows nos. 2 and 3).  
Centrifuged  
at 50 000 g, for  
90 min. (cow no. 2),  
or at 72 000 g, for  
30 min. (cow no. 3)"]
    H --> J["Approx.  
150 ml.  
centrifuged  
at 20 000 g  
90 min."]
    H --> K["Approx.  
400 ml.  
centrifuged  
at 60 000 g for  
30 min."]
    J --> L["Supernatant  
(combined  
with that  
from 400 ml.  
portion)"]
    J --> M["Precipitate"]
    K --> M
    K --> N["Final  
supernatant"]
    M --> O["Combined and  
washed twice  
by suspending  
in sucrose and  
recentrifuging.  
Microsomes  
(cow no. 1)"]
    I --> P["Precipitate.  
Washed twice  
as for cow no. 1.  
Microsomes  
(cows nos. 2 and 3)"]
    I --> Q["Final  
supernatant"]
  
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Fig. 1. Separation of fractions from dispersions of cow mammary gland in 0.25M-sucrose by differential centrifuging. Treatments of the tissue from the cows differed somewhat owing to a mechanical breakdown in a centrifuge. All operations were carried out at 0-4°. 'Sucrose' refers to 0.25M-sucrose at about 0°. The final pellets were suspended in sucrose for enzymic and chemical studies.

Cow 1 was a 6-year-old Friesian-Jersey crossbred which had calved a week before the experiments began. Cow no. 2 was a 5-year-old crossbred which had suffered from mastitis during the previous lactation. However, bacteriological examination showed that there was no active mastitis at the time of these studies. The affected quarter was not used. This cow was also in early lactation. Cow no. 3 was a crossbred in advanced lactation which had been condemned because of a positive tuberculin reaction.

Isolation of milk microsomes

On three different occasions microsomes were isolated from the mixed milk obtained from a Friesian herd. The general procedure followed that of Morton (1954*a*), but differed in the times and speeds of centrifuging. Fig. 2 shows the procedures used.

To enable a direct comparison with gland microsomes, particles were also isolated from the milk of cows nos. 1

and 2. Cow no. 1 was milked twice daily for 4 days before slaughter. On each day the milk samples obtained on that morning and on the previous evening were combined, and microsomes were isolated as shown in Fig. 3. The preparations obtained on each day were held at 0°. They were finally combined and the microsomes again sedimented before use.

The milk obtained from three milkings from cow no. 2 during 36 hr. before slaughter was mixed, and the particles were separated as shown in Fig. 3.

RESULTS

Enzymic activities of microsomes from mixed milk

Table 1 shows the activities of microsomes separated from mixed milk from a herd. Although there is considerable variation in specific activities of the

Fig. 2. Procedure for isolation of particles from mixed milk from a Friesian herd.

microsomes isolated on different occasions, the pattern of the enzymic activities is similar in the three preparations.

Enzymic activities of microsomes from milk and mammary gland of the same cow

Table 2 gives the activities of mitochondria and microsomes from mammary gland, together with the activities of microsomes from milk of the same cow. The results show that the pattern of enzymic activities of microsomes from mammary gland is quantitatively very different from that of microsomes isolated from the milk. In particular, milk microsomes have very much lower DPNH-cytochrome *c* reductase and DPNH-diaphorase

activities, and higher xanthine dehydrogenase activity, as compared with microsomes from the gland. The results for the two cows differ in that the alkaline phosphatase activity of the milk microsomes was lower than that of the gland microsomes for cow no. 1, and higher for cow no. 2.

Effect of incubation on enzymic activities of gland microsomes

The secreted milk is stored in the mammary gland in the lumina of the alveoli, in the small collecting ducts of the lobules and in the milk cistern. The extracellular milk may thus be held at body temperature for up to 12 hr. The

Fig. 3. Procedure for isolation of particles from milk from cows nos. 1 and 2. 'Sucrose' refers to 0.25M-sucrose at about 0°. Final precipitates were suspended in 0.25M-sucrose and held at 0° for use.

effect of incubation for this period on the enzymic properties of gland microsomes was therefore investigated.

Initially, 0.1 ml. of a suspension of gland microsomes from cow no. 1 was mixed with 0.2 ml. of milk serum (see Fig. 3) and incubated at 38° for about 12 hr. The microsomes were then sedimented from the suspension by centrifuging at 30 000 *g* for 1 hr. and suspended in a small volume of 0.25M-sucrose. Activities determined before and after incubation are shown in Table 3. The incubation caused a marked decline in DPNH-cytochrome *c* reductase activity, and a considerable increase in xanthine dehydrogenase activity.

The microsomes and mitochondria from the gland of cow no. 2 were incubated for 12 hr. at 38° with (a) veronal-sodium acetate-HCl buffer, pH 6.6, and (b) milk serum. The pH of the milk serum (6.7) did not change during incubation, indicating that bacterial growth was negligible. After incubation the particles were sedimented from the suspensions by centrifuging and resuspended in 0.25M-sucrose. Enzymic activities were determined before and after incubation and are shown in Table 3. It is seen that the results for particles from cow no. 2 confirm those obtained with cow no. 1, although the activation of the xanthine dehydrogenase activity was less striking.

Table 1. *Enzymic activities of microsomes isolated from mixed herd milk on three different occasions*

Microsomes were isolated as shown in Fig. 2 and enzymic activities were estimated as described in Methods.

Enzymic activity	Results for milk-microsome preparations		
	M ₁	M ₂	M ₃
Alkaline phosphatase			
(a) Hydrolysis of phenyl phosphate*	28.6	31.1	11.6
(b) Transfer to glucose†	—	—	17
(c) Transfer to glycerol‡	—	—	56
Xanthine dehydrogenase‡			
(a) Determined aerobically	5.3	6.8	1.20
(b) Determined anaerobically	—	—	1.60
Xanthine oxidase§	—	—	0.43
DPNH-diaphorase‡	—	—	0.074
DPNH-cytochrome <i>c</i> reductase	—	—	0.004
Succinic dehydrogenase‡	—	—	0

* Inorganic phosphate liberated (μmoles)/mg. dry wt./hr. (at 38°).

† 100 × μmoles of ester P synthesized/μmoles of phenol liberated.

‡ 2,6-Dichlorophenolindophenol reduced (μmoles)/mg. dry wt./hr. (at about 19°).

§ Oxygen reduced (μmoles)/mg. dry wt./hr. (estimated spectrophotometrically at about 18°).

|| Cytochrome *c* reduced (μmoles)/mg. dry wt./hr. (at about 19°).

Table 2. *Comparative enzymic activities of particles isolated from mammary gland and from milk from the same cow*

Cytoplasmic particles were isolated from a dispersion of cow mammary gland as shown in Fig. 1 and from cow's milk as in Fig. 3. Enzymic activities were estimated as described in Methods and are expressed as in Table 1.

Cow no.	Particles	Enzymic activities				
		Alkaline phosphatase*	Xanthine dehydrogenase†	DPNH-diaphorase‡	DPNH-cytochrome <i>c</i> reductase‡	Succinic dehydrogenase‡
1	Milk microsomes	1.14	1.15	0.13	0	0
	Gland microsomes	3.42	0.040	0.58	3.28	0
2	Milk microsomes	9.35	0.670	0.110	0.088	0
	Gland microsomes	2.82	0.046	1.58	7.20	0
	Gland mitochondria	1.81	0.026	1.40	10.10	0.180
3	Gland microsomes	2.05	0.034	—	—	0
	Gland mitochondria	1.72	0.042	—	—	0.086

* Inorganic phosphate liberated (μmoles)/mg. dry wt./hr. (at 38°).

† 2,6-Dichlorophenolindophenol reduced (μmoles)/mg. dry wt./hr. (at about 19°).

‡ Cytochrome *c* reduced (μmoles)/mg. dry wt./hr. (at about 19°).

Table 3. *Effect of incubation on the enzymic activities of cytoplasmic particles from mammary gland*

Microsomes from the gland of cow no. 1 were incubated for 12 hr. at 38° in serum from the milk of the same cow, prepared as shown in Fig. 3. They were then sedimented by centrifuging and suspended in 0.25M-sucrose before testing. Microsomes and mitochondria from cow no. 2 were incubated for 12 hr. at 38° in either veronal-sodium acetate-HCl buffer, pH 6.6, or in milk serum, pH 6.7. In each case the stoppered tube contained chloroform vapour to retard bacterial growth. After incubation the particles were sedimented by centrifuging and suspended in 0.25M-sucrose.

Enzymic activities were determined before and after incubation.

Cow no.	Particles	Incubation treatment	Enzymic activities				
			Alkaline phosphatase†	Xanthine dehydrogenase†	DPNH-diaphorase†	DPNH-cytochrome c reductase‡	Succinic dehydrogenase†
1	Microsomes	*	—	0.4	—	32.8	—
		In milk serum	—	5.0	—	0.9	—
2	Microsomes	*	18.4	0.3	11.0	52.5	—
		In milk serum	29.9	0.9	0.18	0.04	—
		In buffer	29.4	0.6	—	0.04	—
	Mitochondria	*	—	—	—	85.2	1.5
		In milk serum	—	—	—	0.6	0
		In buffer	—	—	—	—	0

* Initial activities before incubation. † Inorganic phosphate (μmoles)/mg. of N/hr. (at 38°).
‡ 2:6-Dichlorophenolindophenol reduced (μmoles)/mg. of N/hr. (at about 18°).
§ Cytochrome c reduced (μmoles)/mg. of N/hr. (at about 18°).

Table 4. *Comparative dehydrogenase activities of microsomes from milk and mammary gland*

Estimations were carried out with microsomes from the milk and mammary gland of cow no. 2 as described in Methods. Conditions for each determination were varied as indicated. The results are expressed as μmoles of dye [2:6-dichlorophenolindophenol (DCIP) or of potassium ferricyanide] reduced/mg. of N/hr. at room temp.

Test conditions			Activities		Quotient Milk activity Gland activity
Substrate	Dye	Other additions	Milk microsomes	Gland microsomes	
Xanthine	DCIP	—	5.3	0.6	9
Xanthine	K ₃ Fe(CN) ₆	—	10.9	0.9	12
Hypoxanthine	DCIP	—	9.1	0.4	23
Hypoxanthine	DCIP	Pteridine*	0	0	—
DPNH	DCIP	—	0.9	11.0	0.1
DPNH	DCIP	Pteridine*	0.9	11.0	0.1

* 2-Amino-4-hydroxy-6-formylpteridine (approx. 7.5 μM).

Comparison of the xanthine dehydrogenase activities of microsomes of milk and mammary gland

Table 4 shows comparative dehydrogenase activities of microsomes from milk and mammary gland as determined with a number of different substrates and acceptors. Owing to the low activity of the gland particles, the activities for these are less reliable than for milk microsomes and this may contribute to the variation in the quotients expressing the relationship between the activities of the two preparations. The results suggest that the xanthine dehydrogenases of the particles from the two sources are identical.

Glutamyl transferase activities of milk and gland microsomes

Activities were estimated as described under Methods. The suspensions of microsomes contained about the same amount of protein. Inspection of

the chromatograms showed that (a) hydrolysis of γ-glutamyltyrosine, (b) synthesis of γ-glutamylglycine, and (c) synthesis of γ-glutamyl-leucine were each catalysed by microsomes from both milk and mammary gland. The amount of product formed in both cases appeared to be about the same, suggesting that the specific activities of the two preparations were similar.

Cytochrome components of milk and gland microsomes

The properties of cytochrome b₅, a haemprotein observed in milk microsomes and subsequently in microsomes from mammary gland and calf intestine, have already been described (Baillie & Morton, 1955). The microsomes from the two sources have very different appearances. When packed as a precipitate, those from milk have a distinct red colour whereas those from mammary gland are buff-coloured. When observed with a

low-dispersion microspectroscope, microsomes from milk show only a weak and diffuse band at about 552–555 $m\mu$, and a very weak α -band of reduced cytochrome b_5 at 557 $m\mu$. The intensities of these bands are unchanged on addition of sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$). In contrast, gland microsomes show no absorption band when freshly isolated. However, addition of $\text{Na}_2\text{S}_2\text{O}_4$ reveals a strong α -band of reduced cytochrome b_5 at 557 $m\mu$. The broad band of the reduced pyridine haemochromogen at 555–558 $m\mu$ formed by addition of alkaline pyridine and $\text{Na}_2\text{S}_2\text{O}_4$ is much more intense in gland than in milk microsomes. No other cytochrome components could be detected in milk or gland microsomes. However, when reduced with $\text{Na}_2\text{S}_2\text{O}_4$, mitochondria from cow mammary gland show typical absorption bands of cytochromes of the a , b and c types.

DISCUSSION

Although it would have been desirable to use milk and mammary glands from mice for this investigation, with the facilities available it was not possible to obtain sufficient mouse milk for the isolation of milk microsomes. Undoubtedly milk microsomes occur in mouse milk as in cow's milk, but as yet they have been isolated only from the latter source (Morton, 1953, 1954*a*; Bailie & Morton, 1955 and this paper; Zittle, Della Monica, Custer & Rudd, 1956).

The close similarity of sedimentation characteristics of the smaller casein particles to those of microsomes makes it difficult to isolate microsomes free of casein. No simple means for assessment of the amount of contamination of the microsome fraction by casein has yet been found. Attempts were made to separate casein particles in milk from microsomes by adjusting milk to pH 9.6 with N-NaOH , by chelation of Ca^{2+} ions with sodium ethylenediaminetetra-acetate at pH 7, and by coagulation with rennin. These methods were only partly successful. Hence the milk microsomes were isolated from the cream fraction essentially as previously described (Morton, 1954*a*). However, dispersion of casein particles by removal of Ca^{2+} ions with sodium citrate or with sodium ethylenediaminetetra-acetate may be useful for isolation of the microsome fraction from mouse milk. The considerable variation in the specific enzymic activities of microsomes isolated from different milks (cf. Tables 1 and 2) may partly be due to varying contamination with casein. As far as possible, extracellular milk was washed from the pieces of secretory tissue but undoubtedly milk remained in the cells of the alveoli. Thus it is probable that the gland microsomes were also somewhat contaminated with casein particles.

In general, the enzymic activities of the microsomes isolated from mixed milk (Table 1) conform with the pattern of activities described by Morton (1954*a*). The alkaline phosphatase activity is rather higher, and the xanthine dehydrogenase activity lower, than that found in microsomes isolated from Cambridge milk. The much lower alkaline phosphatase activity of the microsomes isolated from the milks from cows nos. 1 and 2 (Table 2) may be due to the fact that both these cows were in early lactation. Folley & Greenbaum (1947) and Moore & Nelson (1952) have shown that activities of a number of cytoplasmic enzymes of guinea-pig mammary glands change considerably during pregnancy, lactation and involution of the gland. In the present investigation it was observed that microsomes obtained from the gland of a cow not in lactation had very low alkaline phosphatase activity (about 0.4 μmole of inorganic phosphate liberated/mg. dry wt./hr. at 38°) and negligible xanthine dehydrogenase activity.

Relationship between xanthine dehydrogenase and reduced diphosphopyridine nucleotide-diaphorase activities of milk

It is considered by Corran, Dewan, Gordon & Green (1939) and by Mackler, Mahler & Green (1954) that milk xanthine dehydrogenase catalyses oxidation of both xanthine and DPNH. However, the results in Table 4 confirm earlier findings of Lowry, Bessey & Crawford (1949) that 2-amino-4-hydroxy-6-formylpteridine very strongly inhibits activity with hypoxanthine but does not affect activity with DPNH. It must be concluded therefore that either the one protein has two different substrate-activating centres (Mackler *et al.* 1954) or that two different enzymes are involved in the activation of hypoxanthine and of DPNH (Morell, 1952). This latter view is very strongly supported by the finding that the xanthine dehydrogenase activity of gland microsomes increases during incubation, whereas the diaphorase activity falls very considerably (Table 3).

Relationship between microsomes from mammary gland and from milk

The pattern of the enzymic activities of the microsomes from mammary gland is so different from that of the microsomes from milk (Table 2) that it might be supposed that there is little relationship between the two. In particular, milk microsomes have very low DPNH-cytochrome c reductase and DPNH-diaphorase activities as compared with gland microsomes. However, as shown by Table 3, these enzymic activities could be almost completely lost during incubation of extracellular milk within the gland (or after ejection of the milk).

Of greater interest is the very high xanthine dehydrogenase activity of the microsomes from milk, as compared with those from mammary gland (Table 2). There is little doubt that the enzyme of milk is identical with that of mammary gland (see Table 4). Surprisingly, the xanthine dehydrogenase activity of mammary gland is fairly low, the specific activity of the whole dispersion of the gland of cow no. 2 being only about $0.07 \mu\text{mole}$ of 2:6-dichlorophenolindophenol reduced/mg. dry wt./hr. Preliminary studies of the distribution of activity indicated that about 50 % of the activity was localized in the 'final supernatant' fraction (cf. Fig. 1), and the remainder was distributed among each of the other particulate fractions. However, in view of an unavoidable time delay during fractionation of the gland dispersion, these results require confirmation.

The high activity of the microsomes of milk could arise from adsorption of 'soluble' xanthine dehydrogenase. However, such adsorption seems unlikely since the casein particles of milk, which would present a much greater surface for adsorption, have very low activity (Morton, 1954*a*). An increase in xanthine dehydrogenase activity during incubation of gland microsomes was obtained with particles from both cows (Table 2). This activation could be due to (a) removal of a natural inhibitor associated with the enzyme in the gland or (b) to non-specific effects of incubation on the enzyme protein. There is no evidence that the gland enzyme is associated with a natural inhibitor, although this cannot be excluded, particularly in view of the finding of Swartz, Kaplan & French (1956) that many bacterial enzymes are associated with inhibitors which are dissociated by heat. However, it is well established that the activity of xanthine oxidase in milk is markedly influenced by physical factors. Dixon & Kodama (1926) first reported that the activity of freshly drawn milk increases on storage. Investigations by French workers (see Robert & Polonovski, 1955) have shown that this and similar activations induced by cooling of milk, addition of detergents, use of ultrasonic vibrations and other factors are due to a change in the physico-chemical state of the enzyme protein. The association of the milk microsomes with milk-fat globules (Morton, 1954*a*; Zittle *et al.* 1956) greatly influences the behaviour of the xanthine dehydrogenase (Robert & Polonovski, 1955). In view of the increased xanthine dehydrogenase activity obtained on incubation of gland microsomes (in contrast with the decline of activity of the other dehydrogenases studied), and the findings of Robert & Polonovski (1955), it may be concluded that the amount of xanthine dehydrogenase protein in gland microsomes is not necessarily different from that in milk microsomes. Adsorption of

gland microsomes to the fat globules, and other changes occurring after secretion and before removal of the milk from the udder, could lead to the high xanthine dehydrogenase activity characteristic of milk microsomes.

Table 2 shows that the alkaline phosphatase activity of milk microsomes is not very different from that of gland microsomes, being somewhat less (cow no. 1) or more (cow no. 2) active. In one other case there was little difference in the activities of the particles from the two sources. Although a slight increase of phosphatase activity of gland microsomes was caused by incubation (Table 3), this effect is very much smaller than occurs with xanthine dehydrogenase. The alkaline phosphatase activity of cow mammary gland is mostly associated with the cytoplasmic particles (Morton, 1954*b*).

The above considerations show that the differences in enzymic activities of the microsomes from milk and mammary gland could arise after release of gland microsomes into the secreted fluid, milk.

The absence of succinic dehydrogenase activity in the particles isolated from milk was formerly considered as evidence that mitochondrial fragments do not contribute to the microsome fraction of milk (Morton, 1953, 1954*a*). However, Table 3 shows that the succinic dehydrogenase activity of gland mitochondria is destroyed during incubation. Hence this negative finding does not exclude that mitochondria (or fragments of mitochondria) could contribute to the microsome fraction of milk. However, the occurrence of cytochrome b_5 and the absence of other cytochromes in milk microsomes clearly establishes that these are largely, if not entirely, derived from gland microsomes, since cytochrome b_5 is localized in this fraction of the gland, and does not occur in mitochondria (Bailie & Morton, 1955; see also Martin & Morton, 1957). Much of the cytochrome b_5 of gland microsomes is denatured by incubation, forming a denatured protein haemochromogen with diffuse absorption bands similar to those seen in the milk microsomes. The red-brown colour of isolated milk microsomes may partly be due to this denatured cytochrome b_5 . However, since xanthine dehydrogenase forms 3-5 % of the protein of milk microsomes (see Morton, 1954*a*; Morell, 1955) it seems more likely that the colour arises from the very high concentration of this red flavoprotein and the particular physical state of the milk microsomes.

SUMMARY

1. Microsomes were isolated from mixed milk from a commercial herd and from milkings from individual cows before slaughter. Microsomes and mitochondria were also isolated from the mammary glands of the same cows.

2. Enzymic activities of the particles were compared. Whereas milk microsomes have high alkaline phosphatase and xanthine dehydrogenase, together with low reduced diphosphopyridine nucleotide-diaphorase and reduced diphosphopyridine nucleotide-cytochrome *c* reductase activities, gland microsomes have about the same alkaline phosphatase, much less xanthine dehydrogenase, and much more diaphorase and reduced diphosphopyridine nucleotide-cytochrome *c* reductase activities.

3. It was found that incubation of gland microsomes in milk serum or in buffer at pH 6.7 caused changes in enzymic activities to a pattern similar to that of milk microsomes. The considerable increase in xanthine dehydrogenase activity is probably due to changes in the physical state of the enzyme.

4. Cytochrome *b₅* occurs in milk microsomes, although most of the haemprotein is denatured. Since this cytochrome is restricted to the microsome fraction of mammary gland, milk microsomes must arise mainly from this source. Changes occurring during storage of the extracellular milk in the gland could account for the different pattern of enzymes in milk and gland microsomes.

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Comparative Properties of Microsomes from Cow's Milk and from Mammary Gland

2. CHEMICAL COMPOSITION*

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In the preceding paper (Bailie & Morton, 1958) it was shown that the differences in the enzymic activities of microsomes isolated from milk and from mammary gland are probably caused by changes which occur after secretion of the milk.

* Part 1: Bailie & Morton (1958).

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Adsorption of the microsomes by the fat globules, and the action of enzymes of the milk microsomes themselves as well as of enzymes of the milk serum, probably account for the changes in enzymic activities.

The chemical compositions of the particles from the two sources were compared at the same time. The milk and mammary-gland microsomes differed considerably in their content of nucleic acid. However, it was found that this difference could be due