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THE BIOCHEMICAL AND ULTRASTRUCTURAL LOCALISATION OF PHOSPHOPROTEIN-CONTAINING PARTICLES IN LACTATING MAMMARY GLANDS OF RATS

By

W. C. Tan, Stretton Young, I. J. Goldsmith and D. C. Livingston

ABSTRACT

Portions of rat mammary glands taken during lactation were subjected to homogenisation and to electron microscopy. Biochemical evidence after homogenisation indicated that the phosphoprotein content was highest in the cellular supernatant fraction. Electron microscopy revealed numerous spherical structures in the supernatant fraction and in milk. These spherical structures were similar in size to the protein droplets that are found in the cytoplasm of mammary epithelium during lactation and which have been shown to contain abundant phosphoprotein. This is further evidence for the hypothesis that the spherical protein particles contain casein.

Casein is a phosphoprotein that forms about 80 per cent of the protein content of milk. It is synthesised by the mammary epithelium and during lactation can be detected intracellularly and within the lumena of the mammary acini. The exact localisation of sites of formation is important when studying the reactions of this protein.

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Compact particles inside the cytoplasmic vacuoles of lactating mammary epithelium have been confirmed to be “protein droplets” (Wellings & Philp 1964), but reports on histochemical, ultrastructural and biochemical studies of these protein droplets have so far been lacking. Young & Nelstrop (1969, 1970) have detected casein in rat mammary glands by immunofluorescence microscopy. During lactation fluorescence was found along the acinar border of the secretory epithelium and on the granular material within the acini. Since casein is a phosphoprotein, acid phosphatase can cause the phosphate to be released which in turn forms a precipitate of lead phosphate in the presence of lead. Recently, we have followed this reaction by electron microscopy to localise casein droplets in the secretory epithelium (Tan et al. 1971). In this report, we attempt to correlate biochemical, ultrastructural and histochemical findings concerning the cellular localisation of casein particles.

MATERIALS AND METHODS

Electron microscopy

Mammary glands were removed from Sprague-Dawley rats during the 3rd–7th day of lactation. Portions of the tissue were fixed in 2.5 per cent glutaraldehyde in cacodylate buffer at 2–4°C and post fixed in 1 per cent osmium tetroxide buffered at pH 7.4 with veronal-acetate buffer. The tissue was dehydrated in increasing concentrations of alcohol, and embedded in Araldite. Ultrathin sections were cut with a diamond knife on an LKB ultramicrotome and silver sections were stained for 6 min with lead citrate (Reynolds 1963). Aliquots of tissue homogenates (see below) and of milk were negatively stained on carbon-coated grids by a two per cent solution of sodium phosphotungstate at pH 7.2. Grids were viewed with a Siemen’s Elmiskop 1 electron microscope at 80 kV and photographs were taken on Ilford N50 plates.

Histochemistry

A solution of acid phosphatase was prepared at about 0.5 unit per ml using 0.1 M acetate buffer at pH 5 as solvent and for treating control sections. The sections were first stained with lead citrate. Copper grids supporting the lead-stained sections were then placed on the surface of the acid phosphatase solution for 20 min at room temperature with the tissue side downward. The sections were rinsed with three successive changes of triple distilled water each of 5 min by floating the grid on the surface of the water with the tissue side downward. They were then dried and examined (see Tan et al. 1971).

Cell fractionation

Buffer solution was prepared containing potassium chloride 0.15 M, sodium phosphate 0.004 M and imidazole 0.01 M at a pH of 6.6. Lactating mammary glands were homogenised (Potter-Elvehjem) at 2–4°C in this buffer in the ratio of 1 g of tissue to 20 ml of buffer solution. The homogenate was centrifuged at 800 g for 10 min, 7000 g for 15 min and 105 000 g for 60 min to remove the cellular fractions and fat.
layers. Pellets of each cellular fraction were extracted with chloroform-methanol (2:1) to remove lipids.

Casein assay based on the method of Juergens et al. (1965) and phosphoprotein assay on tissue based on the improved method of Decker (1958) and Decker & McMahon (1969) have both been described in detail in a previous publication (Tan et al. 1972).

**RESULTS**

**Casein and phosphoprotein assay on cellular fractions**

The phosphoprotein assay was used for the estimation of the casein content of all the cellular fractions from the mammary glands. As in the case of the supernatant fraction, the rennin Ca++ method was used to precipitate the proteins, but, not all the proteins precipitated were phosphoprotein or casein. Since only a fraction of the total precipitate was phosphoprotein (Tan et al. 1972) phosphoprotein determination on the total protein precipitates of the supernatant fraction was essential.

The total phosphoprotein content was higher in glands from lactating than from non-lactating pregnant rats which in turn had values higher than those from virgin animals (Tables 1 and 2). About half of the total cellular phospho-

**Table 1.**

Phosphoprotein content expressed as mg per g of tissue, in mammary gland cellular fractions from rats in various physiological states.

<table>
<thead>
<tr>
<th>State of rats</th>
<th>800 × g (nuclei)</th>
<th>7000 × g (mitochondria)</th>
<th>105 000 × g (ribosomes)</th>
<th>Supernatant fractions</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virgin</td>
<td>1.15</td>
<td>0.09</td>
<td>0.56</td>
<td>1.67</td>
<td>3.47</td>
</tr>
<tr>
<td>Virgin</td>
<td>2.37</td>
<td>0.04</td>
<td>0.02</td>
<td>2.59</td>
<td>5.02</td>
</tr>
<tr>
<td>Pregnant</td>
<td>2.78</td>
<td>0.50</td>
<td>2.23</td>
<td>3.29</td>
<td>8.81</td>
</tr>
<tr>
<td>3 days lactation</td>
<td>2.16</td>
<td>0.80</td>
<td>1.95</td>
<td>6.67</td>
<td>11.58</td>
</tr>
<tr>
<td>3 days lactation</td>
<td>4.09</td>
<td>0.97</td>
<td>2.48</td>
<td>7.50</td>
<td>15.04</td>
</tr>
<tr>
<td>7 days lactation</td>
<td>0.82</td>
<td>1.02</td>
<td>3.35</td>
<td>14.46</td>
<td>19.65</td>
</tr>
<tr>
<td>7 days lactation</td>
<td>3.62</td>
<td>1.78</td>
<td>7.94</td>
<td>11.39</td>
<td>24.73</td>
</tr>
<tr>
<td>7 days lactation</td>
<td>3.24</td>
<td>4.11</td>
<td>5.97</td>
<td>12.29</td>
<td>25.61</td>
</tr>
<tr>
<td>20 days lactation</td>
<td>0.49</td>
<td>0.59</td>
<td>2.59</td>
<td>11.24</td>
<td>14.91</td>
</tr>
</tbody>
</table>

All the values were obtained from supernatant fluid after centrifugation of homogenate at 105 000 g for one hour. At least 3 rats were used for each experiment. Tubes in triplicate were used in assay.
Table 2.
Phosphoprotein content expressed as percentage of total phosphoprotein in mammary gland cellular fractions from rats in various physiological states.

<table>
<thead>
<tr>
<th>State of rats</th>
<th>$800 \times g$ (nuclei)</th>
<th>$7000 \times g$ (mitochondria)</th>
<th>$105,000 \times g$ (ribosomes)</th>
<th>Supernatant fractions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virgin</td>
<td>33.14</td>
<td>2.59</td>
<td>16.14</td>
<td>48.13</td>
</tr>
<tr>
<td>Virgin</td>
<td>47.21</td>
<td>0.80</td>
<td>0.40</td>
<td>51.59</td>
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<tr>
<td>Pregnant</td>
<td>31.59</td>
<td>5.68</td>
<td>25.34</td>
<td>37.39</td>
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<tr>
<td>3 days lactation</td>
<td>18.65</td>
<td>6.91</td>
<td>16.84</td>
<td>57.60</td>
</tr>
<tr>
<td>3 days lactation</td>
<td>27.19</td>
<td>6.45</td>
<td>16.49</td>
<td>49.87</td>
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<td>7 days lactation</td>
<td>14.64</td>
<td>7.20</td>
<td>32.10</td>
<td>46.06</td>
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<tr>
<td>7 days lactation</td>
<td>12.65</td>
<td>16.05</td>
<td>23.31</td>
<td>47.99</td>
</tr>
<tr>
<td>20 days lactation</td>
<td>3.29</td>
<td>3.95</td>
<td>17.37</td>
<td>75.39</td>
</tr>
</tbody>
</table>

All the values were obtained from supernatant fluid after centrifugation of homogenate at $105,000 \times g$ for one hour. At least 3 rats were used for each experiment. Tubes in triplicate were used in assay.

Protein was found in the supernatant fraction. The remainder was distributed in the nuclear, ribosomal and mitochondrial fractions. In the case of lactating mammary glands the ribosomal fraction seemed to have the next highest phosphoprotein content. This suggests that the bulk of the casein is present in the epithelial cell cytoplasm.

Electron microscopy
Protein droplets with diameters ranging from 100 to 300 nm were normally seen inside the cytoplasmic vacuoles of the lactating mammary epithelium and within the alveolar lumina. These droplets had the mosaic appearance regarded as characteristic of casein. Negatively stained aliquots of the supernatant fractions of lactating mammary glands contained spherical particles whose diameters also ranged from 100 to 300 nm (Fig. 1). These particles corresponded in shape and size with the protein droplets inside the vacuoles and free in the cytoplasm of the mammary epithelium (Fig. 2). Similar particles were also seen in similar negatively stained preparations from rat milk (Fig. 3).

Since casein is a phosphoprotein and contains phosphate groups in monooester form its molecules can be hydrolysed by acid phosphatase. The phosphate groups thus released react with lead already present in the stained section to form precipitates of insoluble lead phosphate (Tan et al. 1971). After incuba-
Fig. 1.
Electron micrograph (EM) of negatively stained particles from supernatant fluid of homogenised rat mammary gland 3 days after start of lactation.
Sodium phosphotungstate (S. P.) × 30,000.

Fig. 2.
Rat mammary epithelium 3 days after start of lactation. Protein particles within the cytoplasmic vacuoles. EM. Uranyl acetate. Lead citrate. × 30,000.
Fig. 3.
Negatively stained spherical particles from rat milk. EM, S. P. × 30,000.

Fig. 4.
Protein particles located in cytoplasm and within cytoplasmic vacuoles of rat mammary epithelium 3 days after start of lactation. Increased electron density of protein droplets. EM, L. C., acid phosphatase. × 30,000.
tion of the lead-stained sections with acid phosphatase, the protein droplets in
the mammary epithelium showed increased electron density owing to deposits
of lead phosphate (Fig. 4).

**DISCUSSION**

The biochemical estimation of casein is normally carried out by the rennin
Ca$^{2+}$ method and the precipitated proteins are accepted as the casein content.
In a previous study it has been shown that not all proteins precipitated by this
method are casein (*Tan et al.* 1972). We have therefore carried out further
phosphoprotein determinations to obtain a more accurate assessment of the
casein content. The pellet obtained after centrifugation of the mammary homogenate of 800 g was conventionally designated nuclei and cell debris. Any
phosphoprotein detected in this fraction could be due to nuclear proteins or
unbroken cells. The phosphoprotein content was lowest in the mitochondrial
fraction obtained at 7000 g. The ribosomal fraction, obtained after centrifugation at 105 000 g contained a substantial amount of phosphoprotein especially
during lactation. This reflects not only the increase in the synthesis of casein
that occurs at that time, but also suggests the possible site of its phosphorylation. Most of the detectable phosphoprotein, however, was found in the supernatant fraction, corresponding to a location in the matrix of the cytoplasm
within the cell.

It seems reasonable that casein should be found in the cytoplasm of the
lactating mammary epithelium. The first appearance of protein particles coincides with the start of lactation, and they are found within the vacuoles and
free in the matrix of the epithelial cytoplasm of lactating glands. Tacitly, these
protein droplets have been regarded as casein particles (*Wellings & Philp*
1964). Since these protein particles are present in the cell cytoplasmic matrix,
they may be expected to be found in the supernatant fraction after homogeniza-
tion and their presence there has been detected by electron microscopy after
negative staining. From examination of the supernatant fraction and of milk
by this means the shape and size of the protein droplets have been seen to
correspond to those of the protein droplets observed in intact cells. Thus,
spherical structures with diameters ranging from 100 to 300 nm have been
observed from three sources, the supernatant fraction of homogenised glands,
the milk, and the cytoplasmic vacuoles of lactating mammary epithelium.

The detection of phosphate in casein particles can be achieved by utilising
the enzyme action of acid phosphatase. The phosphate radicles released by
hydrolysis react with lead made available from previous staining to give rise
to deposits of inorganic lead phosphate. These are electron-dense and can be
seen on the cut surfaces of the protein droplets in the epithelial cytoplasm
(*Tan et al.* 1972). Droplets of similar size are found in milk and the super-
natant fluid derived from cell homogenisation. During lactation the latter has by far the highest phosphoprotein content of any of the cell fractions examined. This combined biochemical, ultrastructural and histochemical evidence lends strong support to the belief that the protein droplets seen in the cytoplasm of lactating mammary epithelium are partly or wholly casein in composition.

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REFERENCES

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