

## Effect of Milk Constituents on Hepatic Cholesterogenesis<sup>1</sup>

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### ABSTRACT

Utilization of the liver slice technique enabled the isolation of two inhibitory preparations from bovine skim milk which were active in reducing hepatic cholesterol biosynthesis. One of the inhibitors was in the dialyzable fraction and was identified as orotic acid (OA). The other inhibitor, present in the nondialyzable fraction, was not exclusively identified. Preliminary data indicated that it was of high molecular weight, polar, and was neither classical protein nor lipid. This material migrated as one band in polyacrylamide disc gel electrophoresis. Alkaline hydrolysis followed by formation of the methyl esters, GLC and mass spectral analyses indicated that the fraction contained nitrogenous compounds of high molecular weight. Orotic acid appeared to act primarily by inhibiting cholesterol biosynthesis before the formation of mevalonic acid; whereas the nondialyzable inhibitor exerts its effect beyond the formation of mevalonic acid in the biosynthetic pathway. Human milk was also found to inhibit the incorporation of both labeled acetate and mevalonic acid into cholesterol by the liver. Orotic acid could not be the inhibitor in human milk since the samples employed in the study did not contain this compound. Administration of OA-6-<sup>14</sup>C to rats results in its conversion to uracil by the liver. Subsequent work demonstrated that uracil exerted an inhibitory action on hepatic cholesterol biosynthesis similar to that of OA when both were incubated with rat liver slices.

### INTRODUCTION

Several studies on the effects of milk on hepatic cholesterogenesis and blood cholesterol concentrations have been reported. Shah (16) reported that the incorporation of <sup>14</sup>C-labeled mevalonic acid into non-saponifiable fat (NSF) and digitonin-precipitated sterol was lower in liver homogenates from suckling rats, as compared to that in liver homogenates from weaned rats. He suggested that after weaning, there was an increase in the activities of one or more of the enzymes catalyzing the conversion of mevalonic acid into squalene and squalene into cholesterol. McNamara *et al.* (14) reported that rat milk contained a thermostable, nondialyzable protein which depressed

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the activity of hydroxymethylglutaryl coenzyme A reductase (HMG-CoA) in the liver of adult rat. In 1974, Mann and Spoerry (12) studied adult individuals of Masai African tribes to determine whether consumption of fermented milk affects serum cholesterol levels. They reported a decline in the level of blood serum cholesterol when yogurt was consumed by these individuals. In the same year, Boguslawski and Wrobel (6) reported the presence of a compound in bovine and human milk that inhibited liver cholesterogenesis in the rat. This compound was found to be heat stable, dialyzable and present in the supernatant fraction after protein precipitation with either 5% trichloroacetic acid or 5% lactic acid. Its mode of action was the reduction of the rate of incorporation of both sodium acetate and mevalonic acid into liver cholesterol. These findings were supported by studies reported by Malinow and McLaughlin (11). They reported that when 2-week old rats were fed diets containing bovine skim milk for a period of 4 to 7 weeks, there was a significant reduction in blood plasma cholesterol level, as compared to rats fed similar diets with water only. Subsequent studies were conducted by Bernstein *et al.* (4), using liver homogenates from rats, to identify the inhibiting factor in bovine milk, as was described by Boguslawski and Wrobel (6). They suggested the factor to be OA. Furthermore, these studies showed that when liver homogenates were treated with either cultured buttermilk, bovine milk or OA, there was a significant reduction in the incorporation into cholesterol of only labeled sodium acetate, rather than mevalonic acid. Consequently, these workers suggested that the inhibition occurred at the initial steps in the biosynthetic pathway of cholesterol.

The objectives of these studies reported herein were to isolate and define the factor(s) in bovine milk that inhibits hepatic cholesterol biosynthesis, and to determine at which step in this pathway such factor(s) exert its inhibitory action.

## MATERIALS AND METHODS

### Isolation of cholesterol inhibitors from milk

One hundred ml of bovine pasteurized skim milk was dialyzed in a seamless cellulose dialyzing tube which retained materials of 12,000 and higher molecular weight. The dialysis was performed for 48 h at 4°C against two changes of distilled water (500 ml each). The dialyzable inhibitor (OA) was isolated by a combination of anion exchange column chromatography and silicic acid column chromatography (1). The residue from the latter column, after evaporation of the solvent, was utilized for either incubation purposes or quantitative analysis by GLC as previously described (1). The nondialyzable inhibitor fraction was obtained by the following technique: Casein was removed from the nondialyzable fraction of skim milk by reducing the pH to 4.6 with concentrated HCl and centrifuging at 17,600 g for 20 min. The supernatant was freeze-dried and the resulting product solubilized in 10 ml Tris buffer (pH 7.5). Prior to subsequent fractionation, the solution was centrifuged again in order to insure the removal of precipitated casein.

A glass column (100 cm × 2.5 cm) was packed to a height of 90 cm with Sephadex G-100 (Pharmacia Fine Chemicals, Inc.) and equilibrated with distilled water for 4 h. A 2.5 ml portion of the sample was placed on the column, and Tris buffer, at pH 7.5, was the eluent for the entire run. Flow rate was maintained at 2 to 2.5 ml/min. Twenty ml portions were collected, using an ISCO Model 328 fraction collector. The effluent was monitored at 280 nm with an ISCO UA-5 absorbance monitor and ISCO Type 4 optical unit which was coupled with peak separator and strip chart recorder.

Seven pooled fractions were dialyzed separately against tap water for 48 hrs and against distilled water for another 48 h. Subsequently, the seven fractions were freeze-dried and held until they were reconstituted in 2.5 ml incubation buffer for testing their effect on cholesterol biosynthesis.

### Tissue incubation studies

Rate of Wistar strain, 6–8 weeks old, were fed standard rat chow and water *ad libitum*. Liver tissue was immediately obtained from sacrificed male rats. The excised livers were quickly rinsed in cold 0.9% NaCl and liver slices of 0.5 mm in thickness were prepared with a hand-operated Stadie Riggs tissue slicer. The slices were weighed and immediately transferred to incubation vials with the final volume adjusted to 3 ml with either Krebs-Ringer bicarbonate buffer or a modified buffer of Lakshmanan *et al.* (10). The incubations were conducted in a Dubnoff metabolic shaker incubator of 37°C for 3 h in an atmosphere of 95% O<sub>2</sub> and 5% CO<sub>2</sub>, and atmospheric air when CO<sub>2</sub> was collected.

The incubations of the liver tissue slices were terminated by the addition of 4 ml of 20% KOH in methanol. For the determination of radioactivity associated with cholesterol, 4 mg of nonradioactive cholesterol carrier was mixed with the sample before being saponified for 3 h at 65°C in a Dubnoff metabolic shaker. The NSF was extracted by three successive 15-ml aliquots of hexane which were combined and evaporated to dryness under nitrogen. Subsequently, the NSF residue was dissolved in 5 ml of 1% digitonin in 50% ethanol which was permitted to stand for 12 h at room temperature. The digitonides were recovered by centrifugation for 10 min in a clinical centrifuge. The precipitates were washed once each in acetone: ethyl ether (1:2 v/v), ethyl ether, and petroleum ether, and then dissolved in 3 ml of hot methanol and added to 10 ml of Quantafluor for radioactivity determination.

### Oral administration of Orotic Acid-6-<sup>14</sup>C

In another experiment, 5  $\mu$ ci of OA-6-<sup>14</sup>C in 2 ml of water was administered to each of six rats by syringe. One animal was sacrificed at hourly intervals post feeding. Immediately after sacrificing, the livers were removed and rinsed in Krebs-Ringer bicarbonate solution. Following weighing of the livers, portions were assayed for total radioactivity. The remainder was homogenized in 30ml of Krebs-Ringer bicarbonate solution to which 0.5 mg of nonradioactive OA carrier was added. Subsequently, the samples were dialyzed for 48 h at 4°C against two changes of distilled water (250 ml each). The dialyzate was retained for the determination of radioactivity associated with OA and uracil.

The liver homogenate dialyzate was applied to an anion exchange column for isolation of OA and uracil. The OA-containing fraction was further purified as described elsewhere (1).

The uracil-containing peak from the Dowex column was collected, washed twice with ethyl ether:petroleum ether (1:1 v/v), and concentrated to 4 ml, using a Buchi Rotavaporator. Additional purification of uracil was performed, on a Waters Associates High Pressure Liquid Chromatography (HPLC)\*. The samples were applied to the column and eluted with distilled water. The effluent was monitored at a wave length

\*High-pressure liquid chromatography of waters associates, Model 6000, equipped with a bond pack C<sub>18</sub> column (PN 27324), purchased from Waters Associates, Milford, MA.

of 280 nm. The uracil-containing peak with a retention time of 5 min was trapped manually and counted for radioactivity.

Uracil was the only radioactive compound in this fraction. This finding was confirmed by trapping and counting all of the components separated by GLC. Uracil identity was established conclusively by mass spectrometry.

### Analytical technique\*\*

The quantitation of cholesterol was performed on triplicate liver slices obtained from the same rat liver used in the incubation. Following saponification and extraction with hexane, the material was dried and dissolved in a suitable volume of petroleum ether. Cholesterol was analyzed quantitatively by GLC, employing a glass column (183 cm  $\times$  2 mm) and a silicone-coated packing (SP 2100), 3% on 80/100 mesh Supelcoport (Supelco, Inc., Bellefonte, Pa.). Nitrogen, at 45 ml/min, served as the carrier gas. The analyses were conducted isothermally with a column temperature of 255°C, detector temperature of 290°C and injection port temperature of 270°C. (Hewlett Packard, Model 5750). For each set of samples, a linear standard curve was obtained by injecting known quantities of cholesterol (Supelco, Inc., Bellefonte, Pa.) in a concentration ranging from 0.18 to 1.4 mg.

Radioactive  $\text{CO}_2$  was determined using the method of Baruch and Chaikoff (3). The method of Shapiro *et al* (17) was applied for the isolation and determination of mevalonic acid. Where the amounts of radioactivity in neutral lipids (NL), free fatty acids (FFA), and phospholipids (PL) were determined, the incubations were terminated by immersing the sample vials in dry ice-acetone for 30 min. After thawing at room temperature, the total lipids were extracted by a modified Roesse-Gottlieb method (2). Total PL were isolated by a modified Hirsch and Ahrense (9) for silicic acid chromatographic procedure. The NL and FFA were separated by the method of McCarthy and Duthie (13).

## RESULTS AND DISCUSSION

Liver slices were selected as the system for monitoring various fractions for their inhibitory action of cholesterol synthesis. Liver slices consistently produced greater total incorporation of radioactive substrate than did Liver homogenates. In addition, it was believed that the control incubations should exhibit sufficient metabolic activity before a meaningful interpretation of inhibition could be possible. For this reason, a modified Lakshmanan *et al.* (10) media was used to replace Krebs-Ringer bicarbonate media during the investigation. This change was necessitated when it was found that the new media increased the incorporation of labeled substrate into cholesterol.

Different methods were used for the quantitation of cholesterol, and for determination of its  $^{14}\text{C}$  content. The digitonide method was employed so that tracer contaminants would not give false data on the amount of radioactivity in cholesterol. The GLC method permitted accurate quantitation of small amounts of cholesterol. This latter method could not be used for isolation and subsequent radioactivity measure-

\*\* Radioactive sodium acetate-1- $^{14}\text{C}$ , mevalonic acid-2- $^{14}\text{C}$  and OA-6- $^{14}\text{C}$  were purchased from New England Nuclear Corp., Boston, MA. All chemicals were reagent grade and organic solvents were redistilled in glass. All radioactivity measurements were conducted with a Tri-Carb Liquid Scintillation Spectrometer Model 3330. Quench corrections were made using external standardization.

ments since the cholesterol molecule attaches to the solid support and replaces stationary phase during gas chromatography. This was detected by mass spectrometry which showed that when free cholesterol was injected on the column, a mass spectrum of stationary phase, rather than cholesterol, was obtained when the peak entered the ionization chamber.

Table 1 shows the results of incorporation of labeled acetate into cholesterol by rat liver slices incubated with or without the addition of either 100  $\mu$ l of bovine skim milk, the dialyzable fraction of bovine skim milk, the non-dialyzable fraction of bovine skim milk, human milk or OA. The data indicated that the presence of all additives resulted in an inhibitory effect on the incorporation of acetate into cholesterol. The dialyzable and nondialyzable bovine milk fractions displayed an inhibition of 27.3 and 25.4%, respectively. Similarly, human milk reduced acetate incorporation in the cholesterol by 27.8%. Orotic acid was isolated from bovine milk and quantified through dialysis, anion exchange column chromatography, silicic acid column chromatography, and GLC, as previously described (1). Reference OA was treated as a sample through this isolation technique, omitting only the dialysis step. The product from this treatment and the isolated OA from skim milk were tested for their inhibition of cholesterol biosynthesis along with reference OA. The results are presented in Tables 2

Table 1 Incorporation of sodium acetate-1- $^{14}$ C into cholesterol by 500 mg rat liver slices during 3 hours incubation with or without various additives.

| Additive <sup>a</sup><br>(100 $\mu$ l) | Cholesterol<br>(dpm/mg) | Average<br>inhibition<br>(%) |
|----------------------------------------|-------------------------|------------------------------|
|                                        | (mean $\pm$ SD)         |                              |
| Buffer                                 | 43,892 $\pm$ 530        | —                            |
| Skim milk                              | 35,809 $\pm$ 1,990      | 18.4                         |
| Dialyzable skim milk                   | 31,891 $\pm$ 461        | 27.3                         |
| Non-dialyzable skim milk               | 32,751 $\pm$ 4,876      | 25.4                         |
| Human milk                             | 31,764 $\pm$ 540        | 27.6                         |
| Orotic acid<br>(7.3 mg/100 ml buffer)  | 29,352 $\pm$ 9,598      | 33.1                         |

<sup>a</sup>Each additive was incubated in a total volume of 3 ml Krebs-Ringer bicarbonate buffer, pH 7.4, containing 5  $\mu$ ci of sodium acetate-1- $^{14}$ C (average of 2 replicates).

Table 2 Effect of orotic acid (isolated from skim milk versus reference) on the incorporation of sodium acetate-1- $^{14}$ C into cholesterol by 200 mg rat liver slices during 3 hours incubation.

| Additive <sup>a</sup>               | Amount of<br>additive<br>( $\mu$ g) | Cholesterol<br>(dpm/mg) | Average<br>inhibition<br>(%) |
|-------------------------------------|-------------------------------------|-------------------------|------------------------------|
|                                     |                                     | (mean $\pm$ SD)         |                              |
| Buffer                              | —                                   | 5,857 $\pm$ 112         | —                            |
| Orotic acid isolated from skim milk | 25                                  | 3,672 $\pm$ 40          | 37.3                         |
| Reference-technique orotic acid     | 25                                  | 4,544 $\pm$ 194         | 22.4                         |
| Reference orotic acid               | 25                                  | 4,272 $\pm$ 171         | 27.1                         |

<sup>a</sup>Each additive was incubated in a total volume of 3 ml Lakshmanan (10) buffer, pH 7.2, containing 3  $\mu$ ci of sodium acetate-1- $^{14}$ C (average of 2 replicates).

and 3. These data substantiate the claim that OA is the dialyzable inhibitor in bovine milk (4). The values obtained for the references OA put through the isolation procedure eliminated the possibility of contamination by trace reagent which might interfere with the incorporation of acetate into cholesterol.

The nature of the effects of milk, milk fractions, or OA on  $^{14}\text{C}$ -acetate metabolism in cholesterol biosynthetic pathway was studied by collecting  $^{14}\text{C}$ - $\text{CO}_2$  (Table 4), and measuring the amounts of  $^{14}\text{C}$  incorporation in NL, FFA and PL fractions (Table 5).

Table 4 showed that the pattern of cholesterol inhibition by various additives was similar to that in Table 1. For the control, the incorporation of acetate into cholesterol was considerably less than that obtained in previous trials. Such a low incorporation might be due to the lack of continuous flushing with high  $\text{O}_2$  tension atmosphere. Except for OA, the marked inhibitions of cholesterol biosynthesis by all other additives were associated with two or four folds increases in liberation of  $^{14}\text{C}$ - $\text{CO}_2$ , as compared to the control. These findings suggested that a pure OA has a different inhibitory action on acetate metabolism than actions exerted by the various milk fractions.

Data presented in Table 5 showed that the presence of OA in the media resulted in an increase up to 20 to 32% of acetate incorporation in the NL fraction over that present in either FFA or PL fractions. These results are in agreement with those reported by Standerfer and Handler (18) which showed that rats developed fatty livers when were fed diets containing 1% OA.

The results presented in Tables 1, 2 and 3 and those reported by Bernstein *et al.* (4) suggest that OA exerts its inhibitory action on hepatic cholesterol biosynthesis at the early steps of the enzymatic pathway. These suggestions were further investigated by incubating liver slices with or without 50  $\mu\text{g}$  of reference OA, using  $^{14}\text{C}$ -acetate as a substrate. The quantity of radioactivity transferred into mevalonic acid was determined after 1, 2 and 3 h of incubation. The results are presented in Figure 1. The presence of OA caused a great reduction in the synthesis of mevalonic acid from acetate. Inhibition averages at 1, 2 and 3 h of incubation were 72, 57 and 68%, respectively. These results indicated that OA might exert its inhibitory action at the HMG-CoA reductase level. However, Bernstein *et al.* (5) suggested that OA or bovine milk inhibited cholesterol biosynthesis at the acetyl-CoA synthetase level. The results presented here showed that OA inhibited cholesterol synthesis from acetate but at the same time more carbon was incorporated into  $\text{CO}_2$  (Table 4) and into fatty acids of NL fraction (Table 5). In both cases, the transfer of  $^{14}\text{C}$  would require the initial step of acetyl-CoA formation. When labeled acetate was substituted for by labeled mevalonic acid as a substrate in a subsequent study, the same additives elicited different inhibitory patterns on the incorporation of  $^{14}\text{C}$  into cholesterol (Table 6). Skim milk, the nondialyzable fraction of skim milk and human milk all inhibited the incorporation of mevalonic acid into cholesterol; whereas the presence of the dialyzable fraction of skim milk and OA had no inhibitory action.

The differences between the incorporation of acetate and mevalonic acid into cholesterol indicated that bovine milk contained more than one inhibitory constituent that affected hepatic cholestrogenesis. One inhibitor was a dialyzable compound with low molecular weight (6) and appeared to be OA. Its inhibitory action is exerted at the level prior to the formation of mevalonic acid (4). The second inhibitor was non-dialyzable compound, with high molecular weight, and exerted its inhibitory action after the formation of mevalonic acid. These data conflict with earlier reports. Boguslawski and Wrobel (6) reported the inhibitor in bovine milk to be dialyzable and affecting the incorporation of both acetate and mevalonic acid into cholesterol. Bernstein *et al.*

Table 3 Effect of orotic acid (isolated from skim milk versus reference) and uracil on the incorporation of sodium acetate-1-<sup>14</sup>C into cholesterol by 100 mg liver slices during 3 hours incubation.

| Additive <sup>a</sup>               | Amount of additive (μg) | Cholesterol (dmp/mg) | Average inhibition (%) |
|-------------------------------------|-------------------------|----------------------|------------------------|
|                                     |                         | (Mean ± SD)          |                        |
| Buffer                              | —                       | 126,042 ± 2,478      | —                      |
| Orotic acid isolated from skim milk | 50                      | 57,039 ± 4,167       | 54.7                   |
| Reference-technique orotic acid     | 50                      | 48,479 ± 2,439       | 61.5                   |
| Reference orotic acid               | 50                      | 59,506 ± 4,757       | 52.9                   |
| Reference uracil                    | 50                      | 59,412 ± 5,180       | 52.9                   |

<sup>a</sup>Each additive was incubated in a total volume of 3 ml Lakshmanan (10) buffer, pH 7.2, containing 3 μci of sodium acetate-1-<sup>14</sup>C (average of 3 replicates).

Table 4 Incorporation of sodium acetate-1-<sup>14</sup>C into cholesterol and CO<sub>2</sub> by 100 mg rat liver slices during 3 hours of incubation with or without various additives.

| Additive <sup>a</sup> (100 μl) | Cholesterol (Total dpm) | Average inhibition (%) | Cholesterol (dpm/mg) | Carbon dioxide (Total dpm) |
|--------------------------------|-------------------------|------------------------|----------------------|----------------------------|
|                                | (Mean ± SD)             |                        | (Mean ± SD)          | (Mean ± SD)                |
| Buffer                         | 12,961 ± 1,450          | —                      | 1,672 ± 187          | 13,395 ± 3,193             |
| Skim milk                      | 2,140 ± 229             | 83.5                   | 276 ± 29             | 42,994 ± 5,989             |
| Dialyzable skim milk           | 1,581 ± 48              | 87.8                   | 204 ± 7              | 35,365 ± 9,494             |
| Non-dialyzable skim milk       | 1,811 ± 102             | 86.0                   | 234 ± 13             | 35,312 ± 5,545             |
| Whole milk                     | 2,897 ± 600             | 77.6                   | 374 ± 77             | 47,623 ± 6,161             |
| Yogurt (5 mg/5 ml buffer)      | 2,000 ± 231             | 84.6                   | 258 ± 29             | 25,525 ± 6,743             |
| Orotic acid (5 mg/5 ml buffer) | 176 ± 29                | 98.6                   | 23 ± 4               | 15,224 ± 1,850             |

<sup>a</sup>Each additive was incubated in a total volume of 3 ml Krebs Ringer, calcium-free bicarbonate buffer, pH 7.2, containing 3 μci of sodium acetate-1-<sup>14</sup>C (average of 3 replicates).

Table 5 Effect of orotic acid (isolated from skim milk versus reference) on the incorporation of sodium acetate-1-<sup>14</sup>C into various lipid classes by 200 mg rat liver slices during 3 hours of incubation.

| Additive <sup>a</sup>               | Amount of additive (μg) | Distribution of radioactivity in lipids |                |               |
|-------------------------------------|-------------------------|-----------------------------------------|----------------|---------------|
|                                     |                         | Free fatty acids                        | Neutral lipids | Phospholipids |
|                                     |                         | dmp/200 mg tissue (mean ± SD)           |                |               |
| Buffer                              | —                       | 2,113 ± 239                             | 3,922 ± 960    | 2,047 ± 17    |
| Orotic acid isolated from skim milk | 25                      | 2,152 ± 516                             | 5,188 ± 187    | 1,886 ± 239   |
| Reference-technique orotic acid     | 25                      | 1,753 ± 324                             | 4,721 ± 416    | 1,591 ± 35    |
| Reference orotic acid               | 25                      | 1,637 ± 334                             | 5,165 ± 88     | 1,785 ± 257   |

<sup>a</sup>Each additive was incubated in a total volume of 3 ml Lakshmanan (10) buffer, pH 7.2, containing 3 μci of sodium acetate-1-<sup>14</sup>C (average of 2 replicates).

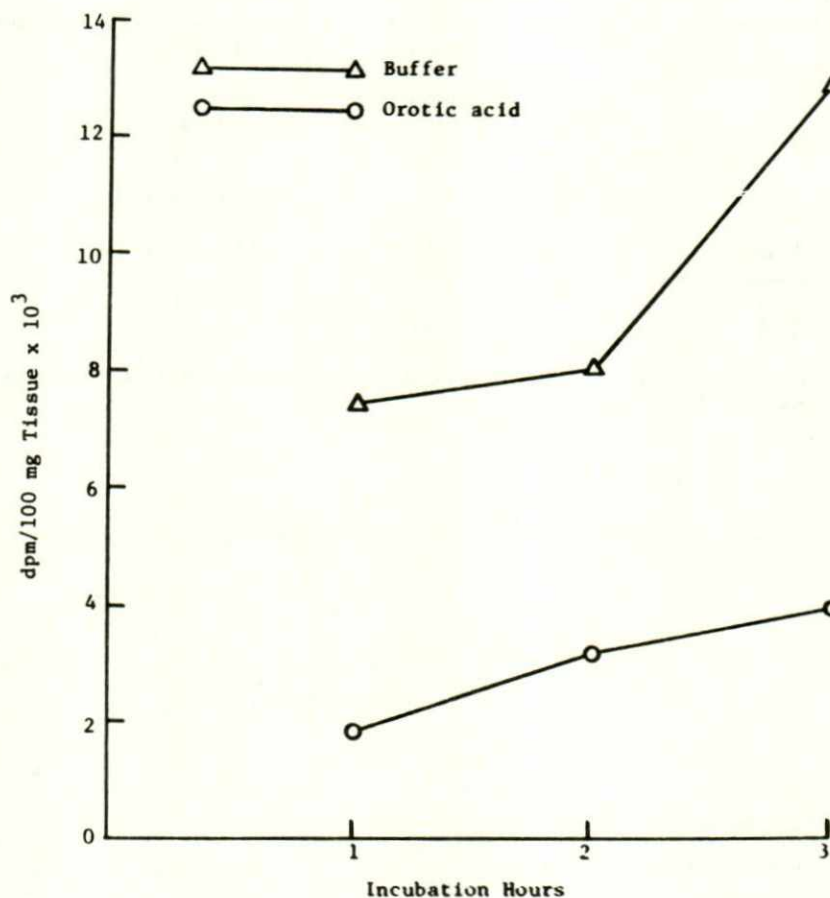


Fig. 1. Effect of orotic acid on the incorporation of sodium acetate-1-<sup>14</sup>C into mevalonic acid by 100 mg rat liver tissue (duplicate analyses).

Table 6 Incorporation of mevalonic acid-2-<sup>14</sup>C into cholesterol by 500 mg rat liver slices during 3 hours incubation with or without various additives.

| Additive <sup>a</sup>              | Cholesterol<br>(dpm/mg) | Average<br>inhibition<br>(%) |
|------------------------------------|-------------------------|------------------------------|
|                                    | (Mean ± SD)             |                              |
| Buffer                             | 158,227 ± 33,170        | —                            |
| Skim milk                          | 123,021 ± 7,481         | 22.2                         |
| Dialyzable skim milk               | 158,363 ± 31,914        | —                            |
| Non-dialyzable skim milk           | 116,568 ± 35,914        | 26.3                         |
| Human milk                         | 126,276 ± 42,541        | 20.2                         |
| Orotic acid<br>(7.3/100 ml Buffer) | 178,411 ± 20,749        | —                            |

<sup>a</sup>Each additive was incubated in a total volume of 3 ml Krebs Ringer bicarbonate buffer, pH 7.4, containing 1  $\mu$ ci of mevalonic acid-2-<sup>14</sup>C (average of 2 replicates).

(4) indicated that bovine milk had no effect on the incorporation of mevalonic acid into cholesterol.

The elution pattern from the liquid-exclusion chromatography of the nondialyzable fraction is illustrated in Figure 2. Seven fractions were collected and their relative inhibitory activity on cholesterol biosynthesis was determined. Preliminary data suggested that most of the inhibitory activity in the nondialyzable portion of the bovine skim milk was in the G100-F<sub>7</sub> fraction. The presence of this fraction in rat liver slices caused a reduction of 44.5% in the amount of cholesterol synthesized during a 3 h incubation rat liver slices (Table 7).

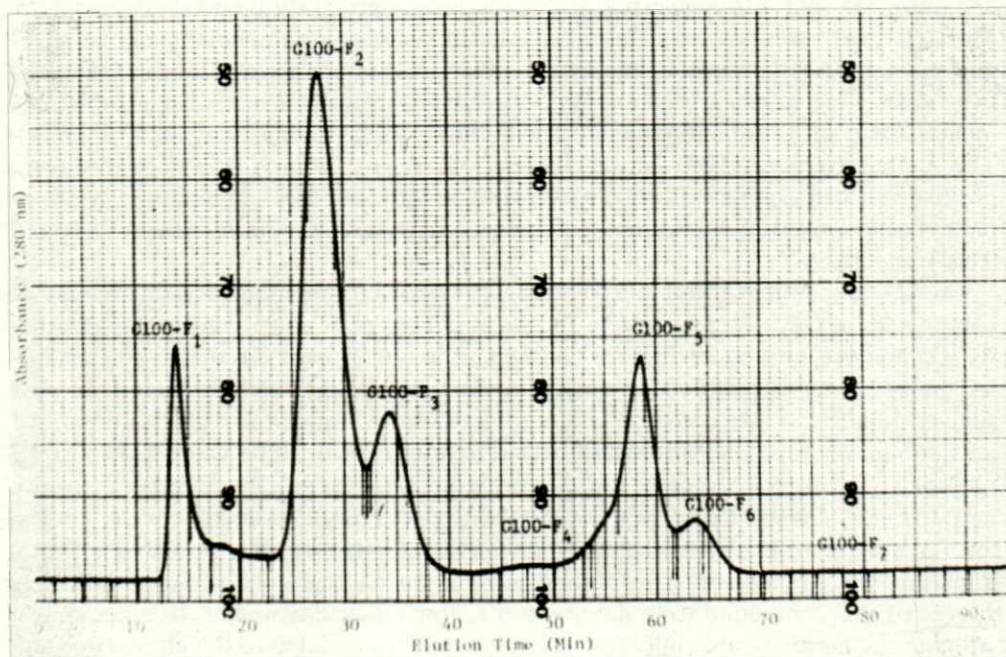


Fig. 2. Elution patterns of the nondialyzable decaseinated fraction of bovine skim milk detected at 280 nm from Sephadex-G100 column chromatography. Fractions G-100 (F<sub>1</sub>-F<sub>7</sub>) were collected separately for incubation.

Table 7 Effect of non-dialyzable skim milk G100-F<sub>7</sub> fraction on the incorporation of sodium acetate-1-<sup>14</sup>C into cholesterol by 100 mg rat liver slices during 3 hours incubation.

| Average <sup>a</sup> | Amount of additive (μg) | Cholesterol (dpm/mg) | Average inhibition (%) |
|----------------------|-------------------------|----------------------|------------------------|
|                      |                         | (Mean ± SD)          |                        |
| Buffer               | —                       | 126,042 ± 2,478      | —                      |
| G100-F <sub>7</sub>  | 200                     | 69,984 ± 4,700       | 44.5                   |

<sup>a</sup>Each additive was incubated in a total volume of 3 ml of Lakshmanan (10) buffer, pH 7.2, containing 3 μCi of sodium acetate-1-<sup>14</sup>C (average of 3 replicates).

In order to characterize the G100-F<sub>7</sub> fraction, several techniques were used including gel electrophoresis, amino acid analysis, gas-liquid chromatography, thin layer chromatography and mass spectrometry. The preparation migrated as one band in polyacrylamide gel. Qualitative analysis indicated the absence of most amino acids, with trace amounts of lysine, proline, cysteine, valine and methionine. Ammonia peak was larger than any of the peaks of the above-mentioned amino acids. When methyl esters of the fraction were compared with methyl esters of conventional fatty acids by GLC, the retention times did not correspond. The nonmethylated G100-F<sub>7</sub> fraction gave no response on GLC. The absence of fatty acids in the methylated G100-F<sub>7</sub> inhibitor fraction was confirmed by GLC-mass spectroscopy. The mass spectra of some of the compounds eluted from GLC showed molecular weights of  $m/e$  311, 325, 339, 353, 367, 381 and 409, suggesting a one methylene group difference between them. Moreover, the mass spectra data indicated that each compound contained one nitrogen molecule. Crude G100-F<sub>7</sub>, methyl esters of G100-F<sub>7</sub>, and methyl esters of standard fatty acids were streaked on TLC plates precoated with silica gel G, using a development solvent of petroleum ether : diethyl ether : glacial acetic acid 180:20:2 (v/v/v). The methyl esters of G100-F<sub>7</sub> and the methyl esters of standard fatty acids gave a positive test with iodine, but the crude G100-F<sub>7</sub> left only white area core. The  $R_f$  of methyl esters of G100-F<sub>7</sub> and methyl esters of fatty acid standards were 0, 0.88, and 0.66, respectively.

The above-mentioned preliminary data suggested that there might be one or several compounds in G100-F<sub>7</sub> responsible for the inhibitory action of cholesterol biosynthesis. Techniques must be devised for additional fractionation of G100-F<sub>7</sub> with each isolate being biologically tested for its activity. As with OA, it should eventually be possible to isolate the active component(s) in G100-F<sub>7</sub>. McNamara *et al.* (14) described an inhibitor in rat milk for HMG-CoA reductase *in vitro* which was nondialyzable, present in the protein fraction (following extraction with an ethanol-ether mixture), and was heat stable. Migicovsky and Madhosingh (15) isolated an inhibitor of cholesterol synthesis from the mitochondria of livers of starved rats by dialysis, tryptic digestion, precipitation at pH 3.0, and column chromatography on Sephadex. They suggested this compound to be lipoprotein of low molecular weight. It is not known whether the nondialyzable inhibitor in bovine milk was related to the above two compounds.

The results presented in Table 1, and those reported by other workers (6) indicated that the inhibition of cholesterol biosynthesis by human milk might be due to the same factor present in the nondialyzable fraction of bovine milk. Such conclusion is further supported by reports of previous workers (1,8) which showed human milk to contain none to only trace amounts of OA.

Figure 3 shows total radioactivity and radioactivity in OA and uracil in the livers of six rats at various times following the oral administration of OA-6-<sup>14</sup>C. Total radioactivity and the radioactivity in OA were at their maximum levels at 2 h post-dosing. After that period, the total radioactivity was stabilized, whereas the OA activity declined throughout the experimental period. The <sup>14</sup>C content of uracil was much greater than that observed for OA throughout the period. The percent of radioactivity in OA of the total activity for the six animals were 0.02 and 0.01% in the 1 and 2 hr rats, respectively, and less than 0.01% in the 3, 4, 5 and 6 hr rats. The corresponding values for radioactive uracil were 35, 17, 14, 66, 22 and 20%. These data indicated that OA was rapidly absorbed from the gut with substantial portion being metabolized by either

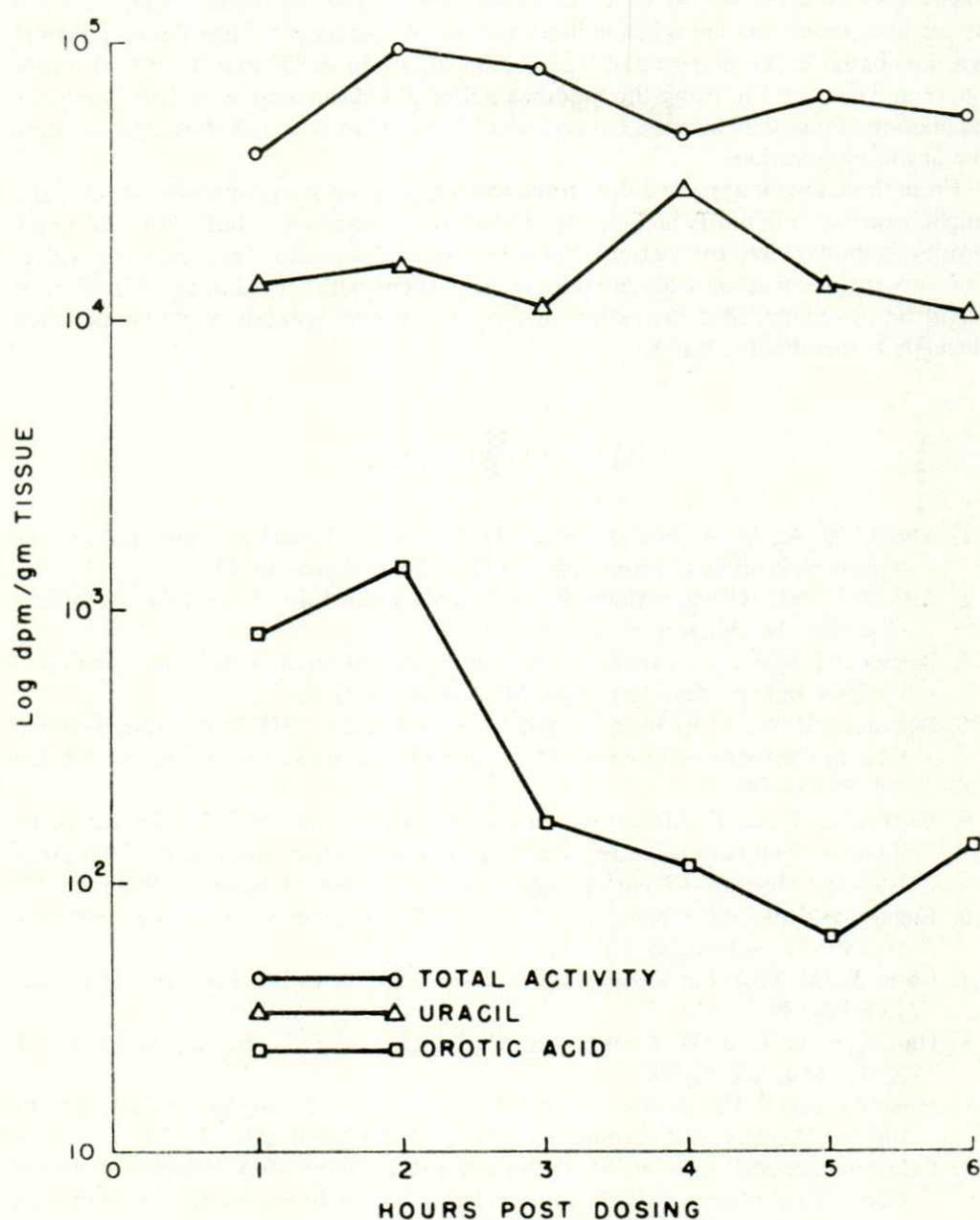


Fig. 3. Radioactivity in orotic acid, uracil and total activity in the livers of six rats at various times following the oral administration of 6  $\mu$ ci orotic acid-6- $^{14}$ C.

incorporation into pyrimidines (7) or being catabolized by the tissues. A high proportion of the administered tracer was recovered as uracil in the liver tissue, which might have occurred via the decarboxylation of OA. The conversion of OA to uracil by rat liver tissue was investigated in an additional experiment. Liver tissues (500 mg) was incubated in the presence of 0.5  $\mu$ ci labeled OA under O<sub>2</sub> and CO<sub>2</sub> (95:5) atmosphere at 37°C for 3 h, using the modified buffer of Lakshmanan *et al.* (10). Duplicate incubations showed an average conversion of 5.2% of OA to uracil, thus substantiating the *in vivo* observations.

From these data it appeared that uracil was an easily derived metabolite of OA, and might exert an inhibitory action on cholesterol biosynthesis. Both OA and uracil produced similar inhibitory actions (Table 3) when each was added at similar concentrations to the incubation vials containing liver slices with <sup>14</sup>C-acetate. Therefore, it could be postulated that the action of OA on cholesterologenesis might be mediated through its metabolite, uracil.

#### LITERATURE CITED

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## تأثير مكونات اللبن على التخليق الحيوي

### للكوليسترول في الكبد

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### المستخلص

أمكن عزل اثنين من المركبات المثبطة ذات القدرة على الحد من تخليق الكوليسترول الكبدي حيويًا ، وجدنا في اللبن البقر والفرز . وقد أمكن تمييز أحد هذين المركبين كحمض الأورتك الذي استقر في الشق القابل للتحليل الغشائي عقب إجراء عملية الفرز بالانتشار الغشائي ، أما المركب المثبط الثاني الذي اتضح عدم قابليته للتحليل الغشائي فقد تعذر التعرف على كنهه ، إلا ان الاختبارات الأولية توحي بأنه مركب قطبي ذو وزن جزئي مرتفع لا ينتمي الى قسم معين من أقسام البروتينات أو الليبيدات . وهذا المركب الأخير يظهر كجزمة واحدة تحركت بتأثير الالكترافوريزس . وبالتحليل المائي القلوي تم تكوين الاسترات المثيلية وإجراء التحليل الكروماتوجرافيا في الغازي والتحليل الطيفي اتضح ان هذا الشق الأخير يحتوي على مركبات نتروجينية ذات وزن جزئي مرتفع . ويبدو أن حمض الأورتك يمارس فعله مبدئيًا بتثبيط التخليق الحيوي للكوليسترول قبل أن يتكون حمض الميفالونيك بينما الشق غير القابل للتحليل الغشائي يمارس نشاطه عقب تكوين حمض الميفالونيك خلال مراحل التخليق الحيوي وقد أمكن اثبات قدرة لبن الأم الادمي على حجب تدخل كلا من الخلايا المشعة وحمض الميفالونيك في تكوين الكوليسترول في الكبد . ولما كانت عينات اللبن الادمي التي استخدمت في هذه

الدراسة تخلص تماما من حمض الاورتك فلا بد وأن يكون العامل المثبط في هذا اللبن  
ذا طبيعة أخرى . وفي حالة اعطاء فيران التجارب حمض الاورتك به كربون مشع  
ك ١٤ يتحول هذا الحامض الى يوراسيل في الكبد ، والآخر أمكن اثبات فعله المثبط  
على تخليق الكوليسترول الكبدي حيويا اسوة بحمض الاورتك بتحضين كلا المركبين  
مع شرائح كبد الفأر .