Galactothermin, a Reversibly Heat-Precipitable Protein of Human Milk at Neutral pH

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1. A protein, aggregating at body temperature and solubilizing when cooled, was isolated from fresh human milk at neutral pH and studied for some of its physical, chemical and immunological properties. The name 'galactothermin' is proposed for this protein. 2. Isolation and purification of galactothermin involved casein removal from skim milk at pH 4.64 followed by centrifugal fractionation of residual protein-containing solutions repeatedly heated and cooled between 40°C and 0°C at pH 7.3. 3. The molecular weight by ultracentrifugal analysis and the minimum molecular weight by sum of amino acid residues were 11400 and 14000 respectively. The sedimentation coefficient $s_{25,w}$ was 1.05S and the diffusion coefficient was 7.15×10^{-7} . Reversible aggregation is favoured by increase in protein concentration, ionic strength, temperature, time and approach to the isoionic point of 7.27 from either acidic or alkaline conditions. 4. Among the amino acid residues, proline predominates and non-polar species account for two-thirds of the total. Cysteine and cystine are absent. Analysis of galactothermin showed it to be essentially free of hexose, sialic acid, calcium and phosphate. 5. Galactothermin is antigenic to the rabbit as evidenced by the passive cutaneous anaphylaxis test. Single precipitin lines are produced in immunodiffusion tests. 6. By electrophoresis in polyacrylamide gel at pH 4.0 only one sharp band is produced.

Preparatory to an investigation of human milk as a bacterial growth medium, we centrifuged a sample at 30000g at 5°C for 30 min to defat it and obtained a clear product suitable for turbidimetric determination of cell population increase during culture. The milk used was a thawed sample from a freshly drawn frozen supply and had pH 7.0. After centrifugation in a plastic tube, the tube was punctured and the cold water-clear product collected in a second tube held in the hand. Examination of the tube contents showed a cloudiness, which increased in density with prolonged holding of the collection tube. We recooled the skim milk and noted that the cloudiness disappeared. By repeated heating to body temperature and re-cooling in ice we found that the alternation of cloudy to clear was a reliably reversible phenomenon. By maintaining the temperature of the skim milk at 37-40°C during subsequent centrifugation at 30000g for 30 min, the insoluble material was obtained as a whitish precipitate, which became a clear gel on cooling in ice. The gel dissolved in cold water to give a solution whose turbidity responded to temperature changes as did the original skim milk. Repeated high-speed centrifugations at 40°C, with an interspersed spin at 5°C

to free the material of trace contamination insoluble at low temperatures, resulted in a purified product from human milk possessing a remarkable negative temperature coefficient of gelation. In recognition of the source of this reversibly heat-precipitable protein of human milk and of its unusual behaviour with change of temperature, we named it 'galactothermin'.

Search of the literature on human milk has failed to reveal any previous report of a milk constituent having the unusual temperature-sensitive properties of galactothermin peculiar to the conditions of its discovery at neutral pH. β -Casein of bovine milk, obtained by pH-controlled fractionation of purified acid-precipitated casein followed by precipitation from aqueous solution at pH 4.5 at 32°C (Hipp, Groves, Custer & McMeekin, 1952), exhibits a temperature-sensitive solubility effect at acid pH in being more soluble at 2°C than at 32°C (Warner, 1944). Prominent among the few other known protein natural products showing a negative temperature coefficient of gelation are sickle-cell anaemia haemoglobin (Murayama, 1957), the A-protein of tobacco mosaic virus (Lauffer, Ansevin, Cartwright & Brinton, 1958), rabbit muscle myosin (Marsland & Brown, 1942) and the

'A' fraction of neutral-salt-soluble collagen from rabbit skin (Fessler, 1960).

The present paper reports the method of isolation of galactothermin and some of its chemical, physical and immunological properties.

EXPERIMENTAL

Isolation of galactothermin

Of 68 samples of human milk examined, 24 (among them a sample directly tested before freezing) gave clear evidence of temperature-reversible turbidity of the skim milk at neutral pH. Details of the isolation and purification of galactothermin from one such milk follow.

A 920ml volume of milk, accumulated over 5–10 days after parturition and frozen immediately, had pH 6.80 when thawed. For the removal of casein (Maeno & Kiyosawa, 1962), we adjusted the milk to pH 4.64 with HCl and kept it at 4°C overnight. After 16 h the treated milk was warmed to 20°C and centrifuged at 78000g for 30 min at 20°C. The clear supernatant was brought to pH 7.3, warmed at 40°C for 15 min and centrifuged at 78000g for 30 min at 40°C. The precipitate was redissolved in 70 ml of water, cooled to 0°C and centrifuged cold at the same speed for 30 min. The warm and the cold treatments were repeated, followed by a final warm centrifugation to obtain the purified galactothermin, which was redissolved in water and freeze-dried. The yield was 250 mg dry wt. of protein.

Physical characteristics

Molecular weight. Ultracentrifugal analysis of a sample of the purified galactothermin at 0.28% concentration demonstrated its homogeneity in a solution of 0.55M-sodium acetate buffer, pH 4.0 and *I* 0.1. The centrifugation was carried out in a valve-type synthetic-boundary cell of a Spinco model E ultracentrifuge at 60000 rev./mi nat 4°C for 2½ h. The sedimentation coefficient $s_{25,w}$ was 1.05S and the diffusion coefficient was 7.15×10^{-7} . Assuming a partial specific volume of 0.73, the molecular weight was calculated to be 11400. When an aqueous solution of galactothermin was exhaustively dialysed for 1 week at 5°C against repeated changes of water, a loss of protein equivalent to 84% of the original weight in the dialysis sac was observed. This loss may be considered as evidence for a molecular weight of the protein less than 14000–15000. Determination of the minimum molecular weight from the sum of amino acid residues gives a value of approx. 14000.

Isoionic point. The isoionic point of galactothermin was determined on an aqueous 1% (w/v) solution of the protein dialysed for 24 h at room temperature against boiled glass-distilled water. The value observed at 5°C was pH 7.27. When this solution was warmed to 37°C, the pH fell to 6.15.

pH determinations were made on a Radiometer type PHM4C instrument standardized at the two temperatures employed, namely 5°C and 37°C. The indicated release of H⁺ ions on heating is associated with the progressive insolubilization of the protein as evidenced by increasing turbidity of the test solution. On resolubilization by a return of the aggregated protein suspension to 5°C, the pH reverted to its original value.

Turbidity studies. The turbidity of a given solution of galactothermin is a function of protein concentration, temperature, pH and ionic strength of the solvent, and of the time of incubation at a set temperature. Table 1 presents results showing the increase in turbidity of a galactothermin solution with increase in ionic strength of the aqueous solvent at each of three temperatures employed. The results also illustrate the attainment of essentially clear solutions on cooling to 0°C with a reversion to the turbid state on reheating to 37°C. Table 2 shows the requirement for time in the realization of maximum turbidity of a solution of this protein at a given temperature. The higher the temperature, the more rapidly is the maximum turbidity attained. The effect of pH on the turbidity of a 0.2% galactothermin solution in aqueous saline, *I* 0.1, is illustrated in Fig. 1. The solution was brought initially to pH 9.02 with dilute sodium hydroxide and then acidified by additions of 0.05M-hydrochloric acid. In each case, readings of E_{700} were made after 10 min incubation at 28°C. It is apparent that maximum turbidity is reached in the approximate region of the isoionic point and that resolubilization occurs as the pH rises or falls on either side of pH 7.27.

Thermal stability. The thermal stability of galactothermin with respect to reversibility of turbidity on cooling was evidenced by a solution of 0.1% protein dissolved in 0.03M-sodium phosphate buffer at pH 7.2 and heated from 40°C to 100°C. The turbidity did not visibly decrease at the higher temperatures, as is observed with Bence-Jones protein (Putnam & Stelos, 1953). After heating at 100°C for 5 min, the turbid solution was chilled in ice with resultant complete resolubilization of the protein. Subsequent rewarming of the chilled solution to 40°C caused the usual turbidity. When boiled and unboiled samples of galactothermin were subjected to electrophoresis on polyacrylamide gel at pH 8.4 with sodium borate buffer, identical patterns of Amido Black-stained protein lines were observed.

Absorption spectra. Spectrophotometric analyses of 0.1% solutions of galactothermin in 0.1M-hydrochloric acid and 0.1M-sodium hydroxide were performed with a Cary model 14 spectrophotometer. Fig. 2 presents the results. In 0.1M-hydrochloric acid, the peak maximum is at 277 nm with $E_{1\text{cm}}^{0.1\%}$

Table 1. *Turbidity of galactothermin solutions at various temperatures and ionic strengths of solvent*

The table shows $E_{320}^{1\text{ cm}}$ values of 80 μg of galactothermin/ml of 0.02 M-phosphate buffer, pH 7.3, plus NaCl to yield the final ionic concentrations given.

<i>I</i>	$E_{320}^{1\text{ cm}}$ after 15 min incubation					
	20°C	28°C	37°C		0°C	37°C
0.05	0.000	0.000	0.022	} chilled to 0°C →	0.000	0.024
0.15	0.000	0.006	0.031		0.001	0.032
0.30	0.000	0.015	0.104		0.005	0.100
0.60	0.024	0.120	0.226		0.017	0.222
					} reheated to 37°C →	

Table 2. *Increase with time in turbidity of galactothermin in a solution of *I* 0.6 at 28°C and 37°C*

The table shows $E_{320}^{1\text{ cm}}$ values of 80 μg of galactothermin/ml of 0.02 M-phosphate buffer, pH 7.3, plus NaCl to adjust the final ionic strength to 0.6.

Time (min)	$E_{320}^{1\text{ cm}}$ (28°C)	Time (min)	$E_{320}^{1\text{ cm}}$ (37°C)
0	0.000	0	0.000
2	0.048	0.5	0.159
4	0.074	4.5	0.205
10	0.110	7.5	0.222
12	0.117	12.5	0.226
14	0.119		
16	0.121		
18	0.121

Fig. 1. pH-turbidity curve for 2mg of galactothermin in 1ml of NaCl solution, *I* 0.1 at 28°C. The pH was adjusted by additions of 0.05 M-HCl to the solution, beginning at pH 9.02.

Fig. 2. Spectral curves of galactothermin (1mg/ml) run at room temperature. —, Solution in 0.1 M-NaOH, read against 0.1 M-NaOH; ----, solution in 0.1 M-HCl, read against 0.1 M-HCl.

0.740. In 0.1 M-sodium hydroxide, two peak maxima were observed: at 290nm, $E_{1\text{cm}}^{0.1\%}$ 0.855, and at 283nm, $E_{1\text{cm}}^{0.1\%}$ 0.815. The two associated minima are at 286nm, $E_{1\text{cm}}^{0.1\%}$ 0.800, and at 270nm, $E_{1\text{cm}}^{0.1\%}$ 0.790.

Electrophoresis. Electrophoresis of galactothermin was performed on cellulose acetate strips and polyacrylamide gels at an acid and an alkaline pH.

We applied to the cellulose acetate strips 89 μ g of the protein in 5 μ l of 0.29M-sodium acetate buffer, pH 4.02, for the acid electrophoresis and 100 μ g in 6 μ l of 0.05M-sodium barbital buffer, pH 8.67, for the alkaline electrophoresis. The strips were run with their appropriate buffers (approx. 15°C) for 1 h at 1.25mA. After electrophoresis, the strips were stained with 0.1% (w/v) Coomassie Brilliant Blue in 10% (w/v) trichloroacetic acid plus 5% (v/v) methanol. Destaining was effected in 10% (w/v) trichloroacetic acid plus 5% (v/v) methanol. Only one line appeared on the acid-run strip at the cathode side of the origin. The alkaline electrophoresis produced one pronounced line in a diffuse area from the origin towards the anode at a distance found with the slow-moving γ -globulins of human serum run as a control.

For acid-gel electrophoresis of galactothermin, 10, 20 and 40 μ g samples were dissolved in 0.1 ml of ice-chilled 0.375M-glycine-0.0534M-acetic acid buffer, pH 4.0, and were run in 7.5% (w/v) polyacrylamide gel with the same buffer for 2 h at 2mA in a 1°C refrigerator with accessory air-cooling circulation. Only one sharp band was produced on staining of the gels with Coomassie Brilliant Blue. Since galactothermin in a solution of tris-glycine buffer at pH 8.8-9.0 on warming produced some visible aggregation, in contrast with its solution in the glycine-acetic acid buffer, pH 4.0, we expected the production of a number of bands in the alkaline polyacrylamide gel because of its sieving effect. Amounts of the protein ranging from 20 to 100 μ g dissolved in 0.1 ml of 0.025M-tris-0.192M-glycine buffer, pH 8.8-9.0, were subjected to electrophoresis in 7.5% (w/v) polyacrylamide gels with this buffer for 2 h at 2mA in the 1°C refrigerator and then stained with Coomassie Brilliant Blue. Two main protein bands were produced in addition to two faint bands farther into the gel and a minor band at the origin. By excision and aqueous extraction of the two main bands, made visible merely by heating the gels to 37°C, we recovered the separated proteins and again subjected them to electrophoresis. From each fraction a similar series of four to five bands were again produced. We consider these results as evidence of the aggregation phenomenon of galactothermin under the conditions of alkaline gel electrophoresis and of its unsuitability for establishment of this protein's purity.

Chemical characteristics

Amino acid composition. Amino acid analyses of galactothermin were performed with a Beckman 120C amino acid analyser equipped for high-sensitivity analysis. Hydrolysis of the protein was carried out in 6M-hydrochloric acid in hydrolysis

tubes repeatedly evacuated and flushed with nitrogen for 30 min. The tubes were finally evacuated, sealed and kept for various times at 110°C. The hydrolysed protein solution was subsequently dried under a nitrogen stream and taken up in 0.01M-hydrochloric acid. Samples containing the equivalents of 50 or 100 μ g of protein were then analysed. Tryptophan was determined in an alkaline hydrolysate by a modification of the standard fluorescence analysis method of Udenfriend (Lovenberg & Williams, 1969). Table 3 summarizes the results on the amino acid composition of galactothermin. The absence of cystine and cysteine was confirmed by analysis of 12.1 mg of the protein by the method of Sullivan *et al.* (1955). Protein hydrolysis was performed by the procedure of Miller & du Vigneaud (1937) by using 20% (w/v) hydrochloric acid in 50% (w/v) formic acid. No cystine was found. Of especial interest is the fact that roughly two-thirds of the amino acid residues are of the non-polar species and that, among these, proline is uniquely predominant. It is not unusual for a protein with so extremely high a proportion of non-polar side chains to show a strong tendency to associate and exhibit a negative temperature coefficient of gelation (Payens & van Markwijk, 1963).

Hexose and sialic acid. The hexose content of galactothermin was determined by the method of Sørensen & Haugaard (1933) as modified by Schultze, Schmidtberger & Haupt (1958). The results showed hexose to be virtually absent, namely 0.65% of the weight of the protein sample or less than 1 mol/2.5 mol of protein. Sialic acid determinations were performed on protein samples by following the recommendation of Schultze *et al.* (1958). No sialic acid was detectable in galactothermin.

Calcium and phosphate. Calcium determination by flame photometry showed 0.25 μ g of calcium/mg of galactothermin, or less than 0.1 mol of calcium/mol of protein. For phosphate determination, the ashing procedure of Ames & Dubin (1960) was employed, followed by the microphosphate analytical method of Chen, Toribara & Warner (1956). Only 0.16 mol of phosphorus/mol of protein was found. From these analyses galactothermin is considered to be essentially calcium- and phosphate-free.

Immunological properties

Defatted whole milk and isolated galactothermin were employed as antigens both intravenously and intradermally with Freund's adjuvant in rabbits to produce antisera for immunological analyses. By application of the antigen and serum-dilution methods for formation of antigen-antibody precipi-

Table 3. *Amino acid composition of galactothermin*

Amino acid	Sample analysis		Average of four analyses
	(nmol)*	(% by wt. of amino acid residues)	(mol/mol of protein)†
Lys	11.3	2.63	3.3
His	8.7	2.15	3.0
Arg	6.3	1.79	1.9
Asp	24.6	5.14	7.0
Thr	15.0	2.76	3.8
Ser	8.5	1.34	1.8
Glu	69.1	16.23	19.1
Pro	97.7	17.23	27.6
Gly	1.5	0.16	0.6
Ala	17.0	2.19	5.5
Val	47.0	8.46	13.4
Met	7.8	1.86	2.1
Ile	22.3	4.58	6.1
Leu	72.6	14.92	20.5
Tyr	11.3	3.35	2.9
Phe	7.3	1.95	2.2
Trp	2.7	0.91	1.0

* In a 55 μ g sample of galactothermin hydrolysed for 16 h.

† The values are derived from samples hydrolysed for 16–20 h and are based on the assumptions that the protein contains seven residues of aspartic acid as standard and that the mol.wt. is approx. 14000.

tates, no gross precipitation was observed with any antiserum. Immunodiffusion analyses of anti-(human milk) serum, anti-galactothermin serum and anti-(human serum) serum against the isolated purified galactothermin showed a single common line of precipitation of antigen with the milk and galactothermin antisera but none with the human serum antiserum. When the passive cutaneous anaphylaxis test in guinea pigs (Ovary *et al.* 1958) was employed, the rabbit anti-galactothermin serum evoked the cutaneous response specific for galactothermin antibodies. In these tests 0.1 ml samples of 1:50-diluted heat-treated antiserum or control normal rabbit serum were injected intradermally at four to six sites in shaved dorsal skin of six albino guinea pigs weighing 200–250 g. Then 3–4 h later 0.5 ml of 0.5% Evans Blue solution was injected intravenously and the sites of antiserum administration were checked for visible reaction. After 30 min 0.1 ml samples containing 10 and 20 μ g of galactothermin were injected intravenously and the subsequent development of visible blue spots and their diameters at the sites of intradermal serum injection were recorded. In all cases strong positive discolorations were evident at the sites where the anti-galactothermin serum had been injected and none at the normal serum control sites. These results are evidence of the antigenicity of the galactothermin.

DISCUSSION

The presence in human milk of a protein aggregating at body heat and solubilizing at lower temperatures is an oddity without immediate explanation. Whether it is a factor in the regulation of milk flow from the gland or serves some other biological function is speculation.

The thermal aggregation of galactothermin as affected by pH and ionic strength of the solvent is grossly similar to the behaviour of poly-L-valyl-ribonuclease investigated by Kettman, Nishikawa, Morita & Becker (1966). With both materials the high proportion of non-polar residues favours apolar interactions leading to aggregation. This aggregation is forwarded by the apparent lowering of repulsive forces among the molecules by higher ionic strengths of the solvent.

The ease of isolation of galactothermin, its ready aggregation at room temperatures in low concentrations, its resistance to heat denaturation as demonstrated by identical gel-electrophoretic patterns of boiled and unboiled samples and their repeatable heat reversibility of aggregation all recommend this protein for investigations of non-polar interactions as involved in the native structure of proteins themselves and in their protein-protein associations.

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