Thermal denaturation of whole cells and cell components of *Escherichia coli* examined by differential scanning calorimetry

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Thermograms of whole cells of *Escherichia coli* obtained by differential scanning calorimetry contained ten main peaks (denoted f , l , m_1 , m_2 , m_3 , n , p , q , r and s) occurring at temperatures of approximately 25, 54, 61, 71, 76, 81, 95, 105, 118 and 124 °C, respectively. After cooling to 5 °C and reheating, peaks denoted f_r , m_r and p_r were observed at 23, 73 and 94 °C, respectively. By examining thermograms of different cell fractions we have identified the following thermal denaturation events. During primary heating there is a broad endotherm (f) beginning below 20 °C and extending to just above 40 °C that is caused by melting of membrane lipids. Superimposed on this is an exothermic process associated with a change of state of the peptidoglycan. The first irreversible denaturation event occurs just above 47 °C, associated with the onset of denaturation of the 30S ribosomal subunit and soluble cytoplasmic proteins. Ribosome melting is a complex process occurring between 47 and 85 °C and is characterized by peaks m_1 , m_2 and n . Peak m_3 at 75–76 °C is of unknown identity but may possibly represent melting of tRNA. Peak p at 95 °C results from melting of a portion of the cellular DNA combined with denaturation of a cell wall component. Peak q at 105 °C is multicomponent and may be caused by melting of a different region of DNA together with denaturation of another cell wall component. The complex events denoted r and s at 118 and 125 °C, respectively, are associated with denaturation of a component of the cell envelope, and possibly also of DNA. Following cooling and reheating there is a broad endotherm with a maximum at 23 °C caused by remelting of membrane lipid and a very broad endotherm extending between 40 and 100 °C caused by the remelting of ribosomal RNA. Peak p_r at 94 °C is caused by the melting of reannealed DNA. Additional features not appearing in whole cells were evident in some cell fractions. These observations should allow us to distinguish events that may lead to loss of viability from those that do not.

Introduction

The inactivation of micro-organisms by heat forms the basis of large sections of the food and pharmaceutical industries. Not surprisingly, the factors affecting microbial heat-resistance and the nature of heat damage are of continuing practical and scientific concern.

The mechanisms of thermal inactivation of micro-organisms have commonly been investigated by using biochemical methods to examine the effect of heat on particular cell structures or processes. Membranes, nucleic acids and certain enzymes have all been identified as cellular sites of heat injury and, in some cases, information is available on the molecular nature of heat damage (Tomlins & Ordal, 1976; Pellon & Sinskey,

1984; Gould, 1989). However, despite this large body of information, we still do not understand precisely how micro-organisms are killed by heat.

Differential scanning calorimetry (DSC) enables denaturation and other thermal processes that occur when samples are heated to be detected and recorded as a time-temperature sequence. The technique has been used to study thermotropic phase changes in membrane lipids (McElhaney, 1982, 1986), activation and germination of spores (Maeda *et al.*, 1974; Verbeke *et al.*, 1981), and the state of water in bacterial cells (Gonda & Koga, 1973).

Verrips & Kwast (1977) examined vegetative cells of *Citrobacter freundii* by DSC and obtained thermograms consisting of eight unidentified endothermic peaks. Two peaks, at 50 and 55 °C, coincided with the temperature over which death was thought to occur but the relationship between temperature and the rate at which viable organisms declined in the calorimeter was not established.

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In previous work, we examined by DSC several species of bacteria having natural differences in heat resistance (Miles *et al.*, 1986). Each species produced a characteristic multi-peaked thermogram but the onset of thermal denaturation occurred at higher temperatures in the more resistant types than in those that were heat sensitive. Additionally, some heat-sensitive organisms displayed thermogram features which were absent in those more resistant. In all organisms the onset of irreversible denaturation occurred at or above the maximum growth temperature but below the temperature at which most cells in the population died (calculated from *D* and *z* values). Cell death and irreversible denaturation thus occurred within the same temperature range.

In order to relate loss of viability to the denaturation of particular cell components, we need to identify the various features observed in thermograms of whole cells. We have identified one substantially reversible process in *Escherichia coli* as the melting endotherm of DNA (Miles *et al.*, 1986; Mackey *et al.*, 1988) but the other peaks remain uncharacterized.

The aim of this work was to identify the major thermogram features that are observed when whole cells of *E. coli* are heated in the calorimeter. To do this we have fractionated the cell into its constituent components and compared the thermogram given by each fraction with that observed in whole cells.

Methods

Organisms and growth conditions. *Escherichia coli* NCTC 8164 lacking ribonuclease I was used in all cell fractionation experiments. *E. coli* DS410 (Dougan & Sherratt, 1977) was used for obtaining minicells. Cells were grown at 37 °C in tryptone soya broth (Oxoid) supplemented with 3.0 g yeast extract l⁻¹. For volumes of up to 3 l, growth was in one or more conical flasks contained in a shaking water bath; for the larger volumes required in ribosome or minicell preparation, growth was in a 10 l fermenter aerated by sparging. Cultures for cell fractionation were grown to an OD₆₈₀ of 1.0; those used for minicell preparation were grown for 18 h. Cells were chilled on ice and harvested by centrifugation at 10000 *g* for 5 min.

Sequential fractionation. Cell pellets from 3 l of culture were washed once and resuspended in 1.5 pellet volumes of a buffer containing 200 mM-potassium glutamate, 10 mM-sodium hydrogen phosphate and 5 mM-magnesium chloride, pH 7.8. The ionic composition of this buffer was intended to approximate that existing within whole cells. The concentrated cell suspension was placed in an X-press (Life Science Laboratories) previously cooled to -25 °C. After holding for 20 min, the frozen cells were disrupted by passing once or twice through the pressure cell. After thawing, the viscous paste of broken cells was stored on ice. Deoxyribonuclease (DNAase) (Sigma, 0.2 mg ml⁻¹) was added and the material centrifuged at 32500 *g* for 30 min. The pellet of whole and broken cells was saved to prepare a crude cell envelope fraction (see below). The supernatant liquid (cell-free extract) was centrifuged at 150000 *g* for 3.5 h to give a pellet of crude ribosomes. The ribosome-free supernatant liquid was retained and, in some cases,

concentrated up to sevenfold by dialysis in a Minicon B-15 concentrator (Amicon). To examine the ribosome content of the cell-free and ribosome-free extracts, the fractions were diluted 1000-fold in glutamate buffer and 3 ml of this suspension was applied to 15 ml of a 10–30% (w/v) linear sucrose gradient in glutamate buffer. The gradients were centrifuged at 90000 *g* for 2.5 h then pumped out by upward displacement with 40% sucrose. Fractions were monitored by measuring *A*₂₆₀. Samples of the following were retained for chemical analysis and examination by differential scanning calorimetry: washed whole cells; broken cells before addition of DNAase; cell-free extract; crude ribosomes; ribosome-free extract; cell envelopes.

Preparation of cell envelopes. The pellet of whole and broken cells from the cell fractionation was resuspended in glutamate buffer and then spun at 8000 *g* for 10 min to remove whole cells. The cell envelopes were then pelleted by centrifugation at 100000 *g* for 1.25 h and washed twice in buffer. Alternatively, cell envelopes were prepared by two or three cycles of low-speed (4000 *g*) and high-speed (30000 *g*) centrifugation.

Preparation of peptidoglycan. Peptidoglycan was prepared as described by Hancock & Poxton (1988). Cells from a 2 l culture were washed once in water and resuspended in 50 ml water. The suspension was kept on ice and added dropwise to 50 ml boiling 8% (w/v) SDS, then boiled for a further 20 min. After cooling overnight at room temperature, the SDS-insoluble material was spun down at 100000 *g* for 1 h. The pellet was washed four times with water, twice with 2 M-NaCl, then four times with water. The pellet was finally washed once in glutamate buffer.

Lipid extraction. Cells from 1 l of culture were harvested by centrifugation and washed twice in 10 mM-Tris/HCl, pH 7.5, containing 10 mM-MgCl₂. The cell pellet was finally resuspended in 4 ml buffer and placed in a stoppered tube with 10 ml methanol and 5 ml chloroform. The mixture was shaken vigorously, kept on ice for 10 min and then 4 ml chloroform was added followed by 4 ml water. After mixing, the suspension was spun in a bench centrifuge to separate the layers. The lower chloroform layer was removed and the chloroform evaporated off using a stream of oxygen-free nitrogen. The resulting lipid was redissolved in a small volume of chloroform and stored at -20 °C under oxygen-free nitrogen.

Preparation of ribosomes and ribosomal subunits. The following buffers were used: basal buffer, containing 10 mM-Tris/HCl, 10 mM-MgCl₂, 5 mM-mercaptoethanol, pH 7.6; gradient buffer, consisting of basal buffer plus 1 M-NH₄Cl and 20% (w/v) sucrose; storage buffer, consisting of basal buffer plus 50 mM-NH₄Cl; subunit buffer, containing 20 mM-Tris/HCl, 1 mM-MgCl₂, 150 mM-NH₄Cl, 5 mM-mercaptoethanol, pH 7.6. To prepare ribosomes, the cell pellet from a 10 l culture was washed once in basal buffer and then resuspended in the same buffer with 0.2 mg DNAase ml⁻¹. The cells were disrupted by passage through the X-press, as described above, and broken cells were removed by centrifugation at 10000 *g* for 5 min. The supernatant was centrifuged at 26500 *g* for 30 min to remove cell debris, then layered onto 2 vols gradient buffer and spun at 140000 *g* for 6 h. This yielded a clear glassy pellet of ribosomes which was resuspended to a volume of 10 ml in storage buffer, dispensed into plastic tubes and stored at -70 °C.

For preparation of subunits, aliquots of ribosomes in storage buffer were thawed and spun down at 100000 *g* for 3 h. The pellet was thoroughly homogenized with subunit buffer and recentrifuged. The pellet was once again homogenized with subunit buffer (5 ml) and dialysed for 24 h at 1 °C against two changes of 200 ml of the same buffer. The dialysate was then layered onto 54 ml of a 10–30% sucrose gradient in subunit buffer. The gradient was spun at 35000 *g* for 16 h at 4 °C and pumped out by upwards displacement using 40% sucrose.

Fractions (1 ml) were collected, and their A_{260} measured. Peak fractions were pooled, diluted with subunit buffer and centrifuged at 140 000 *g* for 19 h. The pellets were resuspended in 5 ml subunit buffer and rerun on two separate 10–30% sucrose gradients as before. Again, the gradients were pumped out and 1 ml fractions collected. The peak fractions were pooled, diluted with subunit buffer and centrifuged at 140 000 *g* for 24 h. The pellets were thoroughly homogenized, washed twice in glutamate buffer and collected by centrifugation at 140 000 *g* for 19 h.

Preparation of minicells. Ten litres of culture was chilled by pumping into flasks surrounded by ice-water, then concentrated to 800 ml by tangential-flow filtration (Millipore). The concentrated suspension was centrifuged at 1000 *g* for 5 min to remove most of the whole cells. Minicells and remaining whole cells were sedimented by centrifugation at 15 000 *g* for 15 min. Pellets were resuspended to 15 ml with M9 medium (Miller, 1972) and 5 ml portions layered onto 54 ml volumes of 10–30% sucrose gradients in M9 medium. The gradients were spun at 5000 *g* for 5 min to separate minicells and whole cells and the top two-thirds of the (upper) minicell band removed. The minicell fractions were pooled, diluted with an equal volume of M9 medium and pelleted by centrifugation at 22 000 *g* for 10 min. The pellet was resuspended to 5 ml with M9 medium then purified by another gradient run as above. The final pellet of minicells was washed twice in glutamate buffer, then examined by DSC.

Nucleic acids. Solutions of DNA, rRNA and tRNA from *E. coli* were obtained from Sigma and diluted to 10 mg ml⁻¹ in glutamate buffer.

Biochemical assays. All the cell fractions sampled for DSC were subsequently freeze-dried. Protein was assayed by the method of Lowry; RNA was assayed using orcinol reagent and DNA was assayed using diphenylamine reagent by the method of Burton, all as detailed by Herbert *et al.* (1971).

Calorimetry. The calorimeter used was a Perkin-Elmer DSC-2C fitted with an Intracooler II, to allow temperature control below 30 °C. The dry box and DSC head were purged with oxygen-free nitrogen supplied via an in-line silica gel drier. Samples up to 20 mg were weighed to ± 0.01 mg, sealed in aluminium pans and heated at 10 °C min⁻¹ from 5 to 130 °C, using an empty pan as the reference. Without adjustment of the DSC or disturbance of the pans, the samples were cooled rapidly (arbitrarily 320 °C min⁻¹) to the lower limit (5 °C) then reheated as before. Data for the run and rerun were collected and the calorimeter was controlled by a Perkin-Elmer 3600 Data Station. The thermogram scans were then transferred to a VAX 11/750 computer for high-resolution plotting, determining peak temperatures and enthalpy measurements. Only those peaks that were consistently present in repeated scans were selected for analysis. Initial transients and some initial step-like features, that appeared to be instrumental artefacts, were ignored. Temperature and power scales were calibrated according to the manufacturer's instructions using indium and ice as standards. The sample dry mass was determined by reweighing the sample, after piercing the pan and drying overnight at 105 $\pm$ 1 °C.

Results

Cell fractionation studies

In the first part of the investigation the bacterial cell was separated into its main constituent parts, with the aim of identifying the major thermogram features seen in whole cells. A simple, and therefore somewhat crude, protocol was used to minimize the number of manipulations

Table 1. *Chemical composition of whole cells and fractions of whole cells of E. coli NCTC 8164*

The results presented are from the sequential fractionation of a single batch of cells as described in Methods. The quoted values are the means ($\pm$ SD) of two samples, each assayed in duplicate. The values for dry matter content were obtained by drying the DSC samples and are the means of three samples.

Sample	Dry matter content (% by mass)	Composition as a percentage of dry matter			Percentage of total dry matter accounted for
		Protein	DNA	RNA	
Whole cells	24 ± 0.4	58 ± 1.0	1.9 ± 0.10	25 ± 1.8	85
Broken cells	19 ± 0.4	54 ± 1.4	1.9 ± 0.07	24 ± 3.1	79
Cell-free extract	16 ± 0.1	54 ± 0.6	0.4 ± 0.11	21 ± 1.4	75
Crude ribosomes	36 ± 0.3	56 ± 1.4	0.3 ± 0.02	38 ± 5.5	95
Ribosome-free extract (concentrated)	11 ± 0.1	66 ± 7.9	0.2 ± 0.04	1.8 ± 3.6	68
Cell envelopes	24 ± 0.3	42 ± 1.0	ND	18 ± 2.4	60

ND, Not detected.

involved, and to enable a complete set of subfractions to be obtained from the same sample of starting material in a reasonable time. Each fraction examined by DSC was analysed for dry matter, DNA, RNA and protein (Table 1). The thermal data are summarized in Table 2.

Whole cells

The composition of the whole cells, i.e. 58% protein, 25% RNA, 1.9% DNA and 15% remainder, was similar to that given by Neidhardt (1987). Whole cells exhibited a multi-peaked thermogram, reproducibly containing the features identified by letter in Fig. 1(a). The labelling of peaks follows that of our previous publications (Miles *et al.*, 1986; Mackey *et al.*, 1988) but is expanded to include l , m_1 , m_2 and m_3 in the region of the main peak (previously labelled m), s in the high temperature region and f in the lower. On rerunning the sample after heating to 130 °C and cooling to 5 °C, three apparently reversible peaks were evident; f_r , m_r and p_r and the level, i.e. the specific heat capacity of heat-denatured cells, was slightly higher than that of undenatured cells in the low-temperature region. To allow comparison between different subfractions, the same notation has been used throughout. Occasionally other features were observed in the rerun: endotherms at temperatures in excess of p_r and indications of a small dip in the m_r endotherm (as in Fig. 1), but these features varied in size and position and were not reproducible.

Table 2. Temperatures of onset of thermal denaturation (T_0) and temperatures of peak maxima ($T_{\max}$) in the thermograms of whole cells and fractions of whole cells of *E. coli* NCTC 8164

The fractionation procedures are described in Methods. The quoted $T_{\max}$ values are the means, maxima and minima of individual peaks each averaged over three different preparations replicated three times except for the dilute ribosomes and ribosome-free extract fractions (two preparations each replicated three times). All peaks were recorded, even when very small in size (e.g. m_1 , m_2 , m_3 in the ribosome-free extract). Two of the three cell wall preparations appeared to be contaminated with ribosomes and this will have affected the data for peaks m_1 to n . It was only in the contaminated specimens that m_r was observed. Some features were present in some but not all of the scans and the number N indicates the frequency of occurrence. f was sometimes obscured by initial transients on the first heating run. Peaks p , q , r and s appeared to be multi-event processes in whole cells because they did not exhibit simple shapes with one obvious $T_{\max}$. In some fractions only a single component of these peaks was apparent; for example, the cell walls gave a sharp peak at q_1 peak only.

Sample		T_0 (°C)	$T_{\max}$ (°C)												
			f	l	m_1	m_2	m_3	n	p	q_1/q	r	s	f_r	m_r	p_r
Whole cells	Mean	47.5	25.4	53.7	60.7	71.0	75.6	81.1	94.8	105.3	118.7	124.6	23.2	72.5	93.9
	N	9	3	9	9	9	8	9	9	9	9	9	9	9	9
	Min.	44.8	24.3	51.7	60.5	70.4	75.2	79.7	94.2	102.5	113.5	122.5	21.7	69.2	93.1
	Max.	50.2	26.0	56.2	61.0	71.9	76.3	82.0	95.8	107.8	119.9	126.9	24.2	74.3	94.8
Broken cells	Mean	45.4	30.7	55.1	62.5	70.9	76.2	80.5	93.5	103.8	117.2	124.6	25.8	71.6	93.5
	N	9	5	5	9	9	3	8	9	8	8	7	9	9	9
	Min.	42.2	27.7	53.1	61.6	70.2	75.7	80.0	91.2	102.7	112.2	123.4	22.5	69.0	92.1
	Max.	45.0	33.8	59.9	63.7	71.5	76.9	81.6	94.4	106.6	119.3	125.6	29.6	74.9	94.3
Cell-free extract	Mean	45.4	—	52.3	62.9	71.0	—	80.2	93.6	103.7	118.2	125.9	23.6	74.2	—
	N	9	—	6	9	9	—	9	6	7	5	7	2	9	—
	Min.	43.4	—	50.3	61.3	70.6	—	79.6	90.3	102.2	114.7	124.1	23.6	70.7	—
	Max.	47.4	—	54.4	64.2	71.6	—	81.1	96.0	106.4	119.8	126.9	23.7	78.9	—
Crude ribosomes	Mean	47.0	—	52.7	62.1	72.8	—	81.2	98.6	—	114.0	123.8	—	72.1	—
	N	9	—	7	9	9	—	9	1	—	1	1	—	9	—
	Min.	44.7	—	52.3	61.5	71.7	—	80.3	—	—	—	—	—	69.5	—
	Max.	48.6	—	53.2	62.7	73.2	—	82.0	—	—	—	—	—	73.4	—
Dilute ribosome 50:50	Mean	47.6	—	—	64.5	73.0	—	80.4	—	—	113.5	125.8	—	71.3	—
	N	6	—	—	6	6	—	6	—	—	1	3	—	6	—
	Min.	45.2	—	—	63.7	72.7	—	79.3	—	—	—	124.8	—	70.0	—
	Max.	51.1	—	—	65.5	73.3	—	81.4	—	—	—	126.7	—	74.0	—
Ribosome-free extract	Mean	46.9	—	—	61.4	69.7	77.2	89.2	95.1	102.9	115.0	126.3	—	71.4	—
	N	6	—	—	3	6	4	1	2	2	1	3	—	1	—
	Min.	45.0	—	—	59.7	69.1	76.7	—	93.0	101.8	—	124.5	—	—	—
	Max.	51.0	—	—	62.6	70.9	77.8	—	97.2	104.0	—	127.4	—	—	—
Cell envelopes	Mean	51.2	33.7	56.7	65.6	72.6	77.8	89.5	96.1	101.9	118.7	123.2	31.2	74.3	—
	N	9	9	2	7	9	6	1	9	9	9	9	9	6	—
	Min.	45.9	30.3	56.2	62.5	71.9	76.6	—	95.4	100.8	117.7	122.2	28.9	71.9	—
	Max.	55.2	35.6	57.2	66.4	74.0	78.7	—	96.8	102.5	119.8	126.3	33.1	79.3	—

Broken cells

Broken cell preparations contained more water than whole-cell preparations because buffer was added in their preparation. The dry matter contained approximately the same amounts of RNA and DNA as whole cells, i.e. nuclease activity was not extensive enough to cause a major decrease (but note the increased SD for RNA in Table 1). The protein content was lower: 54% compared with 58% in whole cells (Table 1).

When cells had been mechanically damaged by freezing and passing through the X-press, there were noticeable changes in the thermogram (Fig. 1*b*). l , m_3 , q , r and s were reduced in size while p increased, and the peaks above p were more variable in shape and position. While reversible features f_r , m_r and p_r were visible in all

samples, p_r was usually smaller than p and less reproducible in shape than the p_r of whole cells.

Cell-free extract

This fraction had been treated with DNAase and centrifuged to remove whole cells and most of the cell wall fragments. The dry matter content was lower than that of the broken-cell preparation and, as expected, the DNA content was also lower (Table 1). The protein content was similar to, and RNA content somewhat less than, that of the broken cells. Removal of cell walls might have been expected to increase the proportion of RNA and protein. This was not observed, probably due to loss of ribosomal material with the cell envelopes and

Fig. 1. Thermograms of whole cells and fractions of whole cells of *E. coli* NCTC 8164. (a) Whole cells; (b) broken cells; (c) cell-free extract; (d) crude ribosomes; (e) ribosome-free extract; (f) cell envelopes. The thicker line of each pair is the thermogram obtained during the first heating run; the thinner line is the thermogram obtained after cooling and reheating the sample. The letters indicate thermogram features described in the text. A subscript 'r' denotes a feature observed during the rerun.

possibly also some loss due to degradative processes. Sucrose gradient analysis showed absorbance peaks typical of ribosomes and a peak near the meniscus which represented small molecules/fragments such as residual tRNA or mRNA and nucleotides from the degradation of DNA and RNA. Absorption of UV by protein would also have contributed to this peak (data not shown).

The main thermogram features of the cell-free extract were peaks similar to m_1 , m_2 and n seen in whole and broken cells (Fig. 1c). There was very little reproducible

structure at temperatures above n or in the low temperature region, i.e. f and f_r were reduced or absent, as were p and p_r , q , r and s . The reversible peak m_r , however, was still present in this fraction. Thermogram features p and p_r are believed to be associated with the melting of DNA (Miles *et al.*, 1986) and their absence in DNAase-treated material is therefore as expected. The absence of f , f_r , q , r and s suggests these features may be associated with denaturation of cell envelope components. However, we believe that DNA melting may also contribute to peak q (see Discussion).

Crude ribosomal fraction

The ribosomes formed a hard pellet and the dry matter content was high, consisting mostly of protein and RNA. Since ribosomes are known to contain 62% RNA (Noller & Nomura, 1987), the 38% RNA content (Table 1) indicates substantial protein contamination.

Thermograms of crude ribosomes (Fig. 1d) showed three major peaks which strongly resembled in shape and position peaks m_1 , m_2 and n seen in whole cells. The peaks at m_1 and m_2 were 1 to 2 °C higher than in whole cells and the peak at m_2 was noticeably sharper. These changes may have been caused by dilution effects and/or changes in the ionic environment on disruption of the cells in buffer. There were noticeable variations between subsamples in the shape of m_1 (compare the crude ribosome preparations in Figs 1 and 2) and when crude ribosome pellets were diluted 50:50 with buffer, there was a more pronounced separation of peaks m_1 and m_2 . A small shoulder corresponding to peak l in whole cells was seen in seven of nine ribosome samples.

On cooling and reheating the sample, a very strong broad clear endotherm denoted m_r was seen (Fig. 1d) (occasionally a small dip was observed in m_r , indicating an exothermic reaction, but its position and size were irreproducible). The other apparently reversible peaks p_r and f_r , present in whole cells, were absent from ribosomes.

Ribosome-free extract

The supernatant remaining after removal of ribosomes by centrifugation was too dilute to yield resolvable peaks in DSC and was therefore concentrated by dialysis. The fraction depicted in Fig. 1(e) had about half the dry matter content of whole cells. The main component of the extract was protein (66%), with much smaller amounts of RNA (1.8%) and DNA (0.2%) (Table 1). Much of the unaccounted dry matter (Table 1) was buffer constituents (the buffer contained 3.7% dry matter). The low level of RNA (1.8%) implies that there was little

Fig. 2. Thermograms of ribosomes and ribosomal subunits from *E. coli* NCTC 8164. (a) Pellet of crude ribosomes; (b) crude ribosomal pellet diluted 50:50 with buffer; (c) purified 70S ribosomes; (d) 50S subunits; (e) 30S subunits. The thicker line of each pair is the thermogram obtained during the first heating run; the thinner line is the thermogram obtained after cooling and reheating the sample.

contamination by residual ribosomes. If we assume that all the RNA was ribosomal in origin, then the concentrated extract contained about 7% of the ribosomes present in whole cells.

UV-absorbing bands were undetectable following sucrose density-gradient centrifugation of this extract, confirming the very low content of ribosomes in this cell fraction.

The thermogram (Fig. 1e) contained a broad multi-component peak with a maximum at a mean temperature of 69.7 °C, but no reproducible peaks above 80 °C or below 60 °C. The rerun showed no reproducible features. The main thermogram feature thus occurred in the same temperature range as features associated with ribosome melting. However, the ribosome content would appear too low to account entirely for this feature and we assume it must also represent denaturation of soluble cytoplasmic proteins.

Cell envelopes

Cell envelopes showed peaks at *f*, *f*_r, *p*, *q*, *r* and *s*, and a broad feature in the region of *m*₃ (Fig. 1f). Some fractions were contaminated with ribosomes as evidenced by a distinct ribosome peak in the thermogram and a high content of RNA in the chemical analysis. The quoted analyses are for samples which were thus contaminated, but the example in Fig. 1(f) was largely

uncontaminated. The most distinctive feature was an endotherm in the region of peak *q* seen in whole cells. This feature was much sharper than *q* and appears to be a single process which we have denoted as *q*₁. In the high-temperature region there was a clear endotherm similar in position to, but larger than, peaks *r* and *s* seen in whole cells. In the low-temperature region there was a broad peak denoted *f* which yielded *f*_r on the rerun. We believe this represents the melting and remelting of membrane phospholipids.

Further examination of cell fractions

Ribosomes. 70S ribosomes showed a multi-event thermogram with the major process having a maximum at 75.9 °C. The extent of the shoulder on the leading edge of that peak varied between samples, and the trailing edge indicated the presence of at least one other process (Fig. 2c). The 50S subunits showed a similar thermogram but no shoulder was apparent on the leading edge and the extent of the process on the trailing edge was increased. The main peak of the 50S subunit occurred at 74.0 °C and was slightly broader than that of the 70S, caused mainly by differences in the steepness of the leading edge (Fig. 2d). The 30S subunit showed a still broader peak, with the main peak at 65.6 °C, a substantial shoulder on the trailing edge at 70.7 °C, and a very broad high-temperature process which followed the line of the rerun

Table 3. Temperatures of onset of thermal denaturation (T_0), temperatures of the peak maxima (T_{max}) and enthalpies of denaturation (ΔH) of ribosomes and ribosomal subunits from *E. coli* NCTC 8164

The quoted values are the means of measurements of two preparations. N indicates the total number of replicate observations. The numbers 1, 2 and 3 heading the columns are not intended to imply that data within a column refer to the same process.

		ΔH of denaturation							
		$T_{\max}$ (°C)					J (g dry matter) ⁻¹		J (g RNA) ⁻¹ *
Sample	T_0 (°C)	1	2	3	Rerun (m_r)	Run	Rerun (m_r)	Rerun (m_r)	
70S	Mean	57.7	68.0	75.9	82.1	70.3	42	23	31
	N	5	4	5	5	5	5	5	—
	Min.	55.8	67.6	75.1	81.5	68.6	40	21	—
	Max.	59.6	68.8	76.7	82.6	71.5	47	26	—
50S	Mean	54.5	—	74.0	81.2	71.7	43	23	—
	N	5	—	5	5	5	4	5	—
	Min.	51.6	—	73.6	80.9	71.1	42	21	—
	Max.	55.8	—	74.6	81.5	72.3	45	25	—
30S	Mean	48.4	65.6	70.7	81.6	71.5	36	22	—
	N	4	4	4	4	4	4	4	—
	Min.	44.3	65.2	70.5	80.4	68.5	36	21	—
	Max.	53.1	66.4	70.9	82.6	75.2	37	24	—

* ΔH [J (g RNA) $^{-1}$] = 100/% RNA by mass $\times \Delta H$ [J (g dry mass) $^{-1}$].

(Fig. 2e). The thermal stability of ribosomal particles, based on either T_{max} of the main peak or T_0 values, thus increased in the order 30S < 50S < 70S. All subunits showed broad peaks on reheating with the same shape and position as peak m_r observed in whole cells. We believe this process is the reversible denaturation of ribosomal RNA (see Discussion). Thermal data for denaturation of ribosomes and subunits are summarized in Table 3.

Sucrose density-gradient analysis revealed that some 30S particles (approx. 17%) were present in the 70S preparation, possibly caused by the effect of pressure on the 30S and 50S association, since there is a large volume increase in association (Van Holde & Hill, 1974). The 30S preparation appeared homogeneous after the second gradient run but the 50S band was much broader and more diffuse. This fraction may therefore have been impure, although we were unable to detect any heterogeneity by separately recentrifuging the upper and lower portion of the band.

The chemical analysis of the different preparations given in Table 4 revealed that the ratio of RNA to protein in 70S, 50S and 30S particles was approximately 2:1. This is higher than the accepted ratio of about 1.6:1 (Noller & Nomura, 1987), possibly indicating some loss of ribosomal protein during preparation and storage. The enthalpy measurements for denaturation of 70S and 50S particles were similar, whilst that for the 30S subunit was

lower. The enthalpy for the m_r endotherm was similar for all three particles. The value of 23 J (g dry matter) $^{-1}$ (70S component) was equivalent to 31 J (g RNA) $^{-1}$.

Cell envelope components

Thermograms of cell envelopes, lipid and peptidoglycan are shown in Fig. 3. If peaks f and f_r were due to the melting of membrane lipid, we would expect the peak maxima to vary depending on growth temperature. When cells were grown at 20 °C, the maximum of peak f was 5 to 8 °C lower than in cells grown at 37 °C (Fig. 3a, b). The rerun endotherms (f_r) were broader than those observed on the first run (f) and occurred at a slightly lower temperature. A difference in T_{max} was still observed, however: 31.6 °C for cells grown at 37 °C, compared with 25.4 °C for cells grown at 20 °C.

Extracted membrane lipid exhibited a sharp peak at 37.9 °C, whereas the rerun peak was broader and had a lower peak maximum (30.8 °C) (Fig. 3c). Thermogram features f and f_r are thus associated with the melting and remelting of membrane lipid. The other main thermogram features (described in the preceding section) were unaffected by growth temperature.

The peptidoglycan preparation, which when viewed under the electron microscope showed the expected 'empty bag' appearance, revealed little structure on the

Table 4. *Temperatures of peak maxima (T_{max}), and enthalpies of denaturation (ΔH) of nucleic acids of E. coli in glutamate buffer*The quoted values are means, maxima and minima of N observations.

Sample		Run		Rerun	
		T_{max} (°C)	ΔH^* J (g nucleic acid) ⁻¹	T_{max} (°C)	ΔH^* J (g nucleic acid) ⁻¹
DNA	Mean	95.3	64	95.3	40
	N	4	4	6	6
	Min.	95.1	53	95.0	24
	Max.	95.7	72	95.7	61
tRNA	Mean	78.7	34	77.6	35
	N	3	2	3	3
	Min.	77.7	28	77.4	31
	Max.	79.3	41	77.9	38
rRNA	Mean	72.6	28	72.4	32
	N	3	3	3	3
	Min.	71.6	25	71.2	29
	Max.	73.1	33	73.3	33

* ΔH [J (g nucleic acid)⁻¹] = 100/% nucleic acid in solution $\times \Delta H$ [J (g solution)⁻¹]. Fig. 3. Thermograms of *E. coli* NCTC 8164 cell envelopes and constituents. (a) Cell envelopes prepared from cells grown at 37 °C; (b) cell envelopes prepared from cells grown at 20 °C; (c) lipid extracted from cell envelopes; (d) peptidoglycan. The thick line is the thermogram obtained during the first heating run; the thinner line is the rerun thermogram.

first scan, except a fairly sharp exotherm at 28 °C which varied in size and appearance between runs (Fig. 3d). Similar small exotherms were occasionally seen in whole cells or broken-cell fractions (Fig. 1). On cooling and rescanning, a broad exotherm was seen at high temperatures. This feature had no obvious relationship with any feature seen in whole cells or envelope fractions.

Nucleic acids

Thermograms of DNA, rRNA and tRNA in glutamate buffer are shown in Fig. 4 and the thermal data are summarized in Table 4. DNA yielded an apparently single endothermic process with a maximum of 95.3 °C and an enthalpy of 64 J (g dry matter)⁻¹. On cooling and

Fig. 4. Thermograms of nucleic acids of *E. coli* in glutamate buffer. (a) rRNA; (b) tRNA; (c) DNA. The thick line is the thermogram obtained during the first heating run; the thinner line is the rerun thermogram.

Fig. 5. Thermograms of (a) normal cells and (b) minicells of *E. coli* DS410.

rescanning the same preparation, an exothermic process occasionally preceded the main endotherm, which was reduced in size to $40 \text{ J (g dry matter)}^{-1}$ but occurred at the same temperature. The melting of DNA was therefore not fully reversible in glutamate buffer, and the

remelting process was more complex than the initial melting.

Ribosomal RNA yielded very broad endotherms on both the run and rerun whilst tRNA yielded a somewhat sharper peak on the run, which became broader on the

Table 5. Temperatures of onset of thermal denaturation (T_0), temperatures of peak maxima ($T_{\max}$), and enthalpies of denaturation (ΔH) of normal cells and minicells of *E. coli* DS410

The data for whole cells are means of three replicates of a single preparation. The minicell data are of two preparations replicated twice and three times. p was small in both minicells and normal cells and measurements of $T_{\max}$ were made with difficulty. N denotes the number of observations for each characteristic.

Sample		T_0 (°C)	$T_{\max}$ (°C)										ΔH		ΔH^*	
			m_1	m_2	m_3	n	p	q	r	s	Rerun		J (g dry matter) ⁻¹	J (g DNA) ⁻¹		
											m_r	p_r			p_r	p_r
Normal cells	Mean	48.1	58.4	67.5	72.9	81.4	95.6	106.2	119.5	122.6	69.4	94.4	1.01	69		
	N	3	3	3	3	3	3	3	3	3	3	3	3	—		
	Min.	46.2	58.2	67.4	72.4	81.3	94.8	106.1	119.1	122.2	67.7	94.1	0.91	—		
	Max.	51.2	58.6	67.7	73.1	81.5	96.1	106.5	119.9	122.9	71.4	94.7	1.11	—		
Minicells	Mean	51.3	63.1	71.1	76.6	—	97.1	106.7	118.5	123.1	71.4	—	—	—		
	N	5	5	5	5	—	4	5	5	5	5	—	—	—		
	Min.	51.1	62.7	70.6	76.1	—	96.1	106.0	117.7	122.8	67.4	—	—	—		
	Max.	51.6	63.4	71.6	77.3	—	97.7	107.4	119.7	123.4	74.3	—	—	—		

* ΔH [J (g DNA)⁻¹] = 100/% DNA by mass $\times \Delta H$ [J (g dry mass)⁻¹].

Table 6. Chemical composition of minicells and normal cells of *E. coli* DS410

The values given are the means ($\pm$ SD) of three (dry matter) or four replicates.

Sample	Dry matter content (% by mass)	Composition as a percentage of dry matter			Percentage of total dry matter accounted for
		Protein	DNA	RNA	
Normal cells	22	66	1.47	11	79
	± 0.3	± 1.8	± 0.02	± 5.4	
Minicells	23	60	0.12	16	76
	± 0.3	± 2.4	± 0.03	± 5.4	

rerun. The $T_{\max}$ for rRNA (72.6 °C) was lower than that for tRNA (78.7 °C). The mean enthalpy for rRNA [30 J (g dry matter)⁻¹] was similar to the value of 31 J (g RNA)⁻¹ calculated from ribosome melting data (Table 3) and slightly lower than the mean for tRNA [35 J (g dry matter)⁻¹]. Both were substantially lower than the value for DNA. No reduction in enthalpy occurred on remelting tRNA or rRNA; the processes were thus substantially reversible.

Minicells

Minicells contain little or no DNA and, by comparison with normal cells, offer a means of identifying thermogram features associated with the intracellular melting of DNA. Thermograms of normal cells and minicells (strain DS410) are compared in Fig. 5 and the thermal data are summarized in Table 6. There were several

differences in detail between the two thermograms but the main finding was that minicells showed no peak p_r on the rerun thermogram whilst, on the first heating run, there was no peak n in the ribosomal region and peaks p and q were much reduced.

The measured $T_{\max}$ value for p was slightly higher in minicells than in normal cells, whereas q and s were very similar and r was slightly lower. However, peak p was very small in both minicells and normal cells, and measurements of $T_{\max}$ were made with difficulty. We cannot be confident therefore that these differences are significant. The enthalpy of peak p_r in normal cells was 69 J (g DNA)⁻¹ (Table 5) which is very close to the value of 64 J (g DNA)⁻¹ obtained when pure DNA was heated for the first time in glutamate buffer (Table 4). Chemical analysis confirmed that minicells contained less than one-tenth the amount of DNA found in normal cells (Table 6). The residual amount may have been due to some small whole cells remaining in the preparation. The absence of peak p_r from minicells confirms that this feature is associated with the melting of intracellular DNA.

Discussion

The purpose of this work was to identify cell organelles or structures whose denaturation was responsible for the characteristic features seen in thermograms of whole bacterial cells. To do this thermograms of various cell fractions have been examined for a correspondence between thermogram features in these fractions and those observed in whole cells.

The processes represented by peaks f and f_r observed in the low-temperature region in whole cells were also seen in envelope fractions but were absent from ribosomes, cell-free extract and ribosome-free extract. Similar features were observed by Melchior & Steim (1976) in calorimetric studies of whole cells and were attributed to the melting of membrane lipids. This conclusion is confirmed by our observation that the peak maxima vary depending on growth temperature, and by the similar melting behaviour of lipids extracted from *E. coli* cells. The observation that the lipid endotherm in cells grown at 37 °C extended to higher temperatures than in cells grown at 20 °C (Fig. 3) concurs with several other reports on the effect of growth temperature on the melting temperature of membrane lipids (Jackson & Cronan, 1978; Halverson *et al.*, 1978; Marr & Ingraham, 1962) and is probably caused by an increased proportion of unsaturated fatty acids in the lipids of the 20 °C sample.

Although the DSC scans were started at 5 °C, observations were limited to temperatures above about 20 °C because of initial instrumental transients, and there was an indication that part of the lipid endotherms occurred below this temperature (see Fig. 5, also Melchior & Steim, 1976). In order to observe the transition at lower temperatures, unobscured by transients or the ice/water transition, it would have been necessary to incorporate an antifreeze agent in the specimen (e.g. Steim, 1972).

In the temperature range studied, the area of f_r was generally larger than f , presumably because part of the lower temperature endotherm of f melted at a higher temperature on reheating. A similar observation was made by Melchior & Steim (1976) on reheating whole cells, and may be caused by different proportions of polymorphic crystalline forms of lipids in unheated and heated cell walls. Alternatively, denaturation of membrane proteins on heating may have affected the packing arrangement of the phospholipids on cooling.

The temperature of peak maxima for melting of membrane lipids or extracted lipids were lower on the rerun than on the run, and this may also be related to changes in the crystalline form induced by heating and rapid cooling.

The rather broad endotherm caused by the melting of membrane lipids quite often contained an additional exothermic process occurring between 25 and 29 °C. This superimposed feature may have been caused by a change in state of the peptidoglycan component of the cell envelope since it was always present in the few peptidoglycan preparations that we examined. This novel feature is particularly interesting because it occurs within the normal growth range of the bacterium.

Peaks m_1 , m_2 and n seen in whole cells are caused by

melting of the ribosomes. Similar, but larger features were present in the crude ribosome fraction but were much reduced or absent in the ribosome-free supernatant. However, it was difficult to make precise correlations between thermogram features observed in purified preparations and those seen in whole cells. It was apparent that changes in the appearance of peaks and in their $T_{\max}$ values occurred during the fractionation procedures. Thus the $T_{\max}$ of the main peak m_2 was about 5 °C higher in purified 70S particles than within whole cells. This may have been caused by differences in melting behaviour in glutamate buffer compared with that in cytoplasm. In crude ribosome preparations, which would have contained both glutamate buffer and cytoplasm, the $T_{\max}$ was of intermediate value. Thermograms of ribosomes were also affected by the concentration of the sample. In pelleted ribosomes, peak m_1 appeared as a shoulder at about 62 °C preceding the main peak m_2 , whereas in material that had been diluted 50:50 with buffer and resuspended, m_1 appeared as a distinct peak with a $T_{\max}$ of 64.5 °C (Fig. 2).

The $T_{\max}$ and shape of m_1 in whole cells are very similar to those of the first peak in the crude ribosome preparation (Fig. 1, Table 2) and the $T_{\max}$ of the first peak observed in diluted crude ribosomes (64.5 °C) is close to that of the purified 30S subunit (65.6 °C). We therefore suggest that peak m_1 seen in whole cells represents melting of the 30S ribosomal subunit.

The main thermogram feature seen in whole cells, peak m_2 , was similar to the main feature seen in crude ribosomes, purified 70S particles and 50S subunits. At first sight it would be tempting to identify m_2 with melting of the 50S subunit, but it is clear that denaturation of the 70S ribosome cannot simply be divided into the separate denaturation of 30S and 50S subunits. The shoulder on the leading edge of the 70S thermograms is too small to represent denaturation of all the 30S component. Part of the melting process of the 30S subunit must therefore occur within the larger, main peak of the 70S thermogram. The temperatures of the shoulder and main peak of the 70S particle are slightly higher than the corresponding maxima in the 30S and 50S subunits. The thermostability of the 70S particle would thus appear slightly greater than the sum of its constituent parts.

Several workers have reported that the 30S ribosomal subunit is more heat-labile than the 50S subunit and Verrips & Brevoort (1979) proposed an inactivation scheme based on denaturation of the 30S subunit followed by hydrolysis of 16S RNA. Exposure of cells of *Salmonella typhimurium* to mild heat (48 °C) in phosphate buffer results in selective destruction of the 30S ribosomal subunit (Tomlins & Ordal, 1976). This was ascribed to an indirect effect arising from loss of

magnesium from the cell followed by ribonuclease attack on the destabilized 30S subunit. However, isolated 30S particles obtained from both wild-type and ribonuclease-negative strains of *E. coli* were also more labile than the corresponding 50S subunits (Tamaoki & Miyazawa, 1966; Tal, 1969). We observed that the thermal stability of the ribosomal particles increases in the order $30S < 50S < 70S$. This is in agreement with the spectroscopic determinations of ribosome melting by Leon & Brock (1967). Their reported T_{max} values for denaturation were 64, 68 and 72 °C for 30S, 50S and 70S particles, respectively. Our corresponding T_{max} values for the main peaks were 65.6, 74.0 and 75.9 °C. Tal (1969) reported that the T_m for 50S subunits was only 1 °C higher than for 30S subunits derived from *E. coli* MRE600 (the original designation of the strain used in the present work). T_m values in that work were determined optically on dilute suspensions or ribosomes in a dilute buffer (1 mM-Tris/acetate, pH 7.2). There is thus some corroborative evidence that the thermal stabilities of the two ribosomal subunits differ from one another and from the complete particle. It is not clear why the associated 70S particle should be more heat stable than its constituent subunits but it is interesting that streptomycin, which inhibits the dissociation process, enhances the heat resistance of ribosomes from streptomycin-sensitive strains of *E. coli* (Wolfe & Hahn, 1968).

tRNA occurs in the cell at a concentration of about 3% of the dry matter, sufficient to cause a discernible peak. The T_{max} of peak m_3 observed in the ribosomal region in whole cells (at 76 °C) is close to that of tRNA (78 °C). Peak m_3 is also reduced on breaking the cells, which could correspond to enzymic breakdown of tRNA. Although this interpretation is consistent with the facts, we propose it only tentatively.

Thermograms of whole cells contained a broad, clear endotherm denoted m_r . This feature was also present, and very obvious, in rerun samples of crude ribosomes and in purified 70S, 50S and 30S particles. We believe m_r represents the remelting of ribosomal RNA for the following reasons: (1) the peak was broad and reversible and hence similar to the peak observed with pure rRNA (Fig. 4); (2) the T_{max} value for m_r in whole cells was 72.5 °C, similar to the T_{max} of the reversible peaks observed in 70S, 50S and 30S subunits (70.3, 71.7 and 71.5 °C, respectively) and similar to the T_{max} of pure rRNA (72.6 °C); and (3) the enthalpy of the reversible peak from 70S subunits [$31 \text{ J (g RNA)}^{-1}$] was similar to the value obtained with pure rRNA [$30 \text{ J (g RNA)}^{-1}$]. A broad endotherm similar to m_r was shown in one of the figures presented by Melchior & Steim (1976) and was attributed to reversible protein denaturation. We believe that interpretation to be incorrect, for the reasons given above.

The enthalpy of 70S ribosome denaturation [$42 \text{ J (g dry matter)}^{-1}$] is greater than that of the RNA component [$23 \text{ J (g dry matter)}^{-1}$]. If we assume that heat-denatured rRNA resumes its original tertiary structure on cooling (which is likely to be at least partially true since deproteinized 16S RNA is known to adopt a similar conformation to that in the 30S subunit and 30S subunits can be reconstituted from heat-denatured RNA; Schlesinger, 1960; Barritault *et al.*, 1979), then approximately half the enthalpy of ribosome denaturation is associated with protein denaturation and/or disruption of higher-order interactions, e.g. protein-RNA, protein-protein and long-range RNA-RNA interactions. The enthalpy change associated with melting of free rRNA [mean value, $30 \text{ J (g RNA)}^{-1}$] is less than that for DNA [$62 \text{ J (g DNA)}^{-1}$], presumably reflecting differences in helical content.

Peak l appeared as a slight inflexion on the leading edge of the ribosome melting process and was seen in whole cells and crude ribosomes. It was absent from purified 70S particles, however, and is therefore not associated with ribosome denaturation. Its presence was somewhat sporadic and we have been unable to identify it with any particular cell structure.

In previous publications (Miles *et al.*, 1986; Mackey *et al.*, 1988) we proposed that peak p at approximately 95 °C and p_r occurring at 94 °C on the rerun, were associated with the intracellular melting of DNA. The evidence identifying p_r with DNA melting is as follows: (1) it is an apparently reversible process, as is the melting of DNA; (2) the T_{max} of p_r in *E. coli* is within a range predicted from its G + C content and internal ionic strength; (3) the T_{max} values of peaks similar to p_r in other microbes were linearly correlated with the G + C content of their DNA; and (4) the mass of DNA calculated from the enthalpy of p_r is in close agreement with that obtained by chemical analysis. The results in this paper support this identification in two respects. First, p_r was absent in minicells but present in whole cells, coinciding with the expected and measured reduction of DNA in minicells. Second, p_r disappeared after DNAase treatment.

We also noted previously (Miles *et al.*, 1986; Mackey *et al.*, 1988) that p_r was larger than p and interpreted this to mean that the melting of DNA during the first heating run was not a single process, possibly because parts of the DNA were stabilized by virtue of differences in physical state or by interaction with other molecules (e.g. proteins or polyamines). Once these stabilizing forces had been disrupted by heating, subsequent remelting occurred as a simpler process giving a sharper and larger peak. In this work we observed that peak p increased in size following breakage of the cells, i.e. it more closely resembled p_r . It would appear that DNA-stabilizing

interactions are disturbed by the physical changes attending freezing and disruption of the cells. Alternatively, stabilizing interactions may be affected by enzymes that are released or activated in the broken-cell preparation.

Although peak p is at least partly caused by the melting of DNA there are two lines of evidence suggesting that other processes may contribute to the endotherm. Firstly, a small peak at 96 °C was observed in cell walls and this could contribute to p in whole cells even though that peak occurs at 95 °C. Secondly, the minicells, which contain little DNA, also showed a peak at 97 °C. We therefore propose that p is a multicomponent peak comprising at least two components, one of which is DNA, and the other a component of the cell wall.

Peak q at around 105 °C in whole cells is also multicomponent. The pronounced peak q_1 at 101.9 °C in cell walls undoubtedly contributes to q , and this component is not DNA or lipid or peptidoglycan. DNA, stabilized within the cell, also probably contributes to q , since on breaking (and disruption of stabilizing interactions) q is reduced and p is increased. Supporting evidence that DNA melting might contribute to peak q was obtained by heating cells to intermediate temperatures and observing changes in the areas of peaks q and p_r : a progressive decrease in area of q was accompanied by a corresponding increase in p_r (data not shown). There is a possible third component of q , because a peak at 104 °C occurred in the cell-free extract and this contained little cell wall component and no DNA since it had been DNAase treated. This component of q is probably caused by protein.

The multicomponent process designated r/s in whole cells was much reduced in the cell-free extract but present and magnified in the cell envelope fraction. This series of endotherms is therefore associated with denaturation of cell wall components that are not peptidoglycan or lipid, because these endotherms were absent in those fractions. Denaturation of a cell-wall-associated thermostable protein may be responsible, but since r/s is reduced following breakage, a possible contribution by nucleic acid cannot be ruled out, by analogy with the reasons given above for peak q . However, r/s is not entirely caused by DNA because the feature was observed in minicells which lack DNA.

On cooling and reheating preparations of peptidoglycan, a large exothermic process occurred between 85 and 125 °C. A similar exothermic process was not observed during reheating of crude envelope fractions. It may have been too small to detect or the peptidoglycan structure may have been physically constrained by interaction with other structural elements of the cell envelope, and thus prevented from undergoing the exothermic process.

The observations recorded in this paper have allowed us to identify some of the main thermogram features associated with the thermal denaturation of *E. coli* cells. We can now begin to place these denaturation events in a time/temperature sequence as follows.

When cells are heated for the first time there is a broad endotherm beginning below 20 °C and extending to just above 40 °C that is caused by the melting of membrane lipid. Superimposed on this is an exothermic process associated with a change of state of the cell wall peptidoglycan. At around 47 °C the first irreversible denaturation events occur, associated with melting of the 30S ribosomal subunit and denaturation of some of the soluble cytoplasmic proteins. Ribosome melting takes place over a broad temperature range between 47 and 85 °C and is characterized by three peaks denoted m_1 , m_2 and n . The process is complex and involves the sequential but overlapping denaturation of 30S, 50S and 70S particles, denaturation and/or loss of ribosomal protein, loss of higher-order structure, and melting of helical regions of the rRNA. A peak denoted m_3 at 75–76 °C is seen in whole cells but not in pure 70S ribosomes. The source of this is unknown but may be associated with melting of tRNA. At around 95 °C, peak p occurs, resulting from the melting of a portion of the cellular DNA combined with denaturation of a component of the cell wall. Peak q at approximately 105 °C in whole cells, is also multicomponent and may also be caused by melting of regions of DNA together with denaturation of a cell wall component.

The complex events denoted r/s with peaks at 118 and 125 °C may be caused by denaturation of thermostable envelope-associated proteins and possibly also by nucleic acid.

Following cooling and reheating there is a peak f_r in the low temperature region corresponding to the remelting of membrane lipid. This is followed by a broad endotherm extending from approximately 40 to 100 °C with a maximum around 73 °C. This is caused by the reversible melting of ribosomal RNA. At 94 °C in *E. coli* is peak p_r caused by melting of reannealed DNA. We have suggested previously that the temperature of this peak may be used to predict the G + C content of bacterial DNA (Mackey *et al.*, 1988). The peptidoglycan also yielded an exothermic process on reheating but this was not discernible in the whole cells.

In other work (Miles *et al.*, 1986; Mackey *et al.*, 1988), involving examination of the thermograms of 63 other species of vegetative cells, we found that it was generally, but not always, possible to recognize peaks corresponding to those labelled m , n , p , p_r and q in *E. coli* (and we now add f and f_r to that list). Therefore, the identifications made here of components of *E. coli* allow at least tentative identification of some peaks in other species.

The work also provides the information required for studying the effects of varying external or internal, and chemical or physical environments on the heat stability of particular cellular components by calorimetric measurements of whole cells and may furnish the means for estimation of their concentration in cells at different stages of growth, or growth under different conditions. Differential scanning calorimetry produces a diagram of the major thermal events underlying the response of micro-organisms to heat, and by identifying thermogram features we are providing points of reference which may be compared with the cardinal temperatures to provide insight into the causes of heat inactivation of bacteria.

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