JOURNAL OF BACTERIOLOGY, Jan. 1969, p. 140-150
Vol. 97, No. 1

Effect of Sublethal Heat on the Metabolic Activity of *Staphylococcus aureus*[1]

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Received for publication 23 September 1968

Cells of *Staphylococcus aureus* MF-31 which have been heat-injured at 52 C have an altered metabolic activity. Analyses of whole-cell preparations by means of the Thunberg technique and Warburg manometry showed decreased dehydrogenase activity and oxygen uptake on a variety of substrates. In cell-free extracts prepared from injured cells, it was demonstrated that the specific activity of fructose diphosphate aldolase, lactate dehydrogenase, and butanediol dehydrogenase was less than that of extracts prepared from normal unheated cells. Recovery of the heat-injured cells in a suitable medium supported a return of the dehydrogenase activity and oxygen uptake, but the activity of the enzymes in cell-free extracts prepared from such partially recovered cells did not fully return to the level of normal (unheated) preparations. Addition of chloramphenicol or actinomycin D to the recovery medium, singly or in combination, retarded the return of the normal metabolic activity. Radiorespirometric experiments indicated that the percentage participation of the Embden-Meyerhoff Parnas and hexose monophosphate pathways remained the same for normal and heat-injured cells. The sublethal heat treatment decreased the catabolic capabilities of *S. aureus* and the production of selected end products associated with the metabolism of glucose.

In recent studies on the effects of sublethal heating of *Staphylococcus aureus* MF-31, it was demonstrated that a number of the normal characteristics of the organism were lost. The changes noted were (i) increased sensitivity to salt (NaCl; 14, 33), (ii) increased lag period upon inoculation of a heated suspension into Trypticase Soy Broth (4), (iii) leakage of intracellular materials (amino acids, material absorbing at 260 nm, proteins, and K^+; 2, 9, 14), and (iv) the heat-induced degradation of ribosomal ribonucleic acid (rRNA; 31). Inoculation of the heat-injured suspension into a suitable recovery medium supported the return of the normal characteristics. During the recovery period, an extended lag phase was encountered which lasted until the injured cells had repaired the thermal lesions. The events occurring during recovery were the return of salt tolerance, which was interpreted as being synonymous with repair of the cytoplasmic membrane (14), and regeneration of the heat-induced degraded rRNA (31). The recovered cells were competent in resynthesizing and reconcentrating the heat-degraded materials, and the recovered cells reproduced at the same rate as a normal (unheated) preparation (4).

Through use of the appropriate inhibitors, the recovery process was found to be independent of cell wall, cell wall mucopeptide, and protein synthesis, but actinomycin D, an inhibitor of RNA synthesis, completely stopped recovery as measured by the return of salt tolerance (14, 33).

During the recovery period of "biological rejuvenescence," the catabolic and anabolic processes of *S. aureus* were directed to repair of the thermal lesions which, when completed, would permit the cells to function in a normal manner. The present study had a twofold purpose: (i) to determine what effect sublethal heating had on the catabolic processes of *S. aureus*, and (ii) to determine to what extent the catabolic processes are repaired during the recovery period and the effect of selected inhibitors on the overall recovery processes.

MATERIALS AND METHODS

Culture preparation and injury procedure. Cultures of *S. aureus* MF-31 were grown in Trypticase Soy Broth for 10 hr on a rotary shaker at 37 C (L. Bluhm, M.S. Thesis, Univ. of Illinois, Urbana, 1967). Approximately 200 ml of the culture was centrifuged at

[1] This report is from a dissertation submitted by the senior author in partial fulfillment of the requirements for the Ph.D. degree in Food Science.

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12,100 × *g* for 10 min at 0 to 2 C. The spent medium was decanted, and the cell pellet was washed twice with cold 0.1 M potassium phosphate buffer (*p*H 7.2). The cells were suspended in 10 ml of 0.1 M phosphate buffer, and this preparation was added to 190 ml of 0.1 M phosphate buffer (*p*H 7.2) pretempered at 52 C. The mixture was heated for 15 min in a thermal death time vessel under constant agitation (12). At the termination of the heating period, the heat-injured cells were harvested by centrifugation at 12,100 × *g* for 10 min (0 to 2 C). Cells that had not been heated were also centrifuged, and the washed pellet was used as representative of normal (unheated) cells.

Assay procedures. Assays were performed to determine the effect of heating on the metabolic processes of *S. aureus*, and the results were compared with those obtained with normal (unheated) preparations. For Warburg manometry, each flask contained, in a total volume of 3.0 ml: 1 ml of 0.1 M phosphate buffer (*p*H 7.2), 0.5 ml of appropriate substrate (10 μmoles/flask), 1.0 ml of concentrated cell suspension [ca. 16 mg (dry weight)/ml], 0.4 ml of water, and 0.1 ml of 20% KOH in center well (35). The data were corrected for endogenous respiration and expressed as micromoles of O_2 consumed per hour at 37 C. For Thunberg analyses, the reaction vessels contained the following: 1 ml of 2.67×10^{-4} M methylene blue, 2.0 ml of 0.1 M phosphate buffer (*p*H 7.2), 1 ml of filter-sterilized substrate (0.1 M), and 0.5 ml of a cell suspension in 0.1 M phosphate buffer at an optical density of 0.446 at 600 nm (35).

In this system, a test tube and syringe apparatus similar in design to that of Palumbo, Berry, and Witter (26) was used for the continuous purging of the samples with oxygen-free nitrogen. The absorption of light was measured at 660 nm in a Klett-Summerson colorimeter (filter no. 66).

Cell-free extracts were prepared from 200-ml amounts of normal (unheated) or heat-injured cell suspensions. The pellet was suspended in 10 ml of cold 0.1 M phosphate buffer introduced into a 45-ml homogenizer flask (Bronwill Scientific, Rochester, N.Y.) together with an equal volume of glass beads (0.11 to 0.012 mm). The preparation was homogenized for 4 min at a motor speed of 4,000 rev/min in a Bronwill homogenizer. Cooling was achieved by the adiabatic expansion of CO_2. Cold 0.1 M phosphate buffer (10 ml) was added to the homogenate, and the suspension was centrifuged at 12,100 × *g* for 10 min. The supernatant fluid was recentrifuged at 27,000 × *g* for 20 min. The resultant supernatant fluid was used as the crude cell-free extract on which the enzyme assays were performed.

Enzyme assays. Enzyme analyses were made, whenever possible, spectrophotometrically by coupling to a pyridine nucleotide-dependent system in such a way that the enzyme to be assayed was limiting. The total volume in each cuvette was 3.0 ml with a 1-cm light path. A unit of enzyme activity was defined as the amount which catalyzes the turnover of 1 μmole of substrate per min (11). Specific activity was expressed as m-units of enzyme per milligram of protein in the extract. A molar extinction coefficient of 6.22×10^6 was used for reduced nicotinamide adenine dinucleotide (NADH; 40), and a molar extinction coefficient of 1.44×10^3 was used for the calculation of phosphoenol pyruvate (29). The following enzymes were assayed by previously published methods: triosephosphate isomerase (5), glyceraldehydephosphate dehydrogenase (19), phosphoglycerate kinase (7), phosphopyruvate hydratase (8), phosphoglycerate phosphomutase (13), pyruvate kinase (29), lactate dehydrogenase (18), butanediol dehydrogenase (16), and acetoin dehydrogenase (16). The methods of Wu and Racker (40) as modified by Lee and Ordal (22) were used for assay of the following enzymes: hexokinase, glucosephosphate isomerase, phosphofructokinase, fructose diphosphate aldolase, glucose-6-phosphate dehydrogenase, and phosphogluconate dehydrogenase. All assays were made at 30 C with a Beckman DU spectrophotometer equipped with a Sargent model SR recorder.

Recovery method. After heat injury of a suspension of cells, the pellet comprised of injured cells was suspended in 10 ml of cold 0.1 M phosphate buffer. The 10-ml suspension was inoculated into 190 ml of the recovery medium according to the method of Iandolo and Ordal (14). The two media used were Trypticase Soy Broth (BBL) and a chemically defined medium (14). Recovery was carried out at 37 C on a rotary shaker for predetermined periods of time as noted in the Results section. If inhibitors were added to the recovery medium, the concentrations were 100 μg/ml for chloramphenicol and 5 μg/ml for actinomycin D. The recovered cells were collected by centrifugation at 12,100 × *g* for 10 min. The pellet was washed once in cold 0.1 M phosphate buffer and resuspended in the appropriate volume of cold phosphate buffer.

Heat treatment of cell-free extracts. Normal extract was dispensed in 3-ml amounts into test tubes (18 × 150 mm) pretempered for 1 hr in a polythermostat (26) set to have a temperature range from 29.4 to 65.2 C. After the extract had been incubated for predetermined periods of time (5, 10, or 15 min), the tubes were cooled in an ice bath. The samples were then centrifuged at 12,100 × *g* for 10 min. Protein determinations and selected enzyme assays were run on the supernatant fluid and on the pellet. The pellets had no detectable enzymatic activity. The enthalpy (ΔH), free energy (ΔF), and entropy (ΔS) were calculated by use of the formulas of Wood (39), Stearn (32), and L. D. Witter (*personal communication*).

Radiorespirometric method. Pellets of the normal (unheated) and heated cells were resuspended in 66.6 ml of cold phosphate buffer [ca. 6.02 mg (dry weight) of cells/ml]. The respirometry flasks were set up to contain the following: 1 ml of cells [6.02 mg (dry weight)/flask], 1 ml of substrate (1 mg of glucose containing 2.5 μc of label), and 4.0 ml of phosphate buffer (*p*H 7.2). Air was used to sparge the flasks at a flow rate of ca. 50 ml/min. The respirometers were placed in an Aminco Warburg bath at 37 C. During the 20-min interval of sampling, the $^{14}CO_2$ was trapped in 10 ml of 0.5 M hyamine hydroxide in absolute methanol. At the end of each time interval, the trap-

ping solution was drained, and the volume was brought back to 10 ml with absolute methanol.

A 1-ml portion of each sample was added to 15 ml of scintillation fluid composed of 5 g of 2,5-diphenyloxazole (PPO) and 0.3 g of 1,4-bis-2-(4 methyl-5-phenyloxazolyl)-benzene (dimethylPOPOP) per liter of toluene. At the termination of the experiment, a 1-ml sample from each flask was added to 15 ml of scintillation fluid composed of 7 g of PPO, 0.3 g of dimethyl POPOP, and 100 g of napthalene per liter of dioxane. The remaining contents of the flask were centrifuged, and 1 ml of the supernatant liquid was added to the second scintillation fluid. The estimation of pathway participation was calculated by use of the formulas of Wang et al. (37).

Counting was performed with a Tri-Carb liquid scintillation spectrometer (model 314-EX, Packard Instrument Co., Inc., Downers Grove, Ill). The specifically labeled substrates (glucose labeled in the 1, 2, 3-4, or 6 position) were obtained from New England Nuclear Corp., Boston, Mass.

Chemical analyses. The following additional analyses were performed: protein (21), glucose (24), lactic acid (4), acetoin and diacetyl (38), and butanediol (23). The analyses were made by use of a Spectronic-20 colorimeter (Bausch & Lomb, Rochester, N.Y.) set at the desired wavelength.

RESULTS

Dehydrogenase activity. For every substrate used, the time required for the reduction of methylene blue was greater for the heat-injured preparations than for preparations of normal cells (Table 1). Incubation of the heat-injured suspension in Trypticase Soy Broth at 37 C supported the return of the ability of the cells to reduce methylene blue (Fig. 1). Such cells regained their ability to reduce methylene blue (< 2 hr) more rapidly than their ability to divide (3 to 4 hr)

TABLE 1. *Reduction of methylene blue by normal (unheated) and heat-injured cells of S. aureus MF-31*

Substrate (0.1 M)	Time required for reduction (min)	
	Normal cells	Injured cells
Ethyl alcohol	13.5	33.0
Glucose	6.3	>120.0
Glycerol	12.0	70.0
Isocitrate	60.0	>360.0
Lactate	5.5	10.0
Maltose	20.0	>120.0
Succinate	90.0	>360.0
Formate	58.0	—[a]
Malate	>120.0	—
Mannitol	58.0	—

[a] Not tested.

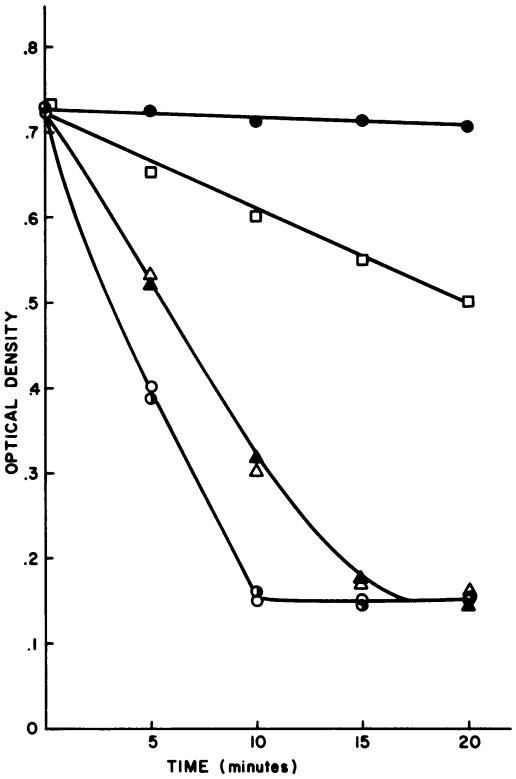

FIG. 1. *Rates of methylene blue reduction on glucose by normal, injured, and recovered cells. Cells recovered in TSB for indicated times. Symbols:* ○, *normal cells;* ●, *zero time injured;* □, *recovered for 0.5 hr;* △, *recovered for 1.0 hr;* ▲, *recovered for 1.5 hr;* ◑, *recovered for 2.0 hr.*

(14, 31). When actinomycin D or chloramphenicol was added to the recovery medium (Fig. 2), the cells regained their ability to reduce methylene blue more slowly than in Trypticase Soy Broth without the inhibitors (Fig. 1). However, the recovery of dehydrogenase activity still took place in the presence of inhibitors. In the recovery experiments of Iandolo and Ordal (14), chloramphenicol had no effect but actinomycin D completely inhibited the overall recovery process as measured by the return of salt tolerance.

Manometry measurements. For all substrates employed, the ability to utilize oxygen was less for the heat-injured cells than for normal cells (Table 2). With two compounds, ribose and succinate, oxygen uptake ceased completely. When the heat-injured cells were inoculated into Trypticase Soy Broth and allowed to recover for 1 hr at 37 C (Table 2), the rates of oxygen uptake were approximately the same as for an uninjured preparation, except when ribose or succinate was

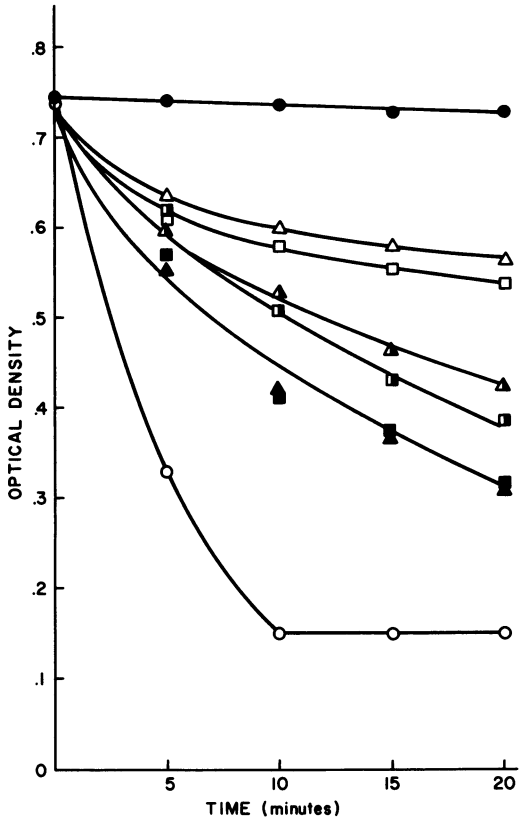

FIG. 2. *Rates of methylene blue reduction on glucose by heat-injured cells recovered in Trypticase Soy Broth containing chloramphenicol or actinomycin D. Symbols:* ○, *normal cells;* ●, *zero time injured;* △, *recovered for 0.5 hr, +100 μg of chloramphenicol per ml;* □, *recovered for 0.5 hr, +5 μg of actinomycin D per ml;* ▲, *recovered for 1.0 hr, +100 μg of chloramphenicol per ml;* ■, *recovered for 1.0 hr, +5 μg of actinomycin D per ml;* ▲, *recovered for 2.0 hr, +100 μg of chloramphenicol per ml;* ■, *recovered for 2.0 hr, +5 μg of actinomycin D per ml.*

the substrate. With these substrates, the ability to utilize oxygen was not regained during the recovery period. The endogenous respiration of the heat-injured preparation was 65% less than that noted for normal cells. After 1 hr of incubation in Trypticase Soy Broth recovery medium, the endogenous rate of respiration was approximately the same as that noted for normal cells. The enzyme(s) involved in endogenous respiration is also heat-labile.

The rates of oxygen uptake of recovered cells were determined with glucose or lactate as substrate after a 1-hr recovery period with actinomycin D and chloramphenicol, singly or in combination, in the recovery medium (Table 3). In the case of glucose, the inhibitors had a negligible effect in suppressing the return of oxygen uptake, as compared with a normal cell preparation. The return of the ability to consume oxygen when lactate was used as the substrate occurred to a lesser degree than with glucose. When recovery medium containing no inhibitors was used, the ability to consume oxygen returned to a level approaching that of normal cells with lactate as substrate; with glucose as the substrate, the rate achieved by the recovered cells was greater than that of the normal control preparation.

Cell-free extract studies. Cell-free extracts were analyzed to provide a comparison of specific activities of heat-injured and normal (unheated) preparations (Table 4). The effect of heat treatment on the enzymes was grouped as follows: those enzymes where no changes in the specific activities were noted (hexokinase, phosphofructokinase, phosphoglycerate phosphomutase, pyruvate kinase, and glucose-6-phosphate dehydrogenase), those enzymes for which specific activities of the preparations from heat-injured cells were greater than those of a normal unheated preparation (triosephosphate isomerase,

TABLE 2. *Oxygen uptake of normal, heat-injured, and recovered cells of S. aureus MF-31*[a]

Substrate	Oxygen uptake		Decrease in O_2 uptake	Oxygen uptake of recovered cells
	Normal cells	Injured cells		
	μmoles/hr	*μmoles/hr*	%	*μmoles/hr*
Glucose	13.03	10.44	20.00	12.80
Glycine	3.27	0.67	79.5	3.19
Sodium lactate	5.75	2.67	54.00	5.63
Ribose	4.42	nil	100.00	nil
Serine	3.43	1.03	69.8	3.13
Succinate	4.91	nil	100.00	nil
Endogenous respiration	9.73	3.44	65.00	9.20

[a] Net oxygen uptake after the subtraction of the endogenous values. All amounts are the average of five determinations.

TABLE 3. *Effect of actinomycin D and chloramphenicol on the recovery of oxygen uptake by recovered[a] cells of S. aureus MF-31*

Type of cells	Oxygen uptake (μmoles/hr)[b]	
	With glucose as substrate	With lactate as substrate
Normal	8.21	4.47
Injured	5.97	1.37
After recovery in		
Trypticase Soy Broth	9.10	3.93
Trypticase Soy Broth + chloramphenicol	8.94	2.41
Trypticase Soy Broth + actinomycin D	8.51	2.35
Trypticase Soy Broth + chloramphenicol + actinomycin D	8.71	2.35

[a] For recovery, heat-injured preparations were inoculated into Trypticase Soy Broth with or without inhibitors and incubated for 1 hr at 37 C. Concentrations of the inhibitors were as follows: chloramphenicol, 100 μg/ml; actinomycin D, 5 μg/ml.

[b] The endogenous uptake has been subtracted. The values are the average of three determinations.

glyceraldehydephosphate dehydrogenase, phosphoglycerate kinase, phosphopyruvate hydratase, and phosphogluconate dehydrogenase), and those enzymes for which the specific activities of the heat-injured preparations were significantly less than those of an unheated preparation (glucose-phosphate isomerase, fructose diphosphate aldolase, and lactate dehydrogenase).

Fructose diphosphate aldolase and lactate dehydrogenase were chosen for further study. A heat-injured preparation was inoculated into Trypticase Soy Broth, and the rate of return of the activity during the recovery process was observed (Table 5). At the designated times of recovery, a suitable sample (150 ml) was taken from the recovery medium and assayed. For both enzymes, there was a return of activity toward the level determined for a normal (unheated) preparation, but at the end of 3 hr of incubation the level attained was never equal to that of the normal cell-free extract specific activity. Tables 4 and 5 show that the activity for fructose diphosphate aldolase decreased from 37.6 to 42.3%, and for lactate dehydrogenase the decrease ranged from 84.5 to 87.2%. After 3 hr of incubation in Trypticase Soy Broth, the aldolase activity recovered to 75% of its normal rate, and lactate dehydrogenase recovered to 51% of its normal rate.

Incorporation of the inhibitors actinomycin D (5 μg/ml) and chloramphenicol (100 μg/ml) in the recovery Trypticase Soy Broth retarded the return of aldolase and lactate dehydrogenase activity (Table 6). The return of activity in Trypticase Soy Broth plus actinomycin D was always greater than that noted in the presence of chlor-

TABLE 4. *Enzymatic activity of cell-free extracts from normal and heat-injured cells*

Enzyme	Specific activity (m-units/mg of protein)	
	Normal extracts	Injured extracts
Hexokinase	59.80	59.16
Glucose phosphate isomerase	61.35	53.27
Phosphofructokinase	35.41	34.07
Fructose diphosphate aldolase	28.69	12.98
Triosephosphate isomerase	1434.88	1532.96
Glyceraldehydephosphate-dehydrogenase	174.90	207.10
Phosphoglycerate kinase	137.35	198.56
Phosphoglycerate phosphomutase	61.31	59.79
Phosphopyruvate hydratase	80.97	118.12
Pyruvate kinase	2.28	2.27
Lactate dehydrogenase	223.07	35.56
Glucose-6-phosphatedehydrogenase	2.92	3.36
Phosphogluconate dehydrogenase	47.90	53.85

TABLE 5. *Enzymatic activity of cell-free extracts[a] prepared from normal (unheated), heat-injured, and recovered cells*

Recovery time (hr)	Specific activity (m-units/mg of protein)	
	Fructose diphosphate aldolase	Lactate dehydrogenase
0	14.61	35.74
0.75	14.93	37.81
1.50	15.21	69.47
2.25	16.22	96.97
3.00	17.54	115.00
Normal activity	23.50	226.70

[a] Extracts prepared from recovered cells taken from Trypticase Soy Broth recovery medium at the designated times.

TABLE 6. *Effect of chloramphenicol or actinomycin D on the recovery[a] of fructose diphosphate aldolase and lactate dehydrogenase of injured cells recovered in Trypticase Soy Broth*

Enzyme	Recovery time (hr)	Inhibitors (μg/ml)		Specific activity (m-units/mg of protein)
		Actinomycin D	Chloramphenicol	
Aldolase	0	—	—	10.82
	2	—	—	14.35
	2	5	—	10.31
	2	—	100	11.13
	3	—	—	23.35
	3	5	—	20.21
	3	—	100	15.20
	Uninjured cells[b]	—	—	25.73
Lactate dehydrogenase	0	—	—	48.34
	2	—	—	126.33
	2	5	—	85.34
	2	—	100	86.40
	3	—	—	165.17
	3	5	—	125.39
	3	—	100	106.56
	Uninjured cells[b]	—	—	292.96

[a] Extracts prepared from cells harvested from the recovery medium at the designated times.
[b] Extract prepared from normal cells.

amphenicol, pointing to the inhibition of de novo protein synthesis in the second case (6).

During recovery in Trypticase Soy Broth, there was an odor characteristic of diacetyl. The odor was more pronounced in the recovery medium containing the heat-injured cells than in the medium inoculated with normal unheated preparations. There were no differences in the acetoin dehydrogenase activity of the two cell-free extracts, but the activity of butanediol dehydrogenase was markedly decreased from 11.90 m-units/mg of protein for normal cells to 1.38 m-units/mg of protein for heat-injured cells. Experiments on the effect of inhibitors on the recovery of butanediol dehydrogenase activity demonstrated the same pattern observed in following the recovery of aldolase and lactate dehydrogenase activity (Tables 5 and 6).

Effect of heat on cell-free extracts. A cell-free extract was prepared from a normal (unheated) cell preparation and incubated at 16 different temperatures in a polythermostat. As the time and temperature of incubation of the extracts were increased, there was a concomitant decrease in the measurable soluble protein. The 35 to 45% decrease in the amount of protein per milliliter noted for extracts prepared from heat-injured cells can be accounted for as an irreversible denaturation of the soluble protein of *S. aureus* heated at 52 C for 15 min. The extracts remaining after centrifugation were assayed for aldolase, lactate dehydrogenase, and butanediol dehydrogenase. From the data on the effect of heating for 15 min, the following thermodynamic values were calculated (Table 7): enthalpy (ΔH), free energy (ΔF), and entropy (ΔS). The large positive ΔF values indicated that the heat inactivation of the particular enzymes did not represent spontaneous reactions; rather, the occurrence of the reactions required a large application of energy. The highest ΔF value coincided with the most heat-stable enzyme, aldolase, of the three enzymes found to have decreased activity after the heat treatment. The values obtained for ΔH were typical of published values for the heat denaturation of proteins (39). The large positive increases in entropy noted for lactate and butanediol dehydrogenases depict an increase in randomness or reorientation of the structure of the enzyme typical of denaturation by heat (15, 39). The negative ΔS value for aldolase suggests that the heat treatment caused an increase in the order of the structure of the enzyme with a subsequent decrease in the activity of the enzyme.

Radiorespirometry, glucose catabolism, and end-product analyses. The data for a normal cell preparation are presented in Fig. 3 where the interval and cumulative rates of $^{14}CO_2$ evolution are plotted versus time in hours. The relative time unit (RTU), as defined by Wang et al. (37), was taken as 5 hr. This unit allows one to compare the relative rates of $^{14}CO_2$ evolution on a unit time basis with the differently labeled substrates. The largest interval and cumulative $^{14}CO_2$ evolution was obtained with glucose labeled in the 3–4 position; lesser amounts were evolved from glucose labeled in the 1, 2, or 6 position. This indi-

TABLE 7. *Thermodynamics of inactivation of aldolase, lactate dehydrogenase, and butanediol dehydrogenase in cell-free extracts heated for 15 min[a]*

Enzyme	Temp (C)	ΔH (cal)	ΔF (cal)	ΔS (cal/degree)
Lactate dehydrogenase.....	55	55,961	22,639	102
Butanediol dehydrogenase.....	43.2	79,745	22,283	182
Aldolase.....	60.1	2,478	24,891	−67

[a] With all three enzymes, the *p*H was 7.2.

cates that glycolysis and subsequent evolution of $^{14}CO_2$ from the 3,4-labeled glucose is the major pathway for glucose utilization. A similar series of experiments were performed with a heat-injured preparation (Fig. 4). A comparable pattern for the evolution of $^{14}CO_2$ was obtained for the two cell treatments. The differences noted between the two types of cell preparation were in the respirometric patterns in which the peak interval level of $^{14}CO_2$ given off by the heat-injured cells was delayed ca. 20 min in comparison with a normal cell preparation. This is indicative of the effect heat injury has on the catabolism of glucose. The interval recovery of $^{14}CO_2$ from glucose labeled in the 6 position was almost negligible for heat-injured cells. At the termination of the experiments, 98.91% or more of the initial label added was recovered (Table 8). The noted decrease in $^{14}CO_2$ evolution can be accounted for in the decreased rate of utilization of glucose as a

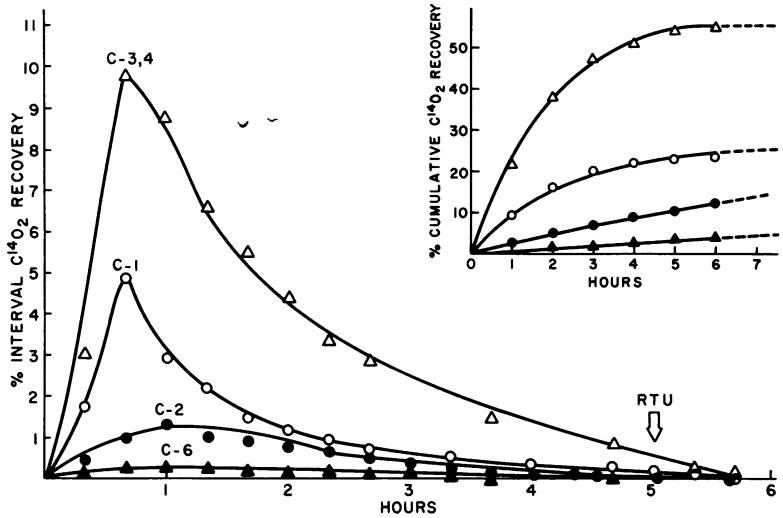

FIG. 3. *Radiorespirometric patterns for the utilization of glucose by normal (unheated) cells of Staphylococcus aureus MF-31. (A) Percentage of interval $^{14}CO_2$ recovery. (B) Percentage of cumulative $^{14}CO_2$ recovery. Symbols: ○, glucose-1-^{14}C; ●, glucose-2-^{14}C; △, glucose-3-^{14}C; ▲, glucose-6-^{14}C; RTU, relative time unit.*

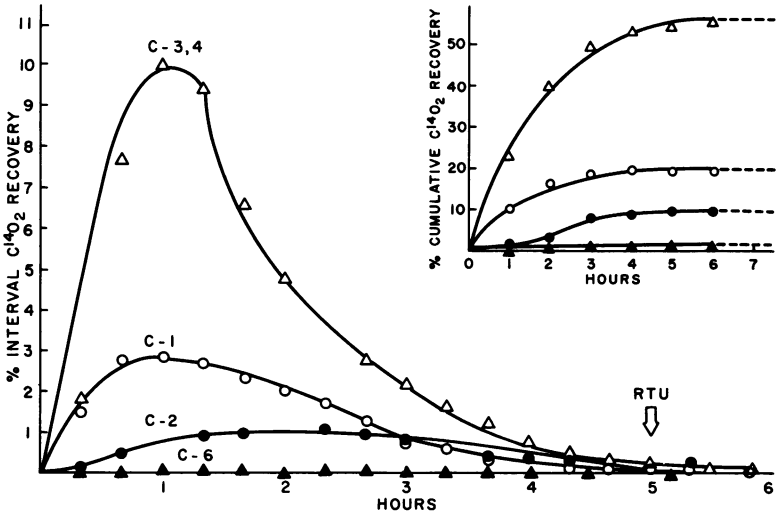

FIG. 4. *Radiorespirometric patterns for the utilization of glucose by heat-injured cells of S. aureus MF-31. (A) Percentage of interval $^{14}CO_2$ recovery. (B) Percentage of cumulative $^{14}CO_2$ recovery. Symbols: ○, glucose-1-^{14}C; ●, glucose-2-^{14}C; △, glucose-3,4-^{14}C; ▲, glucose-6-^{14}C; RTU, relative time unit.*

TABLE 8. *Aerobic incorporation of ^{14}C label into cells, medium, and CO_2 by normal and injured cells of S. aureus*

Type of cells	Substrate	Percentage isotope recoveries			
		CO_2	Cells	Medium	Total
Normal	Glucose-*1*-^{14}C	24.90	3.00	72.25	100.15
	Glucose-*2*-^{14}C	13.46	3.74	82.80	100.00
	Glucose-*3,4*-^{14}C	57.51	3.60	37.80	98.91
	Glucose-*6*-^{14}C	5.80	6.80	87.10	99.70
Injured	Glucose-*1*-^{14}C	20.48	5.88	72.50	98.96
	Glucose-*2*-^{14}C	1.58	9.40	88.96	99.40
	Glucose-*3,4*-^{14}C	54.95	5.19	39.86	100.00
	Glucose-*6*-^{14}C	9.76	5.73	83.51	99.00

result of the sublethal heat treatment. The majority of label from glucose labeled in the 1 and 6 positions was found in the medium, pointing to their incorporation into the end products of glucose metabolism. The calculated percentage participation of the Embden-Meyerhoff Parnas (EMP) and hexose-monophosphate (HMP) pathways for normal unheated cells was 88.7% and 11.3%, respectively. For heat-injured preparations it was 89.5% for EMP and 10.5% for HMP. Although the cumulative recoveries of ^{14}C in the cells, medium, and $^{14}CO_2$ were different for normal and heat-injured preparations, the participation of the two pathways remained fairly constant. Therefore, there was no shift in the pathways for the utilization of glucose as a result of the sublethal heat treatment.

To determine the effect of heat injury on metabolism of glucose and production of end products, experiments were designed to measure the rate of utilization of glucose and the production of lactic acid, acetoin plus diacetyl, and butanediol by normal and heat-injured cells inoculated into chemically defined medium (Fig. 5). The utilization of glucose, as expected, was less for a heat-injured preparation than for the corresponding normal unheated cells (Fig. 5C). The production of lactic acid and butanediol were greater for normal unheated cells (Fig. 5B and 5D). This corroborates the decreased specific activities found for lactate dehydrogenase and butanediol dehydrogenase in the analyses of cell-free extract. If in staphylococci the sequence of conversion is from diacetyl to acetoin to butanediol, the noted decrease in butanediol dehydrogenase activity would cause an increase in the amount of acetoin and diacetyl produced by a heat-injured preparation (Fig. 5C). In Fig. 5, the increase in catabolism of glucose and production of end products after 2 hr of incubation in chemically defined medium is commensurate with the recovery of the heat-damaged activity which, when complete, fits the cells for a more efficient rate of metabolic functionality.

DISCUSSION

The data present a further characterization of a repairable metabolic injury produced in cells of *S. aureus* MF-31 by sublethal heating. The Thunberg and Warburg experiments point to an overall suppression of the dehydrogenase activity and oxygen uptake of heat-injured versus normal cell preparations. The respiratory activity is, therefore, a site of heat-induced lesions in *S. aureus*. Robeson and Morita (28) observed decreased rates of respiration, as evidenced by oxygen uptake, when the marine psychrophile *Vibrio marinus* was heated for various periods of time at 20.4 C. The endogenous respiration of *V. marinus* was also damaged, indicating the heat lability of a number of normal functions of this organism. Ahn et al. (1) reported that lyophilization of a suspension of *S. aureus* cells decreased the rate of oxygen uptake to approximately one-third of the rate of untreated cells. The heat lability of a number of dehydrogenases agrees in part with the observations of Bach (3), who heated suspensions of *S. aureus* to 55 C and found that the dehydrogenase activity decreased to 3% with glucose and 50% with lactate. The earlier work and the present study point to the heat lability of staphylococcal dehydrogenase and respiratory activity when exposed to sublethal temperatures.

The effect of sublethal heating on specific enzymes of *S. aureus* is represented in Table 4. Those enzymes which showed no difference in specific activities between normal and heat-injured preparations were not pursued further. However, the heat lability of fructose diphosphate aldolase and lactate dehydrogenase was of particular interest. The main pathways for the catabolism of glucose by staphylococci are the

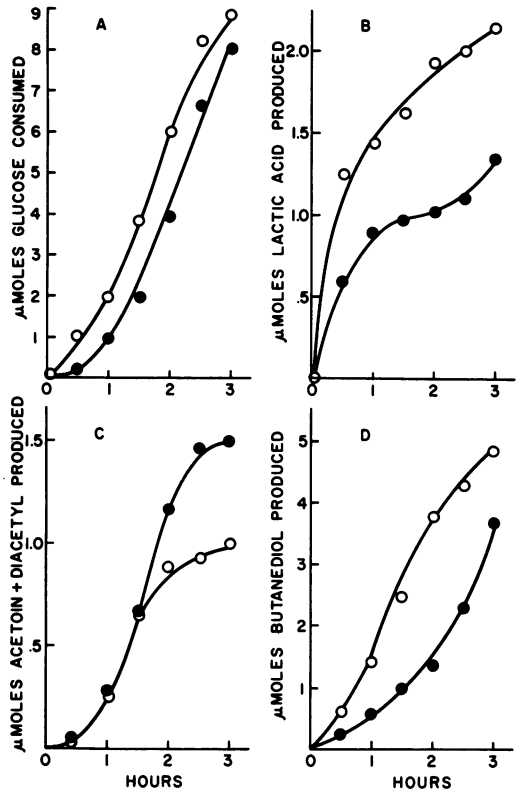

FIG. 5. *Rates of glucose catabolism and end product analyses of normal and injured cells inoculated into Trypticase Soy Broth containing 27.5 μmoles of glucose/ml. (A) Micromoles consumed. (B) Micromoles of lactic acid produced. (C) Micromoles of acetoin plus diacetyl produced. (D) Micromoles of butanediol produced. Symbols: ○, normal cells; ●, heat-injured cells. Normal recovery (end of lag) time for injured cells, 3 to 4 hr.*

EMP and HMP pathways (10, 25, 34), for which the percentage participation is 88.7% and 11.3%, respectively. The aldolase activity was decreased by at least 40% in each comparison of normal (unheated) and heat-injured preparations. Therefore, the overall efficiency of the utilization of glucose via the EMP pathway was decreased by 40%, and the supply of trioses to be metabolized further down the pathway was subsequently decreased. The specific activity of the pyruvate kinase was always the lowest of any of the EMP pathway enzymes assayed. Therefore, pyruvate kinase was the rate-limiting step. A 40% decrease in the aldolase step would seemingly have no significant effect on the subsequent steps in the pathway. However, the injured cell's energy-yielding reactions would also be decreased, with a subsequent loss in the formation adenosine triphosphate via the phosphoglycerate kinase step. When one considers that glucose-grown staphylococci have a minimal or negligible tricarboxylic acid cycle (10, 25), the loss of energy via the EMP pathway takes on more importance.

The loss of 80 to 90% of the lactate dehydrogenase activity affects the cells in two ways. The first effect is a decreased utilization of pyruvate, of which a proportion usually goes to form lactic acid (27). The second effect is a loss of available energy to the cell via a lactic-flavo-oxidase linked to the cytochrome system (25).

The decreased butanediol dehydrogenase activity of a heat-injured preparation accounts for the odor of diacetyl noted in the recovery medium (Trypticase Soy Broth or chemically defined medium). The heat-injured cells were unable to carry out the reactions from diacetyl to butanediol, with a subsequent accumulation of diacetyl and acetoin in the recovery medium. The decreased activities of the above enzymes lead to a decrease in the catabolism of glucose, energy-yielding reactions, and normal end product formation by a heat-injured cell preparation.

The percentage participation of the EMP and HMP pathways in the catabolism of glucose was 88.7% and 11.3%, respectively, for an uninjured cell preparation. The heat-injured preparations had approximately the same percentage pathway participation, indicating that a shift in pathway utilization does not occur because of sublethal heat treatment. The first two enzymes leading into the HMP pathway, glucose-6-phosphate dehydrogenase and phosphogluconate dehydrogenase, had the same specific activities for normal (unheated) and heat-injured preparations, indicating no alterations of the initial steps in the HMP pathway.

When injured cells were incubated in a recovery medium, i.e., Trypticase Soy Broth, a return of enzymatic activity was noted. It was essentially complete after 1 hr for oxygen uptake and 2 hr for dehydrogenase activity. The return of the above characteristics precedes the full return of salt tolerance, which was complete after 3 to 4 hr of incubation (14). Analyses of cell-free extracts prepared from recovered cells demonstrated that there was a return of the enzymatic activity toward the levels determined for a normal cell preparation, but at the end of 3 hr of incubation the level attained never equaled that for uninjured cells. The injured cells are in a state of metabolic imbalance after the heat treatment. This is repaired during incubation in a suitable recovery medium. The recovered cells are then able to function more efficiently during the initial catabolism of glucose and, through anabolic means, are capable of resynthesizing and reconcentrating the heat-degraded material to a

level where the recovered cells are functioning at a nearly normal rate.

The use of chloramphenicol and actinomycin D in the recovery medium did not completely inhibit the return of dehydrogenase activity, oxygen uptake, or specific enzymes in the cell-free extracts. In the presence of the inhibitors, the activity returned toward the level associated with a normal (unheated) preparation, but never equaled it. In the presence of actinomycin D, de novo RNA synthesis was inhibited (17). The rRNA left intact after heat injury [10 to 15% of the normal level of rRNA (31)] might be sufficient to account for the return of dehydrogenase activity, oxygen uptake, or enzyme activity in the recovered cells. The synthesis of enzymes in the recovered cells must function at a reduced rate commensurate with the lower level of undegraded rRNA present after heat injury. The return of activity in the presence of chloramphenicol is quite puzzling. If it is assumed that the enzyme molecule was not totally degraded during the heat treatment, but that only the spatial or conformation arrangement of the active enzyme was altered, then total resynthesis of the enzyme was not necessary for a return of some of the activity. If all that was required in the system was an energy-dependent return of the structural arrangement of the enzyme to the same or nearly the same juxtaposition as the active enzyme molecule, this could possibly account for the negligible effect of chloramphenicol, which does not retard energy-yielding reactions (36).

The loss of soluble protein observed in the cell-free extracts prepared from heat-injured cells agrees with earlier theories on the denaturation of proteins by heat (30). The native protein was coagulated by the heat, and during the initial centrifugation of the homogenate the coagulated protein was sedimented along with the cellular debris and glass beads. This, in part, accounted for the increased specific activities of some of the enzymes obtained from heat-injured cells (Table 4). The enzymes with increased specific activity were heat-stable at the temperature and time of heating. The decreased amount of protein in the injured cell-free extract increased the calculated specific activity, which was reported as m-units of activity per milligram of protein.

The large positive ΔF values indicated that the inactivation of aldolase, lactate dehydrogenase, and butanediol dehydrogenase was not a spontaneous reaction. For inactivation to occur, a large application of energy is needed. The positive ΔS values calculated for lactate dehydrogenase and butanediol dehydrogenase depicted an increase in randomness of the enzyme molecule, as is typical of denaturation by heat (39). The ΔH values for lactate dehydrogenase and butanediol dehydrogenase were typical of those determined for the heat inactivation of enzymes (39). The ΔH calculated for aldolase was lower than other reported values, which range from 4,200 calories for tuna muscle aldolase to 12,000 calories for other tissue aldolases (20).

In summary, the effects of sublethal heat on *S. aureus* are a complex set of events. The structural integrity of the membrane is impaired, as evidenced by the sensitivity to salt and leakage of intracellular materials (14, 33). Other cellular functions are also impaired, as expressed by the extended lag phase (14), the degradation of rRNA (31), the decreased catabolism of a variety of substrates, and the altered production of end products. If the heat-injured cells are given adequate time for recovery in a suitable medium, the impaired structural and cellular functions are repaired to such an extent that the injured-recovered cells can function at a nearly normal rate.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service research grant UI-00174 from the National Center for Urban and Industrial Health.

The technical assistance of Katherine Young is appreciated.

LITERATURE CITED

1. Ahn, T. H., H. Nishihara, C. M. Carpenter, and G. V. Taplin. 1964. Viability and metabolism of *Staphylococcus aureus* after freezing, lyophilization, and irradiation. J. Bacteriol. 88:545–552.
2. Allwood, M. C., and A. D. Russell. 1968. Thermally induced ribonucleic acid degradation and leakage of substances from the metabolic pool in *Staphylococcus aureus*. J. Bacteriol. 95:345–349.
3. Bach, D. 1937. Sur la destruction des déhydrodénases due staphylocoque doié par la chaleur. Action protectrice due substrat. Compt. Rend. 204:158–160.
4. Barker, S. B., and W. H. Summerson. 1941. The colorimetric determination of lactic acid in biological material. J. Biol. Chem. 138:535–554.
5. Bersenberg, G. 1955. Triosephosphate isomerase from calf muscle, p. 387–391. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 1. Academic Press, Inc., New York.
6. Brock, T.D. 1961. Chloramphenicol. Bacteriol. Rev. 25:32–48.
7. Bücher, T. 1955. Phosphoglycerate kinase from brewer's yeast, p. 415–422. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 1. Academic Press, Inc., New York.
8. Bücher, T. 1955. Enolase from brewer's yeast, p. 427–435. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, Vol. 1. Academic Press, Inc., New York.
9. Califano, L. 1952. Liberation d'acide nucleique par les cellules bacteriennes sous l'action de la chaleur. Bull. World Health Organ. 6:19–34.
10. Collins, F. M., and J. Lascelles. 1962. The effect of growth conditions on oxidative and dehydrogenase activity in *Staphylococcus aureus*. J. Gen. Microbiol. 29:531–535.
11. Dixon, M., and E. C. Webb. 1964. Enzymes, 2nd ed. Academic Press, Inc., New York.
12. El-Bisi, H. M., and Z. J. Ordal. 1956. The effect of certain

sporulation conditions on the thermal death rate of *Bacillus coagulans* var. *thermoacidurans*. J. Bacteriol. 71:1–9.
13. Grisolia, S. 1962. Phosphoglyceric acid mutase, p. 236–242. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 5. Academic Press, Inc., New York.
14. Iandolo, J. J., and Z. J. Ordal. 1966. Repair of thermal injury of *Staphylococcus aureus*. J. Bacteriol. 91:134–142.
15. Kauzmann, W. 1959. Some factors in the interpretation of protein denaturation. Advan. Protein Chem. 14:1–63.
16. Kersters, K., and J. De Ley. 1966. Primary and secondary alcohol dehydrogenases from *Gluconobacter*, p. 346–354. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 9. Academic Press, Inc., New York.
17. Kirk, J. M. 1960. The mode of action of actinomycin D. Biochim. Biophys. Acta 42:167–169.
18. Kornberg, A. 1955. Lactate dehydrogenase of muscle, p. 441–443. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 1. Academic Press, Inc., New York.
19. Krebs, E. G. 1955. Glyceraldehyde-3-phosphate dehydrogenase from yeast, p. 407–411. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 1. Academic Press, Inc., New York.
20. Kwon, T. W., and H. S. Olcott. 1965. Tuna muscle aldolase. I. Purification and properties. Comp. Biochem. Physiol. 15: 7–16.
21. Layne, E. 1957. Spectrometric and turbidometric method for measuring proteins, p. 447–4541. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 3. Academic Press, Inc., New York.
22. Lee, C. K., and Z. J. Ordal. 1967. Regulatory effect of pyruvate on the glucose metabolism of *Clostridium thermosaccharolyticum*. J. Bacteriol. 94:530–536.
23. Neish, A. C. 1952. Analytical methods for bacterial fermentations. National Research Council of Canada, N.R.C. No. 2952.
24. Nelson, N. 1944. A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem. 153:375–380.
25. Palkina, N. A., and V. N. Gershanovich. 1964. Respiratory and glycolytic enzymes in *S. aureus*. Mikrobiologiya 33: 777–782.
26. Palumbo, S. A., J. M. Berry, and L. D. Witter. 1967. Culture aeration in a polythermostat. Appl. Microbiol. 15:114–116.
27. Powelson, D. M., P. W. Wilson, and R. H. Burris. 1947. Oxidation of glucose, glycerol and acetate by *Staphylococcus aureus*. Biochem. J. 41:486–491.
28. Robeson, S. M., and R. Y. Morita. 1966. The effect of moderate temperature on the respiration and viability of *Vibrio marinus*. Z. Allgem. Mikrobiol. 6:181–187.
29. Rose, I. A. 1960. Studies on the enolization of pyruvate by pyruvate kinase. J. Biol. Chem. 235:1170–1177.
30. Sizer, I. W. 1943. Effects of temperature on enzyme kinetics. Advan. Enzymol. 3:35–62.
31. Sogin, S. J., and Z. J. Ordal. 1967. Regeneration of ribosomes and ribosomal ribonucleic acid during repair of thermal injury to *Staphylococcus aureus*. J. Bacteriol. 94:1082–1087.
32. Stearn, A. E. 1949. Kinetics of biological reactions with special reference to enzymatic processes. Advan. Enzymol. 9:25–74.
33. Stiles, M. E., and L. D. Witter. 1965. Thermal inactivation, heat injury and recovery of *Staphylococcus aureus*. J. Dairy Sci. 48:677–681.
34. Strasters, K. C., and K. C. Winkler. 1963. Carbohydrate metabolism of *Staphylococcus aureus*. J. Gen. Microbiol. 33:213–229.
35. Umbreit, W. W., R. H. Burris, and J. F. Stauffer. 1957 Manometric techniques. Burgess Publishing Co., Minneapolis, Minn.
36. Vasquez, D. 1966. Mode of action of chloramphenicol and related antibiotics. Symp. Soc. Gen. Microbiol. 16:169–191.
37. Wang, C. H., C. T. Gregg, I. A. Forbusch, B. E. Christensen, and V. H. Cheldelin. 1956. Carbohydrate metabolism in baker's yeast. I. Time course study of glucose utilization. J. Am. Chem. Soc. 78:1869–1874.
38. Westerfield, W. W. 1945. A colorimetric determination of blood acetoin. J. Biol. Chem. 161:495–502.
39. Wood, T. H. 1956. Lethal effects of high and low temperature on unicellular organisms. Advan. Biol. Med. Phys. 4:119–165.
40. Wu, R., and E. Racker. 1959. Regulatory mechanisms in carbohydrate metabolism. III. Limiting factors in glycolysis of ascites tumor cells. J. Biol. Chem. 234:1029–1035.