The Composition of *Staphylococcus aureus* in Relation to the Water Activity of the Growth Medium

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SUMMARY

Staphylococcus aureus organisms, grown in basal medium of water activity (a_w) 0.993 and in basal medium adjusted to several different a_w values by addition of NaCl, were analysed for the following components: sodium, potassium, calcium, magnesium, total amino acids, inorganic phosphate, chloride, water, DNA, RNA, protein. Cell water decreased from 1.66 to 0.88 g./g. dry weight when a_w was decreased from 0.993 to 0.90. Internal concentrations of solutes generally increased with decrease in a_w , potassium and phosphate concentrations being greatest at 0.92 a_w and sodium, chloride, magnesium, and amino acids at 0.90 a_w , the lowest a_w value studied. Increases in potassium and amino acid concentrations resulted largely from the decreased water contents of cocci grown at low a_w values. Intracellular sodium and chloride concentrations were much lower than and proportional to the NaCl concentration in the medium. The predominant cell solute was potassium at 0.92 a_w and above, and sodium below 0.92 a_w . The data are discussed in relation to the inhibition of bacterial growth at low a_w values.

INTRODUCTION

Staphylococcus aureus organisms possess a high osmotic pressure, or low water activity (a_w), when grown in normal media. Mitchell & Moyle (1956) suggested from vapour pressure equilibration of washed cocci that their water activity was equivalent to that of 1.0 molal sucrose solution, namely about 0.98 a_w . Christian & Waltho (1962*b*) obtained a corrected freezing point of unwashed, heated staphylococci of -3.27° , corresponding to about 0.97 a_w . Both values are appreciably lower than the a_w values of the growth media, which were 0.999 and 0.993, respectively. Clearly, these organisms maintain a large turgor pressure when grown in dilute media, and recent data (Christian & Waltho, 1962*a*) suggest that potassium ion and amino acids may be osmotically important components of such cocci, while sodium, phosphate, and chloride are less prominent.

Staphylococci are extremely salt tolerant, growing at a_w values down to 0.88 to 0.86 (Scott, 1958). As bacteria appear to be hypertonic to their growth medium, irrespective of its a_w value (Christian & Ingram, 1959), the concentrations of low molecular weight solutes in staphylococci grown at low a_w values are of some interest. The present paper reports partial analyses of *Staphylococcus aureus* grown

at a_w values of 0.998 to 0.90. Sodium, potassium, calcium, magnesium, total amino acids, inorganic phosphate, chloride, water, DNA, RNA, and protein were determined.

METHODS

Organism and growth conditions. The test organism was *Staphylococcus aureus* strain 49/1974 (Scott, 1953). The basal medium was brain + heart infusion broth of 0.998 a_w , adjusted where required to a_w values of 0.97, 0.94, 0.92, and 0.90 by addition of NaCl, unless otherwise stated in the text. Scott (1953) discussed in detail the control of the a_w value in culture media. Volumes (600 ml.) of medium were inoculated from a 16 hr broth culture and aerated by shaking at 30° until the population reached the stationary phase. The cocci were harvested by centrifugation, washed in a NaCl solution isotonic with the growth medium, and suspended in 40 ml. of the NaCl solution. When harvested from media adjusted to 0.97 a_w with sucrose or to 0.92 a_w with KCl, the washing was omitted and the cocci were re-suspended in 40 ml. of the original supernatant fluid. Eight equal coccal pellets were obtained by centrifuging 5 ml. portions of the concentrated suspension.

Analytical methods. Interstitial space was determined by phosphate dilution and was used to correct water content, dry weight, and chemical analyses for contributions of the contaminating interstitial solution. These techniques were described in detail by Christian & Waltho (1962*a*), as were methods for extraction and analysis of sodium, potassium, total amino acids (as ninhydrin-positive substances), inorganic phosphate, and chloride. In studies of dextran-impermeable water, dextran was determined by the anthrone method (Scott & Melvin, 1953). Additional analyses performed were for magnesium and calcium by atomic absorption spectroscopy and protein by the method of Lowry, Rosebrough, Farr & Randall (1951). Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) were extracted by the method of Burton (1956) and determined with the diphenylamine and orcinol reagents, respectively. The nitrogen content of trichloroacetic acid-extracted cocci was determined by the Kjeldahl procedure. All analyses were performed in duplicate.

The water sorption isotherm at 25° of heated cocci was obtained by the isopeistic technique of Robinson & Sinclair (1934). The coccal preparations were equilibrated with saturated solutions of potassium dichromate, potassium dihydrogen phosphate, potassium nitrate, and barium chloride. The a_w value of saturated potassium dihydrogen phosphate was found in this laboratory to be 0.952 at 25°; those of the other solutions were given by Stokes & Robinson (1949).

RESULTS

Cell water content

When phosphate dilution is used to determine the extent of interstitial space in a centrifuged pellet, it is assumed that the volume found represents that space which is rapidly and passively permeated by other solutes of low molecular weight. In preliminary experiments with 0.02 M-Na,K phosphate buffer and water-washed cocci, the volumes accessible to sodium and potassium were much greater than that accessible to phosphate. It appeared that surface charges or differences in perme-

ability were affecting the distribution of these ions. However, the values for the three ions were not appreciably different with 0.998 a_w buffer (about 0.2M). This concentration of phosphate (with the addition, where necessary, of NaCl to give the solution the same a_w value as the growth medium), was used routinely in the determination of interstitial volume.

Fig. 1

Fig. 2

Fig. 1. Water content of *Staphylococcus aureus* in relation to the external a_w level. ●, Cells grown in broth adjusted with NaCl to a_w levels from 0.993 to 0.900. Standard errors of mean values are shown by vertical lines. △, Cells grown at 0.998 a_w , heated and equilibrated to four a_w levels.

Fig. 2. Amounts of six solutes in cells of *Staphylococcus aureus* grown in broth adjusted with NaCl to a_w levels from 0.993 to 0.90. ▲, Sodium; ○, potassium; +, magnesium; ×, total amino acids; △, inorganic phosphate; ●, chloride. Standard errors of mean values are shown by vertical lines. Sodium, potassium and chloride are expressed as μ -equivalents, the remainder as μ moles.

The water content of staphylococci as a function of the a_w value in the medium is shown in Fig. 1. Reduction of a_w from 0.993 to 0.90 by addition of NaCl decreased cell water by 50 % from 1.66 to 0.83 g./g. dry weight. This change in cell water was related to a_w value, not to NaCl concentration, since similar values were obtained with sucrose (0.97 a_w) and KCl (0.92 a_w) additions (Table 1). Increasing the a_w value to 0.998 by dilution of the medium with water increased the cell water content to 2.44 g./g. dry weight (Table 3).

For comparison, Fig. 1 includes the water sorption isotherm for staphylococci grown in basal medium (0.998 a_w), washed in water and heated to destroy the osmotic barrier. At high a_w values, whole cocci held much less water than heated cocci, suggesting that the a_w within the whole coccus was substantially below that of the growth medium. The situation was reversed at low a_w values, and could be explained if cocci grown at 0.92 and 0.90 a_w had higher solute contents than the heated preparation, which was grown at 0.998 a_w .

Mitchell & Moyle (1956) found the difference between phosphate-impermeable and dextran-impermeable water of staphylococci to be 0.18 ml./g. dry wt. This was about 12 % greater than the phosphate-impermeable or intracellular water, and was interpreted as the water content of the cell wall. In the present studies, similar experiments indicated that the water in the cell wall was 11 % greater than the intracellular water after growth at 0.993 a_w , and 14.5 % greater after growth at 0.90 a_w .

Internal solutes of Staphylococcus aureus

The sodium, potassium, magnesium, amino acid, chloride, and inorganic phosphate contents of cocci grown at various a_w values in NaCl-adjusted media are shown in Fig. 2. Values are expressed on the basis of cell dry wt. Sodium and chloride contents increased with decrease of a_w value to 0.90, probably reflecting the increased NaCl concentration in the medium. The other components decreased between 0.993 and 0.90 a_w , with the exception of the amino acid pool, which increased steeply below 0.92 a_w . Assuming an average molecular wt. of 120 for the amino acids, the solutes of Fig. 2 represent about 16 % of the dry wt. of cocci grown at 0.993 a_w and 18 % when grown at 0.90 a_w .

Figure 3 presents the apparent intracellular concentrations of these solutes, obtained by expressing the values of Fig. 2 in terms of the cell water contents of Fig. 1, and assuming that all of these ions and molecules were in free solution within the cell. The concentrations are *molal*, i.e. moles of solute/kg. cell water. On this basis, intracellular sodium and chloride concentrations increased approximately linearly down to 0.90 a_w . The mean ratios of internal to external concentration were 0.41 for sodium and 0.23 for chloride. Increases occurred in potassium, amino acids, and phosphate concentrations as the a_w value was decreased to 0.92. The ratios of these three concentrations were 100:71:12 at 0.993 a_w and 100:60:11 at 0.92 a_w . Decreasing the a_w value to 0.90 decreased the potassium and phosphate concentrations, while the amino acids increased by over 70 %. As the amounts of these three constituents in the media were constant at all a_w values, the changes observed in intracellular concentrations were due to either the value of a_w or the concentration of NaCl in the growth media. The calcium content of cocci, although not precisely determined, was less than 6 μ -equiv./g. Cocci were much richer in magnesium, which occurred in highest concentration at 0.90 a_w and in lowest at 0.92 a_w . Thus, the trend in magnesium concentration on decreasing the value of a_w was broadly the reverse of that for potassium and phosphate.

The effect on cell composition of the solute used to control a_w level

In experiments described above, a_w was adjusted to desired values with NaCl only. Thus, resulting changes in composition could be ascribed equally to the NaCl concentration as to the a_w value. Table 1 provides comparisons of water content and sodium and potassium concentrations in staphylococci grown at a_w values controlled by the addition of various solutes. Clearly, water content was a function of a_w value, not of the solute used. Cell sodium remained approximately the same at all three a_w values when NaCl had not been added to the medium. Hence it depended primarily on the external salt concentration, not the a_w value. In contrast, cell potassium was influenced both by the a_w value and by high concentrations of KCl in the medium.

Table 1. *Water, sodium and potassium contents of Staphylococcus aureus grown in media* adjusted to several a_w with NaCl, KCl or sucrose*

a_w value	Adjusting solute	Cell water (g./g. dry wt.)	Sodium (mmolal)	Potassium (mmolal)
0.993	Nil*	1.66	100	671
0.97	NaCl	1.35	382	837
0.97	Sucrose	1.37	84	841
0.92	NaCl	0.86	930	1020
0.92	KCl	0.90	78	1922

* Basal medium was brain + heart infusion broth.

Influence of the basal medium on concentrations of potassium and amino acids in staphylococci

The ratio of potassium to amino acids was essentially constant in cocci grown at 0.92 a_w and above (Fig. 3). The concentrations of these solutes in the growth media were also constant. To determine the importance of external concentrations of potassium and amino acids in controlling internal concentrations, cocci were analysed after growth at 0.993 a_w in media whose potassium:amino acid ratio was varied almost 20-fold (Table 2). Very small changes in cell composition resulted from these treatments. Thus at these values, external concentrations of potassium and amino acids did not influence appreciably the amounts accumulated by the cocci. This result contrasts with the effect of very high external potassium concentrations discussed above.

Table 2. *Effect of potassium and amino acid concentrations in the growth medium on the composition of Staphylococcus aureus grown at 0.993 a_w*

	Medium*			
	A	B	C	D
In medium				
Potassium (mm)	23.8	6.0	26.8	6.5
Amino acids (mm)	27.2	6.8	6.8	31.3
Potassium: amino acids	0.87	0.87	3.94	0.21
In cells				
Potassium (μ -equiv./g. dry wt.)	1120	1150	1240	1020
Amino acids (μ mole/g. dry wt.)	588	582	564	503
Potassium:amino acids	1.91	1.91	2.19	2.04

* Media: A, Brain + heart infusion broth (BH); B, 1/4 strength BH + NaCl; C, 1/4 strength BH + NaCl + KCl; D, 1/4 strength BH + NaCl + vitamin-free Casamino acids (Difco). The additions of NaCl were used to bring the a_w value to 0.993 in each case.

Composition of staphylococci grown in dilute media

As most common laboratory media have a_w values well above that of brain + heart infusion broth (0.993 a_w), water, sodium, and potassium contents were determined for comparison in cocci harvested from media of 0.998 and 0.9965 a_w . These were obtained by diluting the basal medium to one-quarter and one-half strength, respectively. The results are compared in Table 3 with those obtained with 0.993 a_w medium. Increase in a_w value resulted in an increase in cell water and a decrease

in cell solute concentrations. However, in spite of the marked fall in sodium and potassium content of the medium as it was diluted to one-quarter strength, both sodium and potassium/g. dry wt. cocci remained relatively constant.

Fig. 3

Fig. 4

Fig. 3. Concentrations of six solutes in cells of *Staphylococcus aureus* grown in broth adjusted with NaCl to a_w levels from 0.993 to 0.90. ▲, Sodium; ○, potassium; +, magnesium; ×, total amino acids; △, inorganic phosphate; ●, chloride. The NaCl concentration in the medium is shown by broken line.

Fig. 4. Protein, DNA, and RNA content of cells of *Staphylococcus aureus* grown in broth adjusted with NaCl to a_w levels from 0.993 to 0.90. ○, Protein; ×, DNA; △, RNA.

Table 3. Water, sodium and potassium contents of *Staphylococcus aureus* grown in media of high a_w value

Dilution of basal medium* with water	a_w value	Cell water (g./g. dry wt.)	Sodium		Potassium	
			(mmolal)	(μ -equiv./g. dry wt.)	(mmolal)	(μ -equiv./g. dry wt.)
Nil	0.993	1.66	100	166	671	1116
1:1	0.9965	1.92	71	137	553	1060
1:3	0.998	2.44	62	151	426	1040

* Basal medium was brain + heart infusion broth.

Some macromolecular constituents

The steep increase in the amino acid pool below 0.92 a_w (Fig. 3) suggested that incorporation of amino acids into protein might be inhibited under these conditions. Protein contents of cocci grown at the various a_w values were determined and are shown, together with the DNA and RNA values, in Fig. 4. The average protein content at the five values of a_w was 35.3 % of the dry wt. The most marked change was a 14 % decrease in protein between 0.92 and 0.90 a_w . DNA averaged only 3.13 % over the five a_w values, and, like protein, decreased by 15 % below 0.92 a_w .

RNA contents were similar throughout, averaging 12.9 % of coccal dry wt. At all five a_w values, the total nitrogen content (calculated) of protein constituents was equal to about 64 % of the trichloroacetic acid-insoluble nitrogen as determined by Kjeldahl digestion and titration.

DISCUSSION

The experiments of Mitchell & Moyle (1956) suggested that the 18 % of the dry weight of staphylococci which could be extracted with trichloroacetic acid represented the low molecular weight solutes responsible for the cell osmotic pressure; 18 % is probably a slight underestimate since some solutes (e.g. sodium ions; Christian, 1962), would be removed by the preliminary water washing. The sum of the six solutes studied here was equivalent to 16–18 % of the coccal dry weight, and presumably these form the bulk of osmotically important solutes in the organisms. In view of the relatively low intracellular chloride and phosphate contents, high concentrations of other anions must be present to effect an ionic balance. *Staphylococcus aureus* organisms grown at 0.993 a_w contain sufficient fixed anions (not removed by butanol treatment and water washing) to balance about one-quarter of their potassium (Christian, 1962). Preliminary experiments indicate that other important anions include organic acids and the dicarboxylic amino acids, glutamic and aspartic acid.

It is tempting to calculate from the analytical data the intracellular a_w of cocci grown at the various a_w values. However, this will not be fruitful until the nature of the various anions and amino acids is known. Meanwhile, an estimate of the a_w value in cocci grown in basal medium may be obtained from the data of Fig. 1. Whole cocci contained 1.66 g. water/g. dry wt. Similar cocci, after heat treatment, contained this amount of water when in equilibrium with a solution of 0.967 a_w . This is close to the cell a_w value deduced from cryoscopic experiments with similar preparations (Christian & Waltho, 1962*b*). The curves of Fig. 1 converge and intersect at low a_w values probably because of the much lower sodium content of heat-treated cocci, which were grown at 0.993 a_w and washed in water. At 0.90 a_w , the whole cocci contained about 1.1 molal sodium, which was absent from the heated preparation. Addition of this concentration of a sodium salt similar to NaCl would increase the equilibrium water content of heated cocci at 0.90 a_w by about 0.25 g./g. dry wt. This would slightly more than compensate for the difference between the curves near 0.90 a_w . This indicates that cocci grown at low a_w values maintain a small turgor pressure, as predicted by the cryoscopic determinations of Christian & Ingram (1959).

Of the solutes examined, only sodium and chloride ions were found at lower concentrations in the cocci than in the medium. The only analogous data for bacteria are those of Schultz & Solomon (1961) and Schultz, Wilson & Epstein (1962), who examined exponential-phase organisms of *Escherichia coli* grown in a medium containing NaCl at concentrations below 0.15 M. Intracellular sodium and chloride concentrations were less than and approximately proportional to the external NaCl concentrations. The ratios were about 0.5 and 0.3, respectively, which are similar to those found in the present study.

The low apparent intracellular concentrations of sodium and chloride might be explained if these ions were confined to compartments in which their concentrations

were the same as in the medium. While staphylococci have an appreciable requirement for potassium (Shooter & Wyatt, 1956), it has been reported that they do not require sodium (Shooter & Wyatt, 1957). Also, sodium is readily removed from staphylococci by treatments which leave the potassium content unaltered (Christian, 1962). Thus sodium could reside in a non-specific compartment. In cocci grown at 0.92 a_w , the sums of intracellular sodium and potassium were very similar, whether the medium was adjusted with NaCl or KCl. Presumably, in cocci grown in KCl the excess potassium ions would be in the non-specific compartment. This would divide intracellular potassium into two pools, one non-specific and the other specifically accumulated. There are insufficient data here to test the compartment hypothesis, but this system clearly offers opportunities for further study. However, it should be borne in mind that the apparent sodium and chloride contents of cocci depend upon the position of boundaries determined by studies of phosphate impermeability. Thus they might merely reflect a gradation of sodium, chloride, and phosphate penetration that is governed by ionic properties of the cell surface.

In contrast to the low chloride content of staphylococci grown in high NaCl concentrations are the very high values of chloride found in extreme halophiles (Christian & Waltho, 1962*a*). It may be relevant that, relative to cell potassium content, the latter strains accumulated very small amino acid pools.

Potassium concentrations in staphylococci appeared to be a function of the a_w value whenever the external sodium:potassium ratio was high. Neither large variations in the external potassium:amino acids ratio nor decrease of a_w value to 0.92 by addition of NaCl altered appreciably the potassium:amino acid ratio within the cocci. Hence the coccal amino acid concentration was also related to the a_w value, and the mechanisms which control the accumulation of potassium and amino acids appeared to be linked in some way. The reciprocal changes in concentrations of these solutes observed below 0.92 a_w were therefore evidence of a marked disturbance of metabolism.

Between 0.92 and 0.90 a_w the amino acid pool increased by 336 μ moles/g. dry wt., while protein decreased by 52 mg./g. These changes would be equivalent if the mean molecular weight of the amino acids concerned were 155. This value is of the correct order, suggesting that most of the sharp increase in the amino acid pool resulted from decreased protein synthesis. A low a_w value might inhibit amino acid incorporation directly. However, since decrease of a_w value from 0.993 to 0.90 inhibits respiration by more than 80 % (unpublished data), it is more likely that low respiration rates limit the energy available for protein synthesis. Decreased respiration would also decrease the accumulation of potassium and inorganic phosphate. Influx of amino acids may be decreased also, but a much lower rate of utilization could result in a net increase in pool size. It seems probable that further investigations in this region (0.92–0.90 a_w) would reveal which steps in metabolism are primarily affected by restriction of the a_w value.

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