

THE INFLUENCE OF NUTRITION ON THE WATER RELATIONS OF *SALMONELLA ORANIENBURG*

By J. H. B. CHRISTIAN*

[Manuscript received August 9, 1954]

Summary

The growth rates of *Salmonella oranienburg* have been determined in four different basal media in which the water activity (a_w) was controlled by addition of salts and by addition of sucrose. In three complex media the lower limit for growth was between 0.94 and 0.95 a_w but in a simple defined medium the limiting a_w for growth was between 0.96 and 0.97. When an amino acid mixture containing proline and methionine was added to salt-adjusted simple medium, growth occurred at 0.96 a_w . Subsequent addition of eight water-soluble vitamins extended the growth range to 0.95 a_w .

I. INTRODUCTION

In an earlier paper (Christian and Scott 1953) it was shown that various basal media supported the growth of several strains of salmonellae at water activities down to about 0.945. This lower limit for growth was substantially unaffected by variations in the concentration and type of nutrients in the medium, even though these media supported different rates of growth at the optimum a_w of 0.995. When the observations were extended to a simple medium of inorganic salts and glucose the expected decrease in growth rate at the optimum a_w was accompanied by a considerable reduction in the range of a_w 's permitting growth. This result indicated that growth at a_w 's of the order of 0.95 to 0.96 could only be realized when certain nutrients, absent from the simple medium, were provided. This paper reports the results of experiments which have defined some of these supplementary nutrients.

II. METHODS

The strain of *S. oranienburg* (206) used in all experiments was one of the 16 strains previously studied by Christian and Scott (1953). The methods for controlling a_w and for measuring rates of growth have been described by Scott (1953). The media used were quarter-strength brain heart broth (B.H., basal a_w 0.998); casamino acids-yeast extract-casitone (C.Y.C., 0.999 a_w); nutrient broth (N.B., 0.999 a_w); and a simple glucose-inorganic salts medium (G.S., 0.999 a_w) containing glucose (0.01m), $(\text{NH}_4)_2\text{SO}_4$ (0.005m), MgSO_4 (0.0005m), Na_2HPO_4 (0.014m), and KH_2PO_4 (0.006m). B.H. and C.Y.C. were described by Scott (1953). The solutes used to obtain the desired a_w 's were a mixture of NaCl, KCl, and Na_2SO_4 in the molal ratio 5 : 3 : 2 (Scott 1953) and

* Division of Food Preservation and Transport, C.S.I.R.O., Homebush, N.S.W.

sucrose. To test substances for stimulation of growth, 0.02-0.06 ml of aqueous stock solution was added aseptically to 10 ml of medium immediately prior to inoculation. Such additions altered the a_w of the medium by less than 0.001.

III. RESULTS

(a) Growth in Complex Media

The rates of growth of *S. oranienburg* in three complex media, B.H., N.B., and C.Y.C., in which the a_w was controlled by addition of the triple salt mixture and sucrose are shown in Figures 1 and 2 respectively. For salt-adjusted media in Figure 1, differences in rates of growth between the three media which were large at high a_w 's became smaller as the a_w was reduced to 0.96. Similarly in

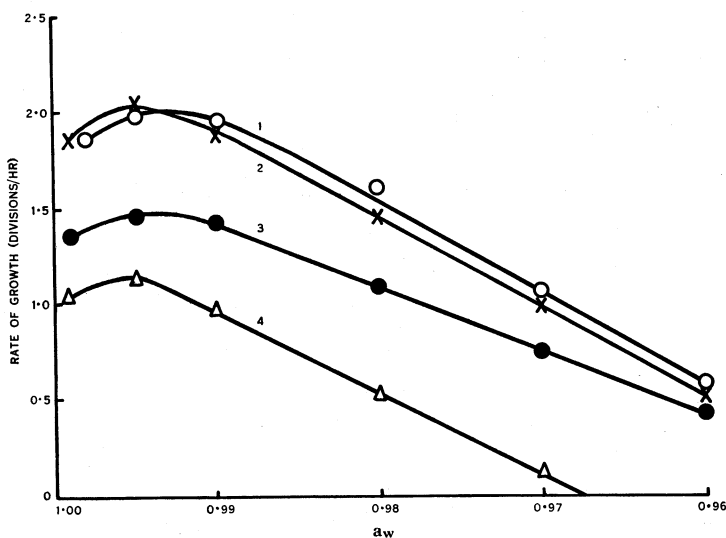


Fig. 1.—Relation between rate of growth and a_w for *S. oranienburg* in four media in which a_w was controlled with NaCl:KCl:Na₂SO₄ mixture in ratio of 5 : 3 : 2 moles. Curve 1: B.H. medium. Curve 2: N.B. medium. Curve 3: C.Y.C. medium. Curve 4: G.S. medium.

Figure 2 the curves for sucrose-adjusted media converged at about the same a_w . Although the absolute rates of growth at 0.96 a_w were about four times as high in salt-adjusted media as when sucrose was the principal solute, it has been shown previously (Christian and Scott 1953) that the lowest a_w 's permitting growth were virtually the same for both sucrose and the mixture of salts.

(b) Growth in G.S. Medium

In G.S. medium much lower rates of growth were observed at 0.995 a_w than in any of the three complex media and these rates decreased rapidly as the a_w was reduced to 0.97. The results were similar whether the a_w was controlled by the salts mixture or by sucrose as shown in Figures 1 and 2. No growth

occurred at 0.96 a_w within 28 days. Clearly, therefore, the range of a_w supporting growth in this medium is much smaller than in any of the three complex media.

(c) *Growth in Supplemented G.S. Medium*

The large differences in rates of growth and in osmotic tolerance of *S. oranienburg* in C.Y.C. and G.S. media were apparently due to differences in the nutrients supplied. C.Y.C. and G.S. media both contained inorganic salts and glucose, but the former was supplemented with yeast extract (Y.E.), casamino acids (C.A.), and casitone (C.T.). Therefore these three supplements were tested individually as growth stimulants in basal G.S. medium at 0.999 a_w and in salt-adjusted medium at 0.97 a_w .

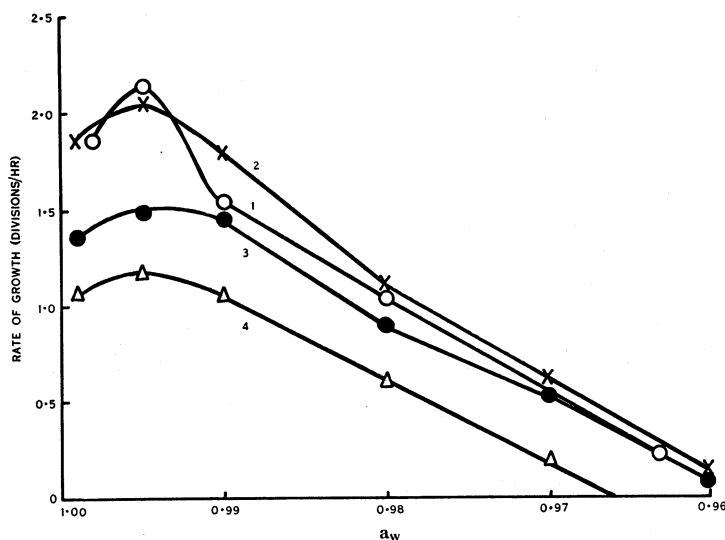


Fig. 2.—Relation between rate of growth of *S. oranienburg* in four media in which a_w was controlled with added sucrose. Curve 1: B.H. medium. Curve 2: N.B. medium. Curve 3: C.Y.C. medium. Curve 4: G.S. medium.

All three supplements increased the growth rate from 1.1 to 1.5 divisions/hr at 0.999 a_w at final concentrations of 500 mg/l. At 0.97 a_w Y.E. stimulated the growth rate from 0.1 to 0.95 divisions/hr and this initial rate was followed by two lower values of 0.58 and 0.35, as shown in Figure 3. C.A. and C.T. both increased the rate from 0.1 to 0.6 at 0.97 a_w , a result which was also obtained with a vitamin-free casamino acid preparation (V.F.C.A.). No stimulation was given by the ash of any of the supplements at either a_w or by an ether extract of Y.E. The ether-insoluble residue retained full activity. This suggested that stimulation was due to amino acids with the possibility of some additional substances present in Y.E. having an effect at 0.97 a_w .

A mixture of 22 amino acids duplicated the effect of V.F.C.A. but omission of proline reduced the stimulation to a very low level. Proline was therefore paired with each of the other 21 amino acids and four combinations were found

which produced greater stimulation of growth than did proline alone. When all of these effective amino acids, proline, methionine, serine, glutamic acid, and histidine, were tested together the rate of growth was very close to that observed with V.F.C.A. A summary of the results with various combinations of these five amino acids is given in Table 1. The final concentrations of these

TABLE 1
STIMULATION OF GROWTH OF *S. ORANIENBURG* BY AMINO ACIDS AT 30° C
Basal medium: G.S. medium adjusted with added salts to 0.970 a_w

Amino Acids Added*					Growth Rate (divisions/hr)
DL-Proline	DL-Serine	L-Glutamic acid	DL-Methionine	L-Histidine	
Basal medium only					0.10
+					0.27
+	+				0.45
+		+			0.35
+			+		0.52
+				+	0.37
+	+	+	+		0.59
+	+	+		+	0.42
+	+		+	+	0.54
+		+	+	+	0.53
	+	+	+	+	0.21
+	+	+	+	+	0.58
Vitamin-free casamino acids†					0.61

* Each amino acid was tested at a final concentration of 40 mg/l. The following did not give appreciable stimulation when coupled with proline: DL- α -alanine, DL- β -alanine, L-arginine, DL-aspartic acid, L-cysteine, glycine, L-hydroxyproline, DL-isoleucine, L-leucine, L-lysine, DL-norleucine, DL-norvaline, DL-phenylalanine, DL-threonine, DL-tryptophan, L-tyrosine, DL-valine.

† Final concentration 500 mg/l.

+ Indicates addition of appropriate amino acid.

five amino acids when supplied by V.F.C.A. (7 per cent. total N) at 500 mg/l, based on an analysis of casein (McMeekin and Polis 1949), were: L-proline 29 mg/l, L-methionine 8 mg/l, L-serine 15 mg/l, L-glutamic acid 55 mg/l, L-histidine 8 mg/l. The growth rates observed when salt-adjusted G.S. medium was supplemented with the mixture of five amino acids at a_w 's from 0.999 to 0.96 are shown in Figure 4.

As Y.E. had stimulated the growth rate to 0.9 divisions/hr in salt-adjusted G.S. medium it was possible that some of the B vitamins were involved. A mixture of eight vitamins was added to the salt-adjusted G.S. medium to give the following final concentrations: thiamin, 0.8 mg/l; riboflavin, 0.2 mg/l; biotin, 4 μ g/l; folic acid, 8 μ g/l; pyridoxine, 0.8 mg/l; Ca pantothenate, 0.8 mg/l;

nicotinic acid, 2 mg/l; *p*-aminobenzoic acid, 2 mg/l. No response was obtained at any a_w in the absence of the five amino acids but in their presence stimulation occurred at lower, but not at higher a_w 's, as shown in Figure 4. Tests of the individual vitamins and of combinations of seven in salt-adjusted G.S. medium at 0.96 a_w with added amino acids all showed some stimulation and it was not possible to assign the effects of the mixture to any particular vitamin or vitamins.

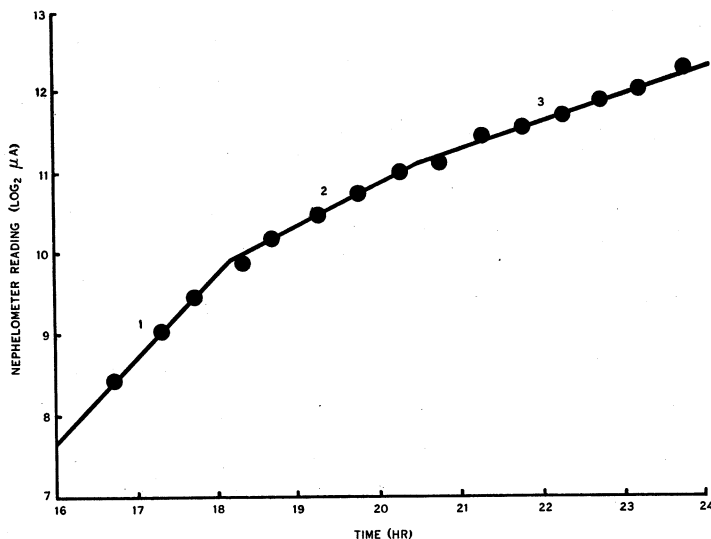


Fig. 3.—Typical growth curve for *S. oranienburg* in G.S. medium adjusted to 0.970 a_w with triple salt mixture and supplemented with 500 mg/l yeast extract. Rates: Curve 1: 0.95 divisions/hr. Curve 2: 0.58 divisions/hr. Curve 3: 0.35 divisions/hr.

A purine-pyrimidine mixture of adenine, guanine, uracil, and xanthine each at a final concentration of 2 mg/l did not affect the growth rate in salt-adjusted G.S. media containing five amino acids and eight vitamins at any a_w .

Stimulation by V.F.C.A. in G.S. medium adjusted to 0.97 a_w with sucrose was of the same order as in salt-adjusted medium. However, the addition of Y.E. to sucrose-adjusted medium produced only a small increase in growth rate from 0.18 to 0.36 divisions/hr, and was not investigated further.

(d) Minimum a_w 's for Growth

The lower limits of a_w supporting growth of *S. oranienburg* within 28 days at 30°C are given in Table 2. Irrespective of the method employed to control a_w , all complex media supported growth at 0.95 a_w , while in G.S. medium growth was recorded at 0.97 but not at 0.96 a_w . In salt-adjusted G.S. medium supplementation with five amino acids permitted growth at 0.96 a_w and with the subsequent addition of eight vitamins growth occurred at 0.95 a_w .

(e) Training Experiments

The possibility of training salmonellae to grow at increased rates under somewhat difficult osmotic conditions has been examined in two experiments. In C.Y.C. medium in which complex nutrients were provided, no evidence of increased growth rates was found during 20 transfers at $0.96 a_w$ using the triple salt mixture to control a_w . Growth rates made on each alternate transfer showed no significant regression with the number of transfers, the mean rate being 0.38 divisions/hr and the range for all values 0.35-0.40. In G.S. medium to which no complex nutrients were added, a similar experiment at $0.975 a_w$ showed slight evidence of a progressive increase in growth rate, the positive regression between rate of growth and number of transfers being significant ($P < 0.05$). The absolute increase in growth rate was, however, small, all values being within the range of 0.28-0.33 divisions/hr.

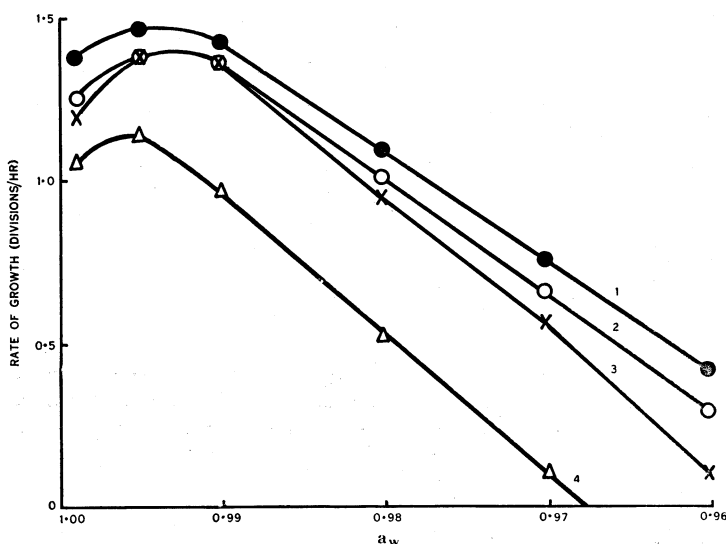


Fig. 4.—Relation between rate of growth and a_w for *S. oranienburg* in four media in which a_w was controlled with NaCl:KCl:Na₂SO₄ mixture in ratio of 5:3:2 moles. Curve 1: C.Y.C. medium. Curve 2: G.S. medium + five amino acids + eight vitamins. Curve 3: G.S. medium + five amino acids. Curve 4: G.S. medium.

IV. DISCUSSION

These results provide another example of the influence of the environment on the nutritional requirements of microorganisms. In respect of oxygen tension, Richardson (1936) has shown that the requirement of *Staphylococcus aureus* for uracil is limited to anaerobic conditions while Knight (1936) has also shown that some strains of *Salmonella* require tryptophan for anaerobic but not for aerobic growth. The effect of temperature has been demonstrated by Hills and Spurr (1952) for *Pasteurella pestis* which required several substances at 36° but not at 28°C. Similarly Beadle and Tatum (1945) have described the pro-

duction of *Neurospora* mutants whose nutritional needs are a function of temperature. Instances in which the osmotic environment modified the nutritional pattern do not, however, appear to have been reported previously.

The amino acid and vitamin requirements for growth of a number of exacting strains of *Salmonella* have been described by Knight (1936) and by Johnson and Rettger (1943) but these have little in common with the nutritional pattern of *S. oranienburg* under adverse osmotic conditions. In the pre-

TABLE 2

MINIMUM a_w 's FOR GROWTH OF *S. ORANIENBURG* IN VARIOUS LIQUID MEDIA AT 30°C AFTER 28 DAYS' INCUBATION

Basal Medium	Solutes Added	a_w	
		Growth	No Growth
B.H.	Salts mixture	0.95	0.94
B.H.	Sucrose	0.95	0.94
N.B.	Salts mixture	0.95	0.94
N.B.	Sucrose	0.95	0.94
C.Y.C.	Salts mixture	0.95	0.94
C.Y.C.	Sucrose	0.95	0.94
G.S.	Salts mixture	0.97	0.96
G.S.	Sucrose	0.97	0.96
G.S. + five amino acids	Salts mixture	0.96	0.95
G.S. + five amino acids + eight vitamins	Salts mixture	0.95	0.94

sent investigations, vitamins were essential for growth at 0.95 a_w and amino acids for growth at a_w 's below 0.97. However, while amino acids stimulated growth appreciably at high a_w 's the vitamins did not. It is of note that the vitamins were not effective in the absence of amino acids.

The observation that sucrose is more inhibitory than the salts mixture in complex media at a_w 's below the optimum without affecting the lower limit for growth has not been investigated. Obviously high sucrose concentrations have viscosities which might be expected to reduce the diffusion rates of nutrients in the solution. However, there is no evidence that this is important. Indeed, observations of a brewers' strain of *Saccharomyces cerevisiae* in complex medium showed the reverse effect—rates were lower in the salt-adjusted medium.

The results of the training experiments suggest that mutation to more osmotically tolerant forms will not be simple. This might be expected from the nutritional studies, which showed the need for a number of substances for growth at low a_w 's. The supplementary substances which increase the rate of growth of *S. oranienburg* at low a_w 's are also responsible for increasing the range of a_w 's over which growth will occur. Analogous studies with other organisms are needed to decide whether or not these nutrients have a general importance in the water economy of bacteria.

V. REFERENCES

- BEADLE, G. M., and TATUM, E. L. (1945).—*Neurospora*. II. Methods of producing and detecting mutations concerned with nutritional requirements. *Amer. J. Bot.* **32**: 678-86.
- CHRISTIAN, J. H. B., and SCOTT, W. J. (1953).—Water relations of salmonellae at 30°C. *Aust. J. Biol. Sci.* **6**: 565-73.
- HILLS, G. M., and SPURR, E. D. (1952).—The effect of temperature on the nutritional requirements of *Pasteurella pestis*. *J. Gen. Microbiol.* **6**: 64-73.
- JOHNSON, E. A., and RETTGER, L. F. (1943).—A comparative study of the nutritional requirements of *Salmonella typhosa*, *Salmonella pullorum* and *Salmonella gallinarum*. *J. Bact.* **45**: 127-35.
- KNIGHT, B. C. J. G. (1936).—Bacterial nutrition. Spec. Rep. Ser. Med. Res. Coun. Lond. No. 210.
- McMEEKIN, T. L., and POLIS, B. D. (1949).—Milk proteins. *Advanc. Protein Chem.* **5**: 202-28, Table 5.
- RICHARDSON, G. M. (1936).—The nutrition of *Staphylococcus aureus*. Necessity for uracil in anaerobic growth. *Biochem. J.* **30**: 2184-90.
- SCOTT, W. J. (1953).—Water relations of *Staphylococcus aureus* at 30°C. *Aust. J. Biol. Sci.* **6**: 549-64.