KUCZYNSKI-HALMANN, M., AVI-DOR, Y. & MAGER, J. (1958). *J. gen. Microbiol.* **18**, 364-368

Turbidity Changes in Suspensions of Gram-Positive Bacteria in Relation to Osmotic Pressure

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SUMMARY: The turbidity of suspensions of Gram-negative micro-organisms is known to be influenced by the tonicity of the suspending medium. In contrast to this, suspensions of Gram-positive micro-organisms have the same optical density in 0.2 M-NaCl as in distilled water. At higher salt concentrations, however, occasionally some batches were found to display changes in turbidity. Correlation was shown between the concentration of the amino acid pool of the organisms and their response to changes in osmotic pressure of the suspending medium. Depletion of the amino acid pool resulted in an increase in the changes in turbidity, with the simultaneous susceptibility of Gram-positive bacteria to plasmolysis. A possible correlation between this optical effect and the separation of cytoplasmic membrane from the bacterial cell wall is discussed.

It was reported in preceding communications (Mager, Kuczynski, Schatzberg & Avi-Dor, 1956; Avi-Dor, Kuczynski, Schatzberg & Mager, 1956), that variations in the osmotic pressure of the suspending medium elicited corresponding changes in turbidity of Gram-negative bacteria. The light-scattering ability of suspensions of Gram-positive micro-organisms was found, however, to be independent of the prevailing tonicity. The object of the present study was to investigate the cause of the different response of these two classes of micro-organisms to changes in the osmotic pressure.

METHODS

Culture media. *Escherichia coli* was grown on nutrient agar (Difco) at 37°. *Bacillus megaterium* was cultivated in a 2% (w/v) solution of an enzymic casein hydrolysate (N.B.C.), fortified with 0.1% (w/v) Marmite and incubated at 30° with shaking. *Staphylococcus aureus* was grown under the same conditions as *B. megaterium* with the addition of 1% (w/v) glucose to the culture medium.

Inoculum. A volume of 0.1 ml. of 24 hr. slope culture of bacteria suspended in 5 ml. saline was used for inoculation of 100 ml. medium.

Duration of incubation: 18 hr.

Chemicals. All chemicals used were of reagent grade.

Determination of ninhydrin-reacting substances released by boiling. A 2 ml.

sample of the bacterial suspension (100 mg. wet weight/ml.) was boiled for 10 min., cooled, centrifuged and 0.2 ml. of the supernatant fluid analysed for ninhydrin-positive substances (Yemm & Cocking, 1955); a similarly treated unboiled sample served for a blank determination. The amount of ninhydrin-positive substances was expressed as μ mole glutamate.

Procedure for measuring the optical effect. The optical effect (o.e.) was measured as described before (Mager *et al.* 1956). The magnitude of the effect (E') was expressed as a percentage

$$E' = \frac{E_s - E_w}{E_w} \times 100,$$

where E_w is the light extinction of the suspension in water, and E_s the extinction in the solute.

RESULTS

In a previous communication, Mager *et al.* (1956) reported an increase in the light extinction of Gram-negative bacterial suspensions in isotonic saline or glucose as compared with suspensions in water (optical effect: o.e.). Under these conditions of assay no appreciable change could be demonstrated with Gram-positive bacteria. In further studies, however, when an increased concentration of NaCl was used (0.5M) occasionally batches of Gram-positive

Table 1. *Correlation between optical effect and content of ninhydrin-reacting substances in different batches of Bacillus megaterium*

The amount of ninhydrin-reacting substances released by boiling of the organism was determined as described in 'Methods' and is calculated as 'glutamate'.

Batch no.	E' * value in 0.5M-NaCl	μ mole 'glutamate'/ 100 mg. (wet weight) bacteria
1	0	4.9
2	5	4.0
3	10	4.2
4	10	4.5
5	20	2.9
6	25	3.3
7	32	2.4

* E' denotes percentage increase in light extinction.

micro-organisms were encountered which showed the optical effect in varying degree. It was also observed that such optically-responsive batches of organisms possessed, as a rule, lower values for the concentration of the amino acid 'pool' than the suspensions which displayed no optical effect. Some representative data on the relation between the content of ninhydrin-positive substances of batches of *Bacillus megaterium* and o.e. values are assembled in Table 1. With *B. megaterium* and other Gram-positive species tested, batches showing more than a 20% o.e. invariably contained lower concentrations of the amino acid pool.

In order to test a possible correlation between the content of ninhydrin-positive substances in the bacteria and the o.e. values, *Bacillus megaterium* was incubated under conditions which caused a depletion of the amino acid pool. Among the media tested (distilled water, phosphate and phosphate + glucose), phosphate buffer (pH 7.3) was found to be the most effective. As seen in Table 2, parallel with the depletion of the amino acid pool the o.e. values increased appreciably. With *Staphylococcus aureus* the addition of

Table 2. *Influence of incubation of Bacillus megaterium with phosphate buffer on the optical effect and the content of ninhydrin-reacting substances*

The washed suspension of *Bacillus megaterium*, harvested after 18 hr. growth, was incubated with 0.05 M-phosphate buffer, (pH 7.3) at 28° for 2 hr.

Batch no.	Before incubation		After incubation	
	<i>E'</i> value	'Glutamate content' (μmole)	<i>E'</i> value	'Glutamate content' (μmole)
1	7	4.5	26	1.6
2	11	4.5	31	1.9
3	12	4.9	26	1.5
4	17	2.4	30	1.5
5	18	2.6	31	1.5

Table 3. *Influence of incubation of Staphylococcus aureus with phosphate-glucose medium, on the optical effect and on the content of ninhydrin-reacting substances*

Washed *Staphylococcus aureus* was shaken in a medium containing 0.01 M-phosphate buffer (pH 7.3), 0.01 M-NaCl, 0.15 M-KCl and 0.01 M-glucose at 37°.

Period of incubation (hr.)	<i>E'</i> value	'Glutamate' content, μmole/100 mg. wet wt. organisms
0	0	13.8
2	0	10.2
5	0	7.1
24	23	1.6

glucose to the incubation medium proved to be necessary to lower the content of ninhydrin-positive substances, in accordance with the findings of Gale (1953). The effect of the period of incubation on the changes in the amino acid pool in relation to the o.e. value, was investigated with *S. aureus* (Table 3).

In previous study on the optical effect in Gram-negative bacteria the osmotic nature of the phenomenon was indicated by the finding that different substances, both electrolytes and non-electrolytes, elicited turbidity changes. Urea, known to be highly diffusible through biological membranes, was ineffective in this respect. With *Bacillus megaterium* (with amino acid pool depleted by incubation with phosphate) only electrolytes elicited the optical effects. A comparison of the effects of the addition of increasing amounts of

NaCl, sucrose, or urea to suspensions of *B. megaterium* and *Escherichia coli* is shown in Fig. 1. As can be seen the optical density of the suspension of *B. megaterium* was increased by the addition of NaCl, remained practically unchanged in urea but in contrast to the response of Gram-negative bacteria it showed a negative O.E. value with sucrose.

Gram-positive bacteria are distinguished by their high internal osmotic pressure, due mainly to the accumulation of amino acids (20–30 atmospheres

Fig. 1. Effect of solutes on the light extinction of suspensions of *Escherichia coli* and *Bacillus megaterium*. To washed organisms suspended in distilled water, various concentration of NaCl (○—○, ●—●), sucrose (□—□, ■—■) or urea (△—△, ▲—▲) were added, and the light extinction at 500 mμ. measured. The *B. megaterium* was pretreated with phosphate as described in Table 1. Unshaded marks = *B. megaterium*; shaded marks = *E. coli*.

in *Staphylococcus aureus* in contrast to 5–6 atmospheres in *Escherichia coli*; Mitchell & Moyle, 1956) and also by being refractory to plasmolysis even in concentrated salt solutions (Eisenberg, 1910). It was thought therefore that the depletion of the amino acid pool which made *Bacillus megaterium* optically responsive to changes of osmotic pressure of the medium might render it also susceptible to plasmolysis. Indeed it was found that the characteristic microscopic picture of *B. megaterium* in water suspensions (i.e. the cytoplasm occupying the whole cellular space; see Pl. 1, fig. 1) was changed by the addition of NaCl only in 'depleted' cells showing the optical effect. In this case the shrinkage and the fragmentation of the cytoplasm was noted (Pl. 1,

fig. 2). Similar to the increased light scattering, these structural changes were fully reversed by suspension of the bacteria in water. In contrast to the behaviour of the 'depleted' micro-organisms, organisms showing no optical effects remained as in Pl. 1, fig. 1, in spite the addition of salt.

DISCUSSION

In Gram-positive micro-organisms changes in light extinction due to variations in the osmotic pressure were demonstrated only after the amino acid pool of the organisms was depleted by a suitable treatment. The close correlation between the appearance of the optical effect and the susceptibility of the organisms to plasmolysis suggest that the high internal osmotic pressure prevailing in Gram-positive micro-organisms might be responsible for the lack of optical response and the absence of shrinkage of the cytoplasm. With suspensions of mitochondria, turbidity changes due to osmotic pressure were attributed to swelling (Cleland, 1952). Volume changes in the bacterial cytoplasm might be similarly responsible for the optical effect. This was demonstrated in *Bacillus megaterium* by using the light microscope. A similar technique seems to be inadequate for the observation of the phenomenon in the much smaller Gram-negative bacteria.

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EXPLANATION OF PLATE

Fig. 1. *Bacillus megaterium* pretreated with phosphate to deplete amino acid pool (see Table 1); suspended in water.

Fig. 2. The same organisms as in Fig. 1, after addition of NaCl (to final concentration 0.5M).

(Received 19 August 1957)

M. KUCZYNSKI-HALMANN, Y. AVI-DOR AND J. MAGER—TURBIDITY CHANGES IN
BACTERIAL SUSPENSIONS. PLATE 1

(Facing p. 368)