Synergistic Effects of Salts and Carbon Dioxide on *Dermatophilus dermatonomus*

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SUMMARY

The addition of K and Na salts to cultures of *Dermatophilus dermatonomus* potentiated the effect of carbon dioxide (CO₂) in stimulating hyphal growth and delaying sporulation; the salts had virtually no effect in the absence of CO₂. The maximum production of these effects required the same minimum concentration, about 50 m-equiv. cation/l., of a comparably wide range of salts, as did maximum alteration of the chemotactic response of the zoospores to CO₂. It is suggested that there may be a common mechanism by which salts sensitize both the zoospores and the growing stages to CO₂. If these reactions are induced by the salts and CO₂ in the skin of infected sheep, they would partly account for the observed rapidity of both hyphal penetration and the emergence of zoospores.

INTRODUCTION

Carbon dioxide has been shown to affect both the growth of the actinomycete *Dermatophilus dermatonomus*, and the movement of its zoospores. Roberts (1963*a*) described a negative aerotactic response in which the zoospores moved away from the air/liquid interface in media of low salt content, but accumulated at the interface in media containing 40 or more m-equiv. monovalent salts/l. Roberts (1963*c*) showed that the movement away from the interface was migration towards an optimum concentration of endogenous CO₂. It seems likely that the salts added sensitized the zoospores to CO₂ so that they migrated towards a lower optimum concentration, which they found near the interface where CO₂ was lost by diffusion into the atmosphere. In cultures of *D. dermatonomus* Roberts (1963*d*) showed that CO₂ stimulated germination and growth but delayed development so that sporulation was inhibited and longer hyphae were formed. The present paper describes experiments which indicate that the effects of CO₂ on germination, growth and development are potentiated by concentrations of salts similar to those affecting the chemotactic response of the zoospores.

METHODS

Organism. The two strains of *Dermatophilus dermatonomus* used were strains 18 and 47 of Roberts (1963*d*).

Liquid medium. 0.5% (w/v) Oxoid Lab-Lemco beef extract, 1.0% (w/v) Difco Bacto or Proteose peptone, 0.1% (w/v) Difco yeast extract, and 0.15% (w/v) glucose,

were dissolved in distilled water; NaOH was added so that the medium was at pH 7.2 after autoclaving (120°, 20 min.).

The growth of cultures with agitation. Cultures were sown and incubated by the methods described by Roberts (1963*d*). When sporulation was studied 45 ml. lots of liquid medium in 1 l. Erlenmeyer flasks were inoculated and incubated at 37° overnight without agitation to produce a light growth of mycelium. The salts to be tested, or water in the case of controls, were then added to the medium in 5 ml. volumes, together with 1 ml. volumes of 50 % (w/v) peptone to ensure a sufficiency of nutrients during sporulation.

Before the flasks were sealed, the appropriate concentration of CO₂ was added to the air space. In the case of cultures to be grown without CO₂, 5 ml. 50 % NaOH was added to a test tube standing within the flask to trap CO₂ formed by the culture; 5 ml. water was added to the tubes in the flasks to which CO₂ was added, so that the tubes in all flasks would have the same weight and therefore, since they were free to move as the flasks rotated, the same tendency to disturb the swirling medium. The cultures were then incubated with agitation at 27° overnight.

When early filamentous growth was to be measured, the flasks again contained 45 ml. medium + 5 ml. of the appropriate sterile salt solutions. They were inoculated with standard volumes of suspensions of zoospores separated from the rest of the culture by passage through sterile 'Ekvip DS' filter pads (Industrial Equipment Australasia Pty. Ltd.). No extra peptone was added. The salt concentration and gaseous environment were adjusted at the time of inoculation. At the end of the incubation period growth was stopped by formalin (HCHO, 40 %, w/v) added to a final concentration of 0.5 % (v/v).

Growth of cultures without agitation. One-ml. volumes of solutions of K and Na salts were added to 9 ml. volumes of liquid medium in 1 oz. bottles, to give final concentrations of added cations of 0, 25, 50, or 100 m-equiv./l. Dense suspensions of filtered zoospores were added in 0.2 ml. volumes and the cultures incubated with their lids unsealed at 37° for 3 hr. Growth was then stopped by adding formalin to a final concentration of 1 % (v/v). Differential counts were made to determine the proportion of the zoospores population which had budded at each salt concentration.

Reagents. The salts used were stated by the manufacturers to be of A.R. grade. The CO₂ was obtained from cylinders filled by the Commonwealth Industrial Gases Ltd., Alexandria, N.S.W.

The measurement of growth. The extinction of zoospore suspensions was measured at 670 mμ with a 1 cm. light path in a Unicam S.P. 1400 spectrophotometer, after separation by filtration as described by Roberts (1963*d*). Filamentous growth was measured with the same instrument but after treatment of the culture with a homogenizer to disperse clumped hyphae.

The determination of differences due to synergism between salts and CO₂. 'Synergism' is defined as the difference between the effect of CO₂ and the salt when supplied together, and the sum of the effects of each when provided independently. It was measured in terms of extinction (E) as follows:

Difference due to synergism

$$\begin{aligned}
 &= \text{effect of CO}_2 \text{ and salt together} - \text{effect of CO}_2 - \text{effect of salt alone.} \\
 &= (E \text{ CO}_2 + \text{salt} - E \text{ controls}) - (E \text{ CO}_2 - E \text{ controls}) - (E \text{ salt} - E \text{ controls}) \\
 &= E \text{ CO}_2 + \text{salt} + E \text{ controls} - E \text{ CO}_2 - E \text{ salt.}
 \end{aligned}$$

Where the effect of CO₂ and salts was stimulatory, as in the case of mycelial growth, the synergism gave a positive value. In the case of sporulation the effect was inhibitory and a negative value was obtained.

The significance of the synergistic effect was determined by the application of Student's *t* test to sums and differences, as described by Mather (1949). In some experiments there were significant differences in variance between groups of flasks receiving different treatments. The application of the method of Cochran & Cox (1950) for *t* tests on samples of different variance was found in each case to give the same result as a simple halving of the total number of degrees of freedom. Thus, to simplify tabulation, a corrected number of degrees of freedom has been given in these instances.

RESULTS

The effect of salts on the germination and budding of zoospores

In these experiments the cultures were not agitated during growth. The results in Table 1 show that K and Na salts had no appreciable effect on budding at 25 m-equiv. cation/l., but usually caused a statistically significant increase in the number of budded zoospores at 50 and 100 m-equiv./l. Although CO₂ was not supplied there would have been endogenous CO₂ present since the cultures were not agitated (Roberts, 1963c).

Table 1. *The effect of salt concentration on the budding of zoospores of Dermatophilus dermatonomus in the presence only of endogenous CO₂*

Strain of <i>D. derma- tonomus</i>	Salt	Salt concentration in m-equiv. cation/l.				χ^2 for comparison of pooled results at 0 and 25 with pooled results at 50 and 100 m-equiv./l.	Significance
		0	25	50	100		
		No. of zoospores budded over total counted					
47	NaCl	56	45	67	65	5.7	$P < 0.02$
		106	100	106	110		
18	NaCl	128	125	146	146	7.46	$P < 0.01$
		257	256	253	250		
47	Na ₂ HPO ₄ + NaH ₂ PO ₄ (pH 7.1)	56	57	69	69	5.23	$P < 0.05$
		106	101	103	106		
47	Na ₂ SO ₄	56	53	46	131	7.86*	$P < 0.01$
		106	100	100	206		
47	KCl	113	56	69	139	4.24	$P < 0.05$
		214	102	116	219		

* The results for 0, 25, and 50 m-equiv. cation/l. were pooled for comparison with those for 100 m-equiv./l.

The effect of salt concentration on hyphal growth in agitated cultures containing the same CO₂ concentration

It was necessary to provide a relatively low CO₂ concentration for effects of salt concentration to be demonstrable. Incubation was continued until there were measurable differences in extinction, but growth was stopped before sporulation

began. These precautions were necessary because it was difficult to obtain a satisfactorily measurable difference in extinction, even when microscopy showed that the hyphae were of different length.

NaCl or KBr was added at concentrations extending from 0 to 70 m-equiv./l. Five flasks were used for each concentration. In the five experiments which fulfilled the necessary conditions, endogenous CO_2 only was present in one, 0.1% (v/v) CO_2 was added to the flasks in two, and 0.125% (v/v) CO_2 to the flasks in the other two. Incubation time was from 6 to 17 hr.

Fig. 1

Fig. 2

Fig. 1. The effect of salt concentration on the early growth of *D. dermatonomus* in agitated cultures at 37° . The cultures were homogenized to disperse clumped hyphae before the extinction was measured. O, Sown with zoospores of strain 18 and supplied with KBr and 0.125% (v/v) CO_2 before incubation for 6 hr; ●, strain 47 with NaCl and 0.1% (v/v) CO_2 , and incubated for 11 hr.

Fig. 2. The effect of salt concentration on the sporulation of *Dermatophilus dermatonomus* strain 47 in agitated cultures. After an initial incubation without agitation at 37° to produce mycelium, 1% (w/v) peptone and the appropriate salt and CO_2 concentrations were added to the flasks, which were then sealed and agitated overnight at 27° . Each culture was then divided into two portions. One was homogenized and used to measure the extinction (E) of the whole culture. The extinction of the other portion was measured after filtration to remove mycelium. The yield of zoospores (E) of filtrate/E of whole culture) was then calculated. Δ, NaCl+0.5% (v/v) CO_2 , ▲, KBr+0.5% (v/v) CO_2 , O and ●, NaCl+1% (v/v) CO_2 , □, KBr+2% (v/v) CO_2 , ■, NaCl+2% (v/v) CO_2 .

The extinction was greatest in the flasks to which 40 m-equiv. salt/l. was added in two experiments, 50 m-equiv./l. in two, and 60 m-equiv./l. in the other. Figure 1 illustrates the growth response in the two experiments which had the briefest incubation, and in which the extinction thus depended most exclusively on hyphal length.

The synergistic action of salts and CO_2 on hyphal growth in agitated cultures

To show synergistic effects of added salts and CO_2 on hyphal growth it was necessary to fulfil the conditions stated in the preceding section. In each experiment four groups of 5 or 6 flasks were incubated together. One group (controls) had neither CO_2 nor added salt in the medium; the second group had CO_2 but no added

Table 2. *The synergistic action of salts and CO₂ on hyphal growth of Dermatophilus dermatonomus*

Strain of <i>D. dermatonomus</i>	Salt added to medium (m-equiv./L.)	Initial CO ₂ concentration in the air space % (v/v)	Incubation time (hr)	No CO ₂ or salt added			CO ₂ + salt	Difference in E due to synergism (mean and s.e.)
				Extinction (E; mean and s.e.)				
47	NaCl, 50	0.125	18	0.002 ± 0.002	0.001 ± 0.001	0.332 ± 0.013	0.383 ± 0.021	0.052 ± 0.025
18	KBr, 40	0.10	17	0.016 ± 0.002	0.014 ± 0.001	0.170 ± 0.029	0.231 ± 0.018	0.063 ± 0.035
47	NaCl, 50	0.125	16	0.004 ± 0.002	0.003 ± 0.002	0.370 ± 0.019	0.446 ± 0.016	0.077 ± 0.026

Table 3. *The synergistic action of salts and CO₂ on sporulation in Dermatophilus dermatonomus*

Strain of <i>D. dermatonomus</i>	Salt added to medium (m-equiv./l.)	Initial CO ₂ concentration in the air space % (v/v)	No CO ₂ or salt added			CO ₂ only of filtered cultures	CO ₂ + salt cultures	Difference in E due to synergism (mean and s.e.)	Degrees of freedom	Significance
			Mean extinction (E)							
47	NaCl, 100	5	0.305	0.274	0.262	0.093	—	—0.148 ± 0.043	20	P < 0.01
47	NaCl, 100	3	0.462	0.356	0.395	0.149	—	—0.140 ± 0.050	11	P < 0.02
47	NaCl, 50	2	0.347	0.349	0.230	0.106	—	—0.126 ± 0.034	12	P < 0.01
	NaCl, 100	2	—	0.359	—	—	—	—	—	—
47	NaCl, 40	1	0.413	0.427	0.291	0.209	—	—0.096 ± 0.027	12	P < 0.01
	NaCl, 80	1	—	0.419	—	0.197	—	—0.100 ± 0.023	13	P < 0.001
18	KBr, 40	1	0.062	0.069	0.101	0.050	—	—0.058 ± 0.014	6*	P < 0.01
47	KCl, 40	0.5	0.221	0.224	0.268	0.155	—	—0.116 ± 0.020	11	P < 0.001
	KCl, 80	0.5	—	0.222	—	0.110	—	—0.159 ± 0.022	7.23	P < 0.001

* Number of degrees of freedom halved because of a difference in variance in the results, as discussed in Methods.

salt in the medium; the third had added salt but no CO₂; the fourth had CO₂ + salt, at the same concentrations as the flasks in the second and third groups, respectively.

In the absence of CO₂, growth was slight and gave very low extinction readings. Microscopic examination showed that virtually all of the zoospores had budded but the resulting hyphae were very short. Added salts did not increase the extinction in the absence of CO₂. The introduction of CO₂ stimulated growth significantly. In the presence of CO₂, added salts usually increased the extinction slightly. The synergism was appreciable in the three experiments illustrated in Table 2, but was statistically significant only in the third experiment shown ($t = 2.96$, with 9 degrees of freedom after adjustment as described in Methods; $p < 0.02$).

The effect of salt concentration on sporulation in agitated cultures containing the same CO₂ concentration

A significant effect of salt concentration on sporulation was much more easily measured than its effect on hyphal growth. It was possible to use higher CO₂ concentrations because small differences in concentration had little effect in the absence of added salts. It was not necessary to adjust the incubation time. In the absence of CO₂, sporulation was not affected by added salts. After the introduction of CO₂, however, increases in salt concentration led to a marked decrease in the production of zoospores. Usually fewest zoospores were produced after the introduction of 40 m-equiv. cation/l.

In these experiments there was sometimes considerable variation in the total amount of growth, which was reflected in the number of zoospores produced. It was found, however, that the yield of zoospores (extinction of filtrate divided by extinction of homogenized whole culture) was not greatly affected by the variation in growth and conformed to a simple curve when plotted against salt concentration. The curves in Fig. 2 show that the minimum yield, reached after the addition of about 40 m-equiv. salt/l., was maintained when 1 or 2 % (v/v) CO₂ had been supplied. With 0.5 % (v/v) CO₂ there was a plateau rather than a minimum at this concentration.

The synergistic action of salts and CO₂ on sporulation in agitated cultures

A statistically significant reduction in sporulation, due to synergism between added salts and CO₂, was produced in each of the six experiments (Table 3).

The permeability of growing forms to salts

Since Roberts (1963*a*) showed that the chemotactic response of zoospores was altered only by salts to which they were permeable, the osmotic method used in the studies on zoospores was used to determine whether the growing stages were permeable to some of the salts which affected growth. The refractive index of the cytoplasm was measured by immersion refractometry, and relative changes in protoplasmic volume were calculated, as described by Roberts (1963*a*). At a total osmolar concentration of about 0.1, the average protoplasmic solids content of hyphae and dividing stages of strains 18 and 47 was 27 % (w/v).

When the osmotic pressure was increased to 1 osmole by the addition of NaCl the average solids content of both strains was increased to 35 % (w/v), indicating a

23 % loss of volume. At 1.7 osmoles, with Na₂SO₄, the average solids content of both strains was greater than 44 % (w/v), indicating a reduction in volume of more than 38 %. The increases in solids content were far too great to be accounted for by penetration of salt into the protoplasm. Moreover, the organisms were seen under the microscope to be smaller. There was no apparent restoration of volume or reduction in refractive index within 30 min. with either salt, showing that the organisms had remained impermeable to them both.

DISCUSSION

In liquid media zoospores of *Dermatophilus dermatonomus* accumulate where the CO₂ concentration is at an optimum (Roberts, 1963*c*). Therefore the accumulation of zoospores at the air/liquid interface at increased salt concentrations (Roberts, 1963*a*) is probably due to a decrease in the optimum CO₂ concentration so that the zoospores move to the interface, where the CO₂ content is lowest as a result of diffusion into the atmosphere. The effect of the salt could be described as an increase in the sensitivity of zoospores to CO₂ or, alternatively, as a potentiation of their response to CO₂. Roberts (1963*d*) showed that CO₂ stimulated hyphal growth but inhibited sporulation. In the experiments reported here, increased salt concentrations enhanced these effects. Thus salts seemed to sensitize the organism to CO₂, or to potentiate the action of CO₂ on the organism. This was supported by the fact that the salts had little or no effect in cultures from which all CO₂ was rapidly eliminated, whereas in the presence of added CO₂ increased salt concentration led to effects on growth significantly greater than the sum of the separate effects of CO₂ and added salt.

There was also evidence that the effects of salt on chemotaxis and growth may be mediated by the same mechanism. Both actions were produced by a similar variety of salts. With chemotaxis the effect was produced by those mono- and divalent salts of the alkali metals to which the particular suspension of zoospores was permeable (Roberts, 1963*a*). In the present experiments, germination was stimulated by the chloride, sulphate, and mono- and dihydrogen orthophosphates of Na, and by KCl. Hyphal growth was stimulated, and sporulation inhibited, by NaCl and KBr (no other salts were tested). In addition, very similar concentrations were needed to produce the maximum effect on both chemotaxis and growth. Budding was not affected by 25 m-equiv. added salts/l. but, in four of five experiments, was stimulated to a maximum degree by 50 m-equiv./l. In the case of hyphal growth, the maximum effect was at 40 m-equiv./l. in two experiments, at 50 in two, and at 60 in the other one. A minimum yield of zoospores was always produced at about 40 m-equiv./l. In all of these experiments the salt content of the medium before addition of the specific salts was about 8 m-equiv./l., due mainly to NaOH added to adjust the pH value. It may thus be stated that at each stage the maximum effect on growth was produced by a minimum salt concentration of about 50 m-equiv. cation/l. Roberts (1963*a*) observed that the chemotactic response was altered in only a few zoospores at 20 m-equiv. cation/l. but in virtually every zoospore in the suspension at 40 m-equiv./l. A slight increase in effect on raising the salt concentration above 40 m-equiv./l. was not observed, but would hardly have been recognizable under the experimental conditions. It may therefore be concluded that there was

no real difference between the minimum concentration needed to give the greatest effect on the chemotactic response, and the minimum concentration for the greatest effect on growth.

The chemotactic response of zoospores was only altered by salts to which they were freely permeable (Roberts, 1963*a*), whereas the various growing stages were found to be impermeable to salts which had been shown to potentiate the effects of CO₂ on growth. This casts doubt on the idea that there is a common synergistic mechanism in both cases. It is possible that such a mechanism operates in the interior of zoospores, but at the surface in growing stages. It seems more likely, however, that there are periods of permeability during growth which did not show under the conditions of the permeability experiment, and that the salts act within the organism in both phenomena.

According to Peters & van Slyke (1931) the total concentration of K⁺ and Na⁺ in animal tissue fluids is about 150 m-equiv./l. This is well above the minimum that gives the maximum synergism with CO₂ *in vitro*. If the salts and CO₂ in the lesion which develops in infected sheep had the same action on *Dermatophilus dermatonomus*, the increased hyphal growth would contribute to the rapid penetration which has been observed after artificial infection. Also, sensitization to CO₂ by salts in dried exudate on such lesions would accelerate the emigration of zoospores if, as discussed by Roberts (1963*b, c*), this is a response to the endogenous CO₂ in the wetted scab.

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