

Studies on the Nature of the Killer Factor Produced by *Saccharomyces cerevisiae*

By D. R. WOODS* AND E. A. BEVAN

*Botany School, Oxford, and Botany Department,
Queen Mary College, London, E.1*

(Accepted for publication 20 September 1967)

SUMMARY

Killer strains of yeast, *Saccharomyces cerevisiae*, liberate a killer factor into the medium which kills sensitive yeast strains. The growth conditions necessary for the production of stable high-titre killer solutions and a biological assay for the killer factor are described. Purification was achieved by fractional precipitation with $(\text{NH}_4)_2\text{SO}_4$, dialysis, gel filtration and ultrafiltration. The fractionated killer factor is an unstable macromolecular protein which is inactivated by papain. The death of sensitive cells is not coincident with absorption of the killer factor, but can be delayed or prevented by variations in the environmental conditions. Sensitive cells are most susceptible to the action of the killer factor when in log phase. Treated resting cells on entering log phase are killed immediately.

INTRODUCTION

The killer reaction in yeast, *Saccharomyces cerevisiae*, was discovered by Bevan & Makower (1963), who described how certain strains could be classified into one of three phenotypes which they termed 'killer', 'sensitive', and 'neutral'. When killer and sensitive cells are grown together in the same culture medium, a high proportion of the latter are killed. Neutral cells neither kill sensitive cells nor are they killed by killer cells. Killing can occur without cell contact between killer and sensitive cells. The agent released by the killer cells which causes the death of the sensitive cells has been called the 'killer factor'. Studies described here show it to be a proteinaceous substance with a highly specific action spectrum, dependent on specific pH, temperature and aeration conditions.

The action of the killer factor resembles that of bacteriocins which do not cause lysis of the sensitive organisms and whose action is thus bactericidal, not bacteriolytic (Jacob & Wollman, 1959).

METHODS

Strains. Three haploid strains of *Saccharomyces cerevisiae* possessing the same mating type α , were used in the experiments: D1, a wild-type killer strain was used for the production of the killer factor; D2, a wild-type sensitive strain for the sensitive control experiments; and D3, a marked *ad*₁ mutant sensitive strain which produces a red pigment (Reaume & Tatum, 1949), as the sensitive strain in the tests performed to detect killing.

* Present address: Department of Microbiology, Rhodes University, Grahamstown, South Africa.

Seeded agar phenotype test. The phenotypes of the strains were checked by replica plating (Lederberg & Lederberg, 1952) colonies derived from single cells onto buffered complete agar medium containing 0.003 % methylene blue and killer or sensitive cells (10^5 cells/ml.). Killer colonies were identified by zones of inhibition on the agar plates seeded with sensitive cells. Since methylene blue is a specific stain for dead yeast cells (Lindegren, 1949), the sensitive colonies were identified by a dark zone of dead cells on the agar plates seeded with killer cells. Neutral colonies showed no reaction on either killer or sensitive agar plates.

High-titre killer solutions. Stable high-titre killer solutions were obtained by incubating killer cells in buffered yeast complete or minimal medium (Bevan, 1955) at 22–24° for 3 days. The liquid minimal medium was supplemented with 0.05 % gelatine, and for agar media a 2 % (w/v) concentration of agar was utilized. All media were buffered at pH 4.8 with a 0.077M citrate/phosphate buffer (Gomori, 1959). The solutions were sterilized by filtration through Oxoid membrane filters

Well test. The Wilkins technique (1949) for obtaining inhibition zones in seeded agar was used for the assay of the killer factor in liquid media. 0.05–0.1 ml. aliquots of the solutions to be assayed were added to 4 holes 13 mm. diam. in 10 ml. seeded agar plates (10^8 *ad*₁ sensitive cells/ml.). The plates were kept for 0–2 hr at room temperature before being incubated overnight at 28°.

Agar-layer technique. The technique of assaying for the killer factor in agar medium was evolved by Makower (1964). Plates spread with a lawn of wild-type killer or sensitive (control) cells were incubated for 5–7 hr at 28° before a 10 ml. cooled agar layer was placed on top of the background cells. The plates were then incubated for 6–9 hr before spreading the marked (*ad*₁ red) sensitive cells (150 cells/plate) on the surface. After a further 18 hr incubation the plates were replicated onto fresh agar medium, and the % of marked sensitive cells killed was obtained by comparing the number of red colonies arising on treated (killer background) and control (sensitive background) plates.

Purification. The killer factor was precipitated from minimal medium solutions supplemented with 0.05 % (w/v) gelatine by 30 % $(\text{NH}_4)_2\text{SO}_4$ saturation. The sediments were washed and resuspended in 0.077M-citrate/phosphate buffer at pH 4.8 before being dialysed against the buffer. The killer factor was further fractionated by gel filtration at 0–4° with 1.5×30 cm Sephadex G-150 gel columns prepared by the method of Andrews (1965). The columns were equilibrated and eluted at a flow rate of 20 ml./hr with 0.0853 M-citrate/phosphate buffer (pH 4.8) containing 0.008M- MgSO_4 ; 2 ml. starting samples were applied to the columns, and the effluents collected in 5 ml. fractions. The effluent volume containing the purified killer factor was concentrated by the pressure dialysis method of Hofsten & Falkbring (1960), using Visking tubing 8/32 at 0–4°.

Examination of column effluents. The killer factor was assayed by the Well test; and the proteins and nucleic acids by light-extinction measurements at 280 and 260 $m\mu$. Proteins were also measured by the colorimetric method of Lowry, Rosebrough, Farr & Randall (1951) as modified by Eggstein & Kreutz (1955).

Sucrose density-gradient centrifugation. 0.5 ml. killer factor samples were layered on a 4–18 % sucrose gradient and the tubes centrifuged at 107,000 *g* for 6 hr at 0–4° in an M.S.E. Super 40 swing-out rotor. The contents of the tubes were collected and assayed as 8–10 drop fractions after the bottoms of the tubes had been punctured.

Agar-gel electrophoresis. The electrophoresis was carried out using microscope slides covered by a buffered homogeneous 1% (w/v) Oxoid Ionagar layer 2 mm. in thickness, at a constant current of 2 mA per inch width of agar and at a potential of 30 V for 15–20 hr. After the electrophoresis the agar slides were cut in half longitudinally and each half was characterized for proteins and killer activity respectively. Proteins were stained by the nigrosin method of Weime (1965). The killer factor was identified by pipetting in a line down the centre of the agar strip, 0.075 ml. of a fourfold-strength buffered complete medium containing 0.06% methylene blue and 10^4 sensitive cells/ml. After overnight incubation at 28° the slides were examined for zones of inhibition.

Electron Microscopy. Serial dilutions of the fractionated killer factor were shadowed with chromium and then examined under a Siemens Elmiskop 1 A electron microscope.

RESULTS

Well-test assay technique. Although subject to the effect of well-known variables like duration of incubation (Kavanagh, 1963), type of medium or genotype of the seeded sensitive cells (Woods, 1966), the Well test under standardized conditions was a reliable quantitative assay for the killer factor. The results in Fig. 1 show that at high concentrations of the killer factor there was a linear relationship between the square of the diameter of the inhibition zone and the log of the killer factor concentration.

Effects of physical and chemical agents on the production and stability of the killer factor

Effect of pH. The optimum pH for the production and stability of the killer factor was determined by incubating killer cells at 22° for 2 days in media buffered at different pH values between 3.0 and 5.4. The cells were removed by filtration and the filtrates retained at 28°, 22°, 12° and 4° for 1 hr and 24 hr, before the killer activity was estimated by the Well test.

The results shown in Table 1 indicate that the optimum pH for the production and stability of the killer factor in liquid medium lies within the narrow range pH 4.6–4.8.

Effect of temperature. To determine the effect of temperature on the killer factor in liquid medium, aliquots of a killer solution were kept at 18°, 23°, 25°, 28° and 37° respectively for different time intervals between 0 and 4 hr before estimating the killer activity by the Well test.

The results expressed in Fig. 2 illustrate that in buffered liquid medium the killer factor was inactivated by temperatures above 25°. Such inactivation may be due to a protease derived from the yeast cells and the temperature effect may reflect the Q_{10} for digestion.

In agar medium, on the other hand, the killer factor was markedly more stable and was only inactivated by temperatures above 42° (Fig. 3). This was shown using the agar-layer technique and comparing the percentage killing on killer plates incubated at 28°, 37°, 42°, 43°, 44° and 45° respectively for 5 hr after the addition of the top agar layers and also an initial incubation period for 7 hr at 28°. Makower (1964) showed that the length of this initial period at 28° between the addition of the top agar layers and the spreading of the sensitive cells greatly affected the percentage killing. Thus, a decrease in the percentage killing between the killer plates incubated throughout the experiment at 28° and those incubated at different temperatures following the initial

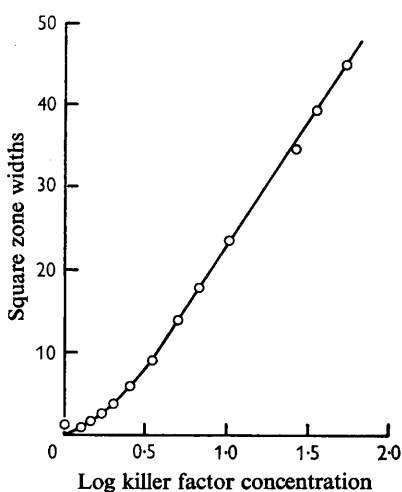


Fig. 1

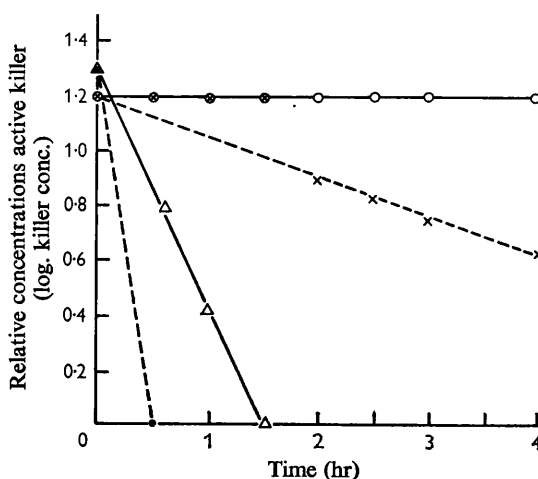


Fig. 2

Fig. 1. Dilution of the killer factor: Well test.

Fig. 2. Effect of temperature on the killer factor in liquid complete medium.

○, 18° and 23°; ×, 25°; △, 28°; ●, 38°.

Table 1. *Effect of pH on the stability of the killer factor*

pH of medium	Relative concentrations of active killer (log killer concentrations)				
	0 hr (cultures harvested)	1 hr 28°	24 hr 22°	24 hr 12°	24 hr 4°
5.4	0	0	0	0	0
5.2	0	0	0	0	0
5.0	0.53	0	0.53	0.53	0.53
4.8	1.40	0.53	1.40	1.40	1.40
4.6	1.40	0.53	1.40	1.40	1.40
4.4	1.40	0	0.75	1.40	1.40
4.0	1.40	0	0	1.40	1.40
3.6	0.53	0	0	0.10	0.53
3.4	0.30	0	0	0	0.30
3.2	0	0	0	0	0
3.0	0	0	0	0	0

incubation period, would indicate that the killer factor produced at 28° was being inactivated.

It can be concluded from these experiments that the inactivation of the killer factor by temperature depends upon the composition of the medium.

Surface inactivation. It was observed that the killer activity of a minimal medium solution disappeared on sterilization by filtration. To determine whether or not the inactivation was due to surface inactivation caused by aeration, the effect of vigorously shaking aliquots of killer complete medium and minimal medium containing increasing quantities of gelatine for 0, 2, 4 and 8 min. at 22° was studied.

It can be seen from Fig. 4 that the killer factor was rapidly inactivated when

vigorously aerated and, like many physiologically active proteins and bacteriophages, this surface inactivation was prevented by gelatine (Adams, 1948).

Effect of yeast extract. The results in Table 2 show that yeast extract enhanced the production of the factor by killer cells. A similar effect of yeast extract on the production of maltases by *Saccharomyces oviformis* was reported by Lewis (personal communication).

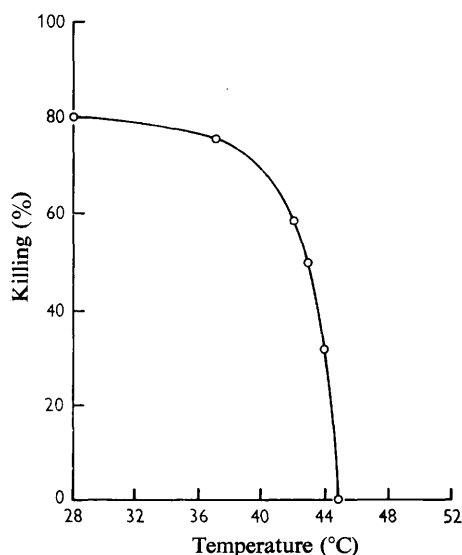


Fig. 3

Fig. 3. Effect of temperature on the killer factor in agar complete medium.

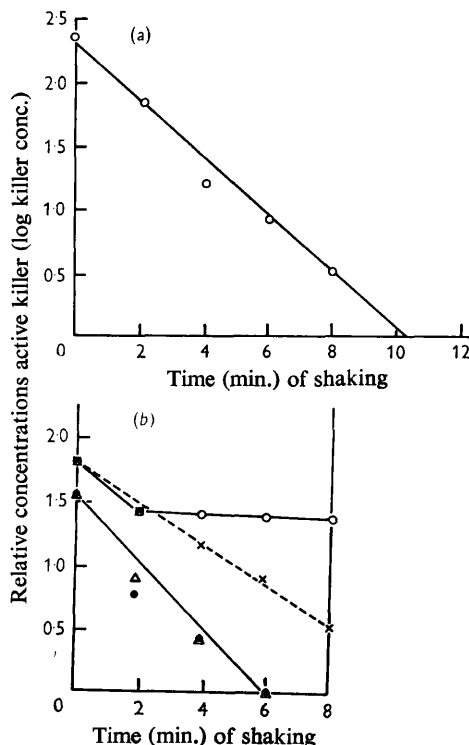


Fig. 4

Fig. 4. Effect of vigorous aeration on the killer factor. (a) Inactivation of the killer factor in complete medium by shaking. (b) Inactivation of the killer factor in minimal medium by shaking in the presence of increasing quantities of gelatine. ○, 0.25% (w/v); ×, 0.05% (w/v); △, 0.005% (w/v); ●, 0% (w/v) gelatine.

Table 2. *Effect of various growth media on the production of the killer factor by killer cells*

Type of killer medium	No. of yeast/ml. at harvesting	Relative concentrations of active killer (log killer concentration)
CM* + gelatine	14×10^7	2.3
MM† + gelatine + yeast extract	12×10^7	2.3
MM + gelatine + peptone	14×10^7	1.59
MM + gelatine + casein hydrolysate	14.5×10^7	1.40
MM + gelatine	10×10^7	1.05

* Complete medium.

† Minimal medium.

PURIFICATION

$(NH_4)_2SO_4$ fractionation. The results of the fractionation of the killer factor from chemically defined media, summarised in Table 3, indicate that it was precipitated by 70–81 % saturation with $(NH_4)_2SO_4$ from complete and minimal media. However, when 0.05 % (w/v) gelatine was added to the minimal medium the factor was precipitated with the gelatine at 25–30 % saturation with $(NH_4)_2SO_4$ and yielded a stable killer sediment with the highest specific activity. Agar-gel electrophoresis studies confirmed that only the killer factor and gelatine were precipitated (Fig. 6). The results

Table 3. *Fractionation of the killer factor by $(NH_4)_2SO_4$ from complete and minimal media*

Killer factor solution	% saturation with $(NH_4)_2SO_4$ required to precipitate killer factor	Stability resuspended sediment		Specific activity sediments in buffer
		Complete medium	Buffer	
Killer factor + complete medium	70–81	+	+	100
Killer factor + minimal medium	70–81	+	–	0
Killer factor + minimal medium + 0.05 % (w/v) gelatine	25–30	+	+	850

+, Stable. –, Unstable.

Table 4. *Concentration by ultrafiltration of the purified-killer-factor peak and a sensitive control sample (effluent volume 75–100 ml.) after gel filtration with Sephadex G-150*

Samples	Relative concentrations of active killer	Absorption		Folin test: absorption 750 μ
		280 μ	260 μ	
Killer <i>a</i>	0.10	0.015	0.025	0.01
Killer <i>b</i>	0.53	0.155	0.1875	0.03
Killer <i>c</i>	0	–0.005	0	0.0125
Sensitive <i>a</i>	0	–0.02	–0.03	0.0075
Sensitive <i>b</i>	0	–0.015	–0.02	0.0075
Sensitive <i>c</i>	0	–0.025	–0.0375	0.0075

a, Effluent volume 75–100 ml. before concentration by ultrafiltration.

b, Sample concentrated by ultrafiltration.

c, Buffer removed during ultrafiltration.

also show that the fractionated killer factor was not stable but could be stabilized by resuspension in a diluent containing gelatine or the other proteins present in complete medium.

Gel filtration. The gel-filtration elution profiles of the dialysed killer factor plus gelatine (Fig. 5) illustrated that the factor was eluted at two peaks: a sharp peak between 15 and 30 ml. effluent volume coinciding with the gelatine peak which was excluded by Sephadex G-150 and was eluted at the void volume; and a second fractionated peak eluted between 70 and 110 ml. The fractionated killer factor was unstable and became inactive after retention at 0–4° for 34 hr. It can also be seen from Fig. 5 that there was no correlation between the second killer peak and the absorption at 260 and 280 m μ . The considerable degree of killer activity of this fraction as indi-

cated by the Well test illustrated the high sensitivity of this biological assay as compared with the physical and chemical methods employed. It was found possible to estimate the killer factor, by physical and chemical tests, only after the fractionated factor had been concentrated by ultrafiltration (Table 4).

The elution of the killer factor in two peaks suggested that either there were two different factors, or polymers capable of causing the killer reaction. Alternatively the fractionation by gel filtration resulted in the separation of only some of the killer

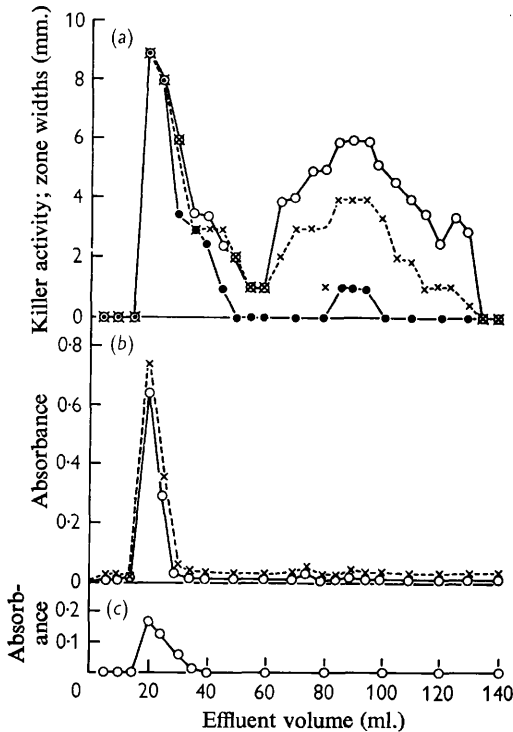


Fig. 5

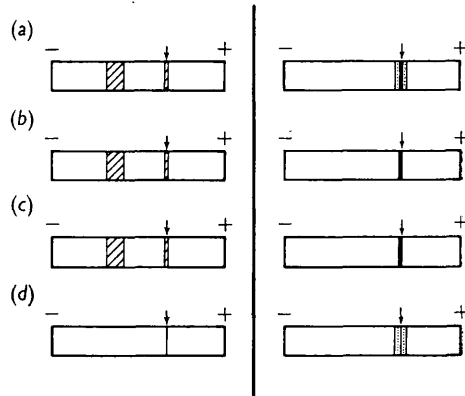


Fig. 6

Fig. 5. Elution profiles of the killer factor and gelatine sample after gel filtration with Sephadex G-150. (a) Killer activity at different time intervals between 0 and 34 hr. after gel filtration. O, 0 hr; X, 12 hr; ●, 34 hr. (b) Absorption at (X) 260 mμ and (O) 280 mμ. (c) Folin-Ciocalteu test for proteins (absorption at 750 mμ).

Fig. 6. Agar-gel electrophoresis. (a) Killer factor + gelatine sample after 30% (NH₄)₂SO₄ fractionation. (b) Sensitive control solution + gelatine sample after 30% (NH₄)₂SO₄ fractionation. (c) Gelatine sample. (d) Purified killer factor sample after concentration by ultrafiltration. ↓, origin; ▨, protein stain (gelatine); ■, purple stain (gelatine at origin + methylene blue); ▩, zone of inhibition (killer factor).

factor from the gelatine. To distinguish between these possibilities an attempt was made to separate the killer factor and gelatine by sucrose density-gradient centrifugation prior to gel filtration. Although the killer factor was dispersed throughout the gradient (Woods, 1966), it was possible to separate the bulk of it from the gelatine, since the latter was confined to a sharp peak at the top of the gradient.

After gel filtration this gelatine-free factor was eluted between 65 and 100 ml. effluent volume only. It was therefore concluded that the killer factor eluted at the

void volume was attached to the gelatine, and was thus not fractionated by gel filtration with Sephadex G-150.

It is interesting to note that the killer factor was hetero-dispersed after centrifugation in a sucrose gradient and also following ultracentrifugation in complete medium for 3 hr at 107,000 g.

The killer factor and gelatine sample eluted at the void volume were recycled but the killer factor was again only eluted at the void volume along with the gelatine. Recycling the fractionated killer factor failed to yield an effluent with any killer activity, presumably due to its instability in the absence of gelatine and to dilution effects.

In fractionations by gel filtration where the separation is governed entirely by the molecular-sieve effect, the distribution coefficient (Kd) cannot be greater than unity, and it is possible to estimate the molecular weights of proteins from the elution volume (Granath & Flodin, 1961; Andrews, 1965). However, the Kd for the separation of the killer factor was 2.59. This indicates that adsorption was also taking place, and it was therefore impossible to estimate the molecular weight of the killer factor by this method.

Agar-gel electrophoresis. This technique was used to demonstrate the fractionation of the killer factor during the purification process. The pherograms of the experiments illustrated in Fig. 6 show that the killer factor remained at the origin and was not affected by electrophoresis at pH 4.6. The purified killer sample, which contained no detectable contaminant proteins, was only identifiable by a zone of inhibition at the origin. The dialysed killer sample, which had not been further purified, contained only the killer factor and gelatine (Fig. 6).

Electrophoresis was carried out at different pH values between pH 4.0 and 5.0 and several conclusions can be made from the results. First, the killer factor was only identifiable by zones of inhibition at the origin between pH 4.6 and 4.8; this was expected as it is unstable at other pH values. Secondly, there was no other migrating protein band at any of the pH values besides that formed by gelatine.

Degree of purification. The results given in Table 4 show that a 40-fold purification of the killer factor was achieved by the procedures described. It should be pointed out, however, that this estimate is a conservative one due to the fact that the killer factor was gradually inactivated once it had separated from the stabilizing gelatine. Thus a certain amount of inactivation always occurred before the assay procedure could be performed.

Electron microscopy. Serial dilutions of the purified killer factor were examined under the electron microscope but it was not detected by the technique adopted.

Effect of papain. Evidence for the proteinaceous nature of the killer factor was obtained by the study of the effect of the plant protease enzyme, papain, which has a maximum enzymic activity between pH 5 and 7.5 (Kimmel & Smith, 1954): 0.2 ml. aliquots of a minimal-medium killer solution and a purified-killer-factor solution were added to 0.2 ml. aliquots of an activated papain (crystalline B.D.H.) solution containing 0.025 M-cysteine and 0.005 M-versene; an inactive papain solution (papain alone); and a solution containing cysteine and versene. The solutions were incubated at 20° and the killer activity determined after 0, 4, 6 and 20 hr.

The results shown in Table 5 indicate that only the killer factor was inactivated by papain. Since it was inactivated by papain only in the presence of the reducing agents it is concluded that the proteolytic action of the enzyme was responsible. The eventual

loss of killer activity by the purified killer control sample was due to the unstable nature of the fractionated killer factor.

Mode of action of the killer factor. The susceptibility of the sensitive cells was studied by comparing the % killing at different stages in the growth cycle. The sensitive cells were most susceptible during active growth but were completely resistant during stationary phase (Woods, 1966). Similar effects associated with changes in growth rate are well known in bacteria (*Topley & Wilsons Principles* 1964).

Table 5. *Effect of the protease enzyme, papain, on the killer factor*

Sample	Relative concentrations of active killer (log killer concentration)		
	0 hr	6 hr	20 hr
Killer factor + MM* + gelatine	1.40	1.40	1.40
Killer factor + MM + gelatine + cysteine + versene	1.40	1.40	1.05
Killer factor + MM + gelatine + papain	1.40	1.40	1.40
Killer factor + MM + gelatine + cysteine + versene + papain	1.40	0.30	0
Purified killer factor	0.53	0.30	0
Purified killer factor + papain	0.53	0.30	0
Purified killer factor + cysteine + versene + papain	0.53	0	0

* Minimal medium.

Table 6. *Effect of temperature and pH on the post-absorption action of the killer factor*

Exp.	Type of medium	Post-absorption treatment		(%) Killing
		pH	Temp.°	
1	Sensitive	4.8	22	0
		5.8	38	0
	Killer	4.8	22	57.6
		5.8	22	43.0
		4.8	28	51.2
		5.8	28	31.0
		4.8	38	25.2
		5.8	38	18.7
2	Sensitive	4.8	18	0
		4.8	38	0
	Killer	4.8	18	78.0
		4.8	24	72.7
		4.8	28	68.5
		4.8	38	60.4

The action of the killer factor was investigated under different pH and temperature conditions. Sensitive cells were added to samples of killer and control solutions. The cells were plated on media buffered at pH 4.8 and/or 5.8 and incubated at 18°, 22° 24° 28° or 38°. The % of sensitive cells killed was estimated by comparing the number of colonies arising on killer and control plates.

The results (Table 6) show that the post-absorption action of the killer depended on the growth conditions, and was not necessarily fatal unless the organisms were exposed to the optimum conditions for the stability of the killer factor. It is interesting to note that the effect of temperature on the post-absorption action of the killer factor, contrasts with that on the action of megacin where post-adsorption death was proportional to the incubation temperature (Holland, 1962).

Treated sensitive yeast cells kept at 0° for 0–72 hr on agar plates buffered at pH 4·8 did not subsequently recover when the cells were allowed to grow at 22° (Woods, 1966).

Preliminary experiments indicated that the action of the killer factor was not reversed by exposing treated cells to papain. Further, washing and shaking treated cells did not affect the post-absorption action of the killer factor. This contrasts with the action of colicins, which is reversed by trypsin, suggesting that they are adsorbed on the cell surface (Nomura, 1964).

DISCUSSION

The killer factor in yeast, *Saccharomyces cerevisiae*, may be compared with any other agents involving killing and heredity, but the analogy between the killer factor and bacteriocins is particularly striking. Both act on strains of the same or closely related species and are proteinaceous in nature. The production and stability of the killer factor and some bacteriocins is very dependent on growth conditions, which must be carefully controlled for optimal titres. The killer factor is markedly more stable in agar medium than in broth. Similarly, many colicinogenic strains which produce good zones of inhibition on agar may show little or no activity in broth (Reeves, 1965).

The proteinaceous nature of the purified killer factor is indicated by its inactivation by papain, its absorption at 260 and 280 m μ , and the Folin–Ciocalteu test (1927) for proteins described here. The presence of aromatic moieties in the killer factor is suggested by both the significant absorption observed at 280 m μ (Layne, 1957) and the adsorption of the factor by Sephadex. It is well known that Sephadex adsorbs predominantly aromatic and heterocyclic compounds (Gelotte, 1960).

The size of the killer factor is at present unknown but the dialysis experiments suggest that it is a macromolecular protein.

The bactericidal action of the killer factor and that of megacin are very similar in many respects. First, growth of the treated sensitive organisms is required before death occurs. Secondly, the physiological state of the sensitive cells affects the amount of killing, and, thirdly, absorption and death of the organisms are separated into two distinct processes. Holland (1962) reported that the decrease in sensitivity, with age, of *Bacillus megaterium* strains to megacin is concurrent with the progressive development of an extensive slime layer. He suggested that the slime layer prevented or retarded the penetration of megacin to those structures where it exerted its lethal activity. At present the reason for the increased sensitivity of the sensitive yeast cells to the action of the killer factor during log phase is not known.

The post-absorption action of both the killer factor and megacin is affected by the growth conditions. The former requires that the organisms be exposed to the optimum conditions for the stability of the killer factor during growth, but the latter is not necessarily fatal unless the organisms are exposed to optimum growth conditions. Holland (1962) suggested the possible involvement of an enzymic system since the post-absorption death rate is proportional to the incubation temperature, and the

Q_{10} (27–37°) for the loss of viability is close to 2. The effect of temperature between 28° and 38° on the action of the killer factor has the opposite effect resulting in a decrease in the % killing.

Another example of the effect of the physiological state of the sensitive and temperature organisms on the activity of the bacteriocin, pesticin, produced by *Pasteurella pseudotuberculosis* was reported by Ben-Gurion & Hertman (1958). They indicated that the activity of pesticin is 20 times greater when tested at 37° than at 30°. However, it should be noted that the sensitive organism *P. pseudotuberculosis* grows more abundantly at 37° than at 30°.

The authors wish to thank Mr J. Kirkham, who carried out the electron microscopy, and the Science Research Council for financial support of this work. One of us (D. R. W.) wishes to acknowledge a Rhodes Scholarship.

REFERENCES

- ADAMS, M. H. (1948). Surface inactivation of bacterial viruses and of proteins. *J. gen. Physiol.* **31**, 417.
- ANDREWS, P. (1965). The gel filtration behaviour of proteins related to their molecular weights over a wide range. *Biochem. J.* **91**, 222.
- BEN-GURION, R. & HERTMAN, I. (1958). Bacteriocin-like material produced by *Pasteurella pestis*. *J. gen. Microbiol.* **19**, 289.
- BEVAN, E. A. (1955). *Investigations on the genetics of yeast (Saccharomyces cerevisiae)*. Ph.D. thesis, Glasgow University.
- BEVAN, E. A. & MAKOWER, M., (1963). The physiological basis of the killer character in yeast. *Proc. 11th int. Congr. Genet.* **1**, 203 (abstract).
- EGGSTEN, M. & KREUTZ, F. H. (1955). Vergleichende Untersuchungen zur quantitativen Eiweissbestimmung in Liquor und eiweissarmen Lösungen. *Klin. Wschr.* **33**, 879.
- FOLIN, O. & CIOCALTEU, V. (1927). On tyrosine and tryptophane determinations in proteins. *J. biol. Chem.* **73**, 627.
- GELLOTTE, B. J. (1960). Studies on gel filtration: sorption properties of the bed material Sephadex. *J. Chromat.* **3**, 330.
- GOMORI, G. (1959). Preparation of buffers for use in enzyme studies. *Meth. Enzymol.* **1**, 138.
- GRANATH, K. A. & FLODIN, P. (1961). Fractionation of dextran by the gel filtration method. *Makromolek. Chem.* **48**, 160.
- HOFSTEN, B. & FALKBRING, S. (1960). A simple arrangement for the concentration of protein solutions. *Analyt. Biochem.* **1**, 436.
- HOLLAND, I. B. (1962). Further observations of the properties of megacin, a bacteriocin formed by *Bacillus megaterium*. *J. gen. Microbiol.* **29**, 603.
- JACOB, F. & WOLLMAN, E. (1959). Colicins and other bacteriocins. In *Bacteriophages*. Ed. by M. H. Adams. New York: Interscience Publishers Inc.
- KAVANAGH. (1963) *Analytical Microbiology*. New York: Academic Press Inc.
- KIMMEL, J. R. & SMITH, E. L. (1954). Crystalline papain. I. Preparation, specificity and activation. *J. biol. Chem.* **207**, 515.
- LAYNE, E. (1957). Spectrophotometric and turbidimetric methods for measuring proteins. *Meth. Enzymol.* **3**, 447.
- LEDERBERG, J. & LEDEBERG, E. M. (1952). Replica plating and the indirect selection of bacterial mutants. *J. Bact.* **63**, 399.
- LINDEGREN, C. (1949). *The Yeast Cell: Its Genetics and Cytology*. Saint Louis, U.S.A.: Educational Publishers Inc.
- LOWRY, B. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, J. (1951). Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193**, 265.
- MAKOWER, M. (1964). *Inheritance of a killer reaction in yeast* D.Phil. thesis. Oxford University.
- NOMURA, M. (1964). Mechanism of action of colicins. *Proc. natn. Acad. Sci. U.S.A.* **52**, 1514.

- REAUME, S. E. & TATUM, E. L. (1949). Spontaneous and nitrogen mustard induced nutritional deficiencies in *Saccharomyces cerevisiae*. *Arch. Biochem.* **22**, 331.
- REEVES, P. (1965). The Bacteriocins. *Bact. Rev.* **29**, 24.
- Topley and Wilson's Principles of Bacteriology and Immunity* (1964). 5th Ed., revised by G. S. Wilson and A. A. Miles. London: Edward Arnold (Publishers) Limited.
- WEIME, R. J. (1965). *Agar Gel Electrophoresis*. Elsevier Publishing Company.
- WILKINS, W. H. (1949). Investigation into the production of bacteriostatic substances by fungi. A revision of the testing method. *Ann. appl. Biol.* **36**, 257.
- WOODS, D. R. (1966). *Studies of the nature of the 'killer factor' in yeast*. D.Phil. thesis. Oxford University.