Biochimica et Biophysica Acta, 298 (1973) 868–875

BBA 76292

YEAST KILLER FACTOR: ATP LEAKAGE AND COORDINATE INHIBITION OF MACROMOLECULAR SYNTHESIS IN SENSITIVE CELLS

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(Received October 30th, 1972)

SUMMARY

The action of the yeast killer factor proteins on sensitive yeast cells has been examined. The killer factor caused a coordinate inhibition of protein synthesis, nucleic acid synthesis and D-[^{14}C]glucose incorporation into macromolecules in growing sensitive cells. During the inhibition period ATP became detectable in the growth medium and the cellular ATP pool level fell to exhaustion. ATP synthesis continued over this period as extracellular ATP accumulated to levels 4–20-fold those found in the cellular pools of control cultures. Leakage studies on other cellular components over the ATP leakage period indicated little loss of macromolecules, but an increased efflux of pools of leucine and glucose. The results are consistent with a killer-induced alteration in the yeast cell membrane.

INTRODUCTION

Killer yeasts make a group of extracellular proteins that are toxic to sensitive strains[1,2]. Previous work on the action of these killer proteins showed a general inhibition of macromolecular synthesis occurring with a lag after binding of the proteins to the cells[2]. We have further examined the killing process and have found a coordinate cessation of macromolecular synthesis which coincides with an increase in efflux of compounds from the cellular pools. Among these compounds is ATP which accumulates in the growth medium in amounts several-fold greater than the total ATP present in control cultures. This ATP leakage is of interest in relation to both the action of the killer, and the control of ATP synthesis.

MATERIALS AND METHODS

Organisms and growth conditions

Saccharomyces cerevisiae killer strain K_{12} (α, *ade*$_{2\cdot5}$, M(k)), and sensitive strains S_{14} (α, *ade*$_{2\cdot5}$, M(o)) and S_{29} (α, wt, M(o)), were obtained from Dr J. M. Somers. Cultures were grown using the medium of Halvorson[3] (Min), or supplemented with yeast extract and peptone each at 0.5% (yeast extract–peptone medium): both media contained 2% glucose. Where indicated glucose was replaced by 4% glycerol.

Preparation of killer

Cell-free extracellular macromolecular extracts with killer activity were obtained from the culture fluid of K_{12} by ultrafiltration and dialysis as described[2]. Killer activity was determined by incubating growing sensitive strain S_{14} with varying killer concentrations for 3 h at 22–24 °C and determining viable cells by plating. The preparations used in these experiments had protein concentrations of 2–2.5 mg/ml and contained $1–4 \cdot 10^{11}$ killing units/ml, calculated from the multiplicity, m, obtained from the relation $S/S_0 = e^{-m}$ where S/S_0 is the survival ratio.

Estimation of cellular pools and macromolecular synthesis

Cultures of S_{14} were grown in yeast extract–peptone medium of approx. $2 \cdot 10^7$ cells/ml at 22–24 °C. To a 5-ml culture, the appropriate labelled compound was added in 10–50 μl to a final concentration of L-[^{14}C]leucine ($5 \cdot 10^{-4}$ M, 0.5 Ci/mole), [^{14}C]-adenine ($2.5 \cdot 10^{-4}$ M, 0.08 Ci/mole), D-[^{14}C]glucose (0.11 M, 0.9 mCi/mole). Killer, 50 μl containing 120 μg of protein, or an equivalent volume of citrate–phosphate buffer (control) was added with the labelled material. Samples, 0.5 ml, were taken at time intervals, filtered, washed, extracted with 60% ethanol and cellular pool levels and incorporated material measured as described previously[4]. Similar levels of incorporation of labelled material into cellular macromolecules were obtained by precipitation of samples with 5% trichloroacetic acid.

Measurement of ATP

ATP was measured using luciferase by the method of Foulds[5]. For total (cellular + medium) ATP extraction, 0.7 ml of culture *plus* 2.0 ml of water were boiled for 15 min and filtered on glass fibre discs. Medium ATP was determined after filtering 0.7 ml of culture on glass fibre discs, washing with 2.0 ml of water and boiling the cell-free filtrate for 15 min. Attempts to measure cellular ATP, after boiling washed, filtered cells, resulted in ATP levels consistently lower than those obtained by subtracting medium ATP from total ATP. The reasons for the reduced levels are uncertain, but because of the problem only total (cellular + medium) and medium ATP levels were measured.

RESULTS

Coordinate inhibition of macromolecular synthesis

Incorporation of [^{14}C]leucine, [^{14}C]glucose and [^{14}C]adenine into growing sensitive cells was measured in the presence of killer to estimate protein synthesis, incorporation of [^{14}C]glucose (which would include polysaccharide)[6] and total nucleic acid synthesis, respectively (Fig. 1). Inhibition starts at 40–50 min, and is complete by 130 min in all cases. After inhibition, the label incorporated into cellular macromolecules remains constant. Such a pattern of inhibition would be consistent with a defect in some process common to these events. Some energy generating system could be a site of action, or membrane transport could be altered as it is with colicins E_1 and K in *Escherichia coli*.

Measurement of pool levels of components, or their derivatives, used to estimate macromolecular synthesis showed no rapid depletion prior to macromolecular inhibition (Fig. 2), and variable depletion over the period of inhibition. Net total uptake

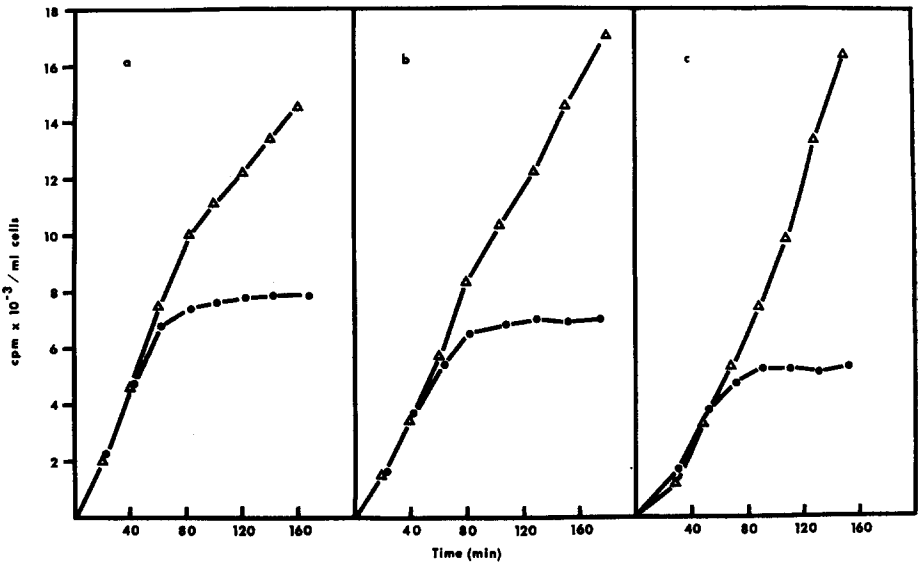

Fig. 1. Effect of killer on macromolecular synthesis. Cultures of S_{14} were grown on yeast extract–peptone medium to approx. $2 \cdot 10^7$ cells/ml at 22–24 °C. Killer was added with the appropriate labelled compound at time zero, and incorporation measured. (a) [^{14}C]leucine; (b) [^{14}C]glucose; (c) [^{14}C]adenine. Killer multiplicity was 9.5–11.0. △, control; ●, *plus* killer.

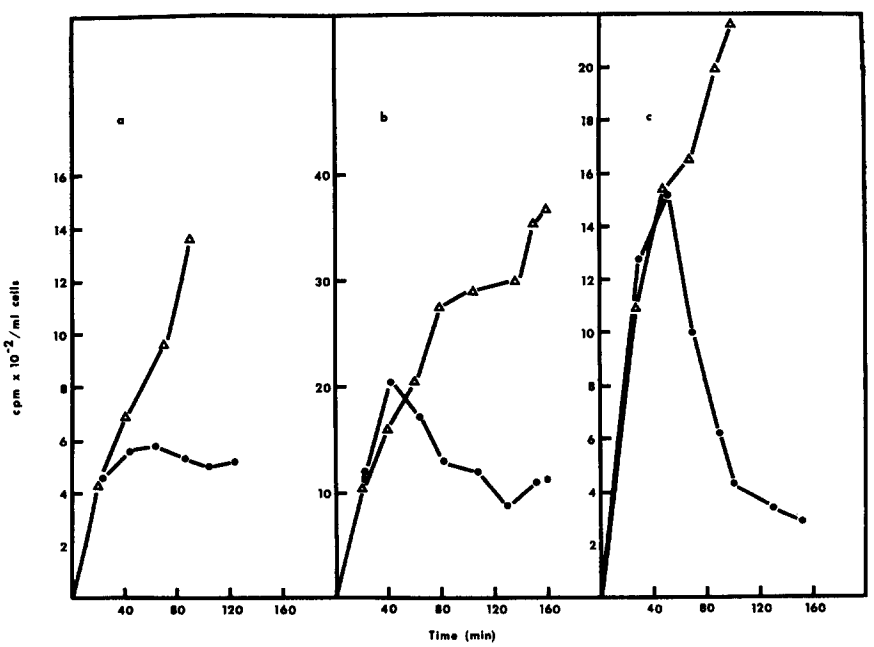

Fig. 2. Cellular pool levels on killer treatment. Strain S_{14} was grown as in Fig. 1. Culture samples were filtered, washed with unlabelled medium and pools extracted by washing with 60% ethanol. Pools and incorporated counts (Fig. 1) were taken in similar experiments, and are thus comparable. Killer multiplicity 9.5–11.0. (a) [^{14}C]leucine pool; (b) [^{14}C]glucose-derived pool; (c) [^{14}C]adenine-derived pool. △, control; ●, *plus* killer.

(sum of incorporated and pool counts in Figs 1 and 2) stopped in the inhibition period, and with [^{14}C]leucine or [^{14}C]glucose no significant loss of material from the cells was detected. In the case of [^{14}C]glucose, the pool fell to one half of its highest value, but this fall was accounted for by incorporation into non-extractable material. Total uptake of [^{14}C]adenine showed a net loss of 6–10% and this was due to a pool depletion of 80% (Fig. 2c). This greater loss of [^{14}C]adenine-derived pool components coupled with the macromolecular inhibitions made ATP metabolism a possible candidate for killer action.

ATP levels on killer treatment

An increase in total ATP concentration occurred when killer was added to an S_{14} sensitive culture (Fig. 3). Cells grown on nutrient medium show approximately a 4-fold increase in ATP level. Corresponding with this increase, the ATP level in the medium rose and by 130 min almost all the ATP was extracellular. An untreated control culture showed little increase in total ATP and negligible ATP in the growth medium.

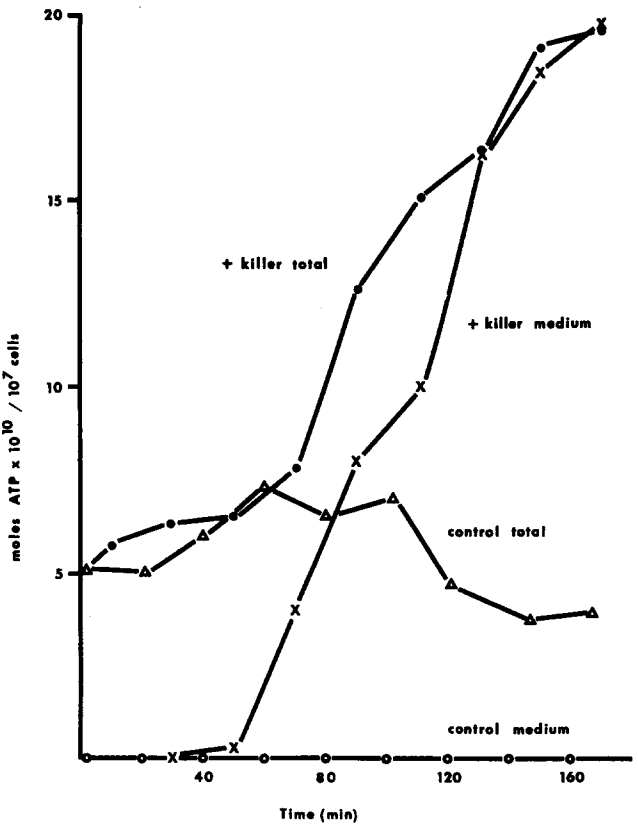

Fig. 3. ATP accumulation and leakage into medium on killer treatment of sensitive strain S_{14}. Cells grown on yeast extract–peptone medium as in Fig. 1. Killer, multiplicity 8.3, was added at time zero. The data are expressed as 10^7 cells/ml at the time of killer addition. ●, *plus* killer total; ×, killer medium; △, control total; ○, control medium.

TABLE I

EFFECTS OF KILLER TREATMENT ON YEAST ATP LEVELS

Strains were grown on the media indicated at 22–24 °C to a cell concentration of $1.5–2.0 \cdot 10^7$ per ml. Killer (240 μg of protein to 5 ml of culture, multiplicity on S_{14} grown on yeast extract–peptone medium, 8.3), was added at the same concentration in all experiments. Total and medium ATP were determined as in Materials and Methods. The ATP levels are normalised to the cell number at the start of the experiment. The data in Row 2 are taken from those given in Fig. 3. Killer strain K_{12} continued to grow at the control rate in the presence of added killer protein.

Strain	*Medium*	*% Survival*	*ATP level 10^{-10} moles/10^7 cells*		
			Control pool	*Final in medium*	*Final total*
S_{14}	yeast extract–peptone medium	control*	5.2	$\leqq$ 0.05	4.0
	yeast extract–peptone medium	$2.5 \cdot 10^{-4}$	5.2	19.7	19.5
	yeast extract–peptone glycerol 4%	$1.0 \cdot 10^{-4}$	0.8	18.5	17.0
$S_{14}\rho A$	yeast extract–peptone medium	10	0.4	5.0	4.9
S_{29}	yeast extract–peptone medium	$1.5 \cdot 10^{-1}$	1.7	5.3	5.4
	minimal	$2.0 \cdot 10^{-1}$	0.9	3.9	4.6
K_{12}	yeast extract–peptone medium	100	5.3	$\leqq$ 0.05	3.6

* No killer added.

Similar data were obtained for strain S_{14} grown on glycerol (Table I, Row 3). While the initial pool level of ATP is lower, the total ATP level after killer treatment is similar to that found with glucose and shows an increase of 20-fold compared with the control.

These data do not explain the previous finding that at lower killer multiplicities glucose-grown cells are more sensitive to killer than those grown on glycerol[2].

To see whether ATP leakage was related to its mode of synthesis, ATP produced in glycolysis was distinguished from that produced in mitochondrial oxidative phosphorylation by the use of an ethidium bromide-induced petite, $S_{14}\rho A$ (obtained from K. Al-Aidroos). Ethidium bromide treatment leads to the loss of mitochondrial DNA[8], and presumably of many mitochondrial functions. The results (Table I, Row 4) indicate that ATP production and leakage occur with ATP made in glycolysis, though the ATP pool and the amount of ATP produced on killer treatment are lower than when most of the ATP production is mitochondrial (Table I, Row 2). The $S_{14}\rho A$ strain was far less sensitive than its parent though the reason for this is uncertain as a spontaneous petite derived from S_{14} was as sensitive as the parent, as were several other ethidium bromide-induced petites (Al-Aidroos, K., unpublished). Strain S_{14} was an adenine auxotroph, and the possiblity that exogenous adenine allowed increased ATP production was tested using a sensitive wild type, S_{29}. An increase in ATP level was found when S_{29} was grown on nutrient or minimal media, (Table I, Rows 5 and 6), the increase corresponded with ATP leakage. It is unknown if the lower ATP pool and the reduced killer sensitivity of the strain are related.

Killer producing strain K_{12} (from which S_{14} had been derived as a spontaneous sensitive) was immune to added exogenous killer proteins at the concentrations used in these experiments. This strain did not leak ATP into the growth medium and maintained an ATP pool comparable to that of an untreated S_{14} control (Table I, Row 7).

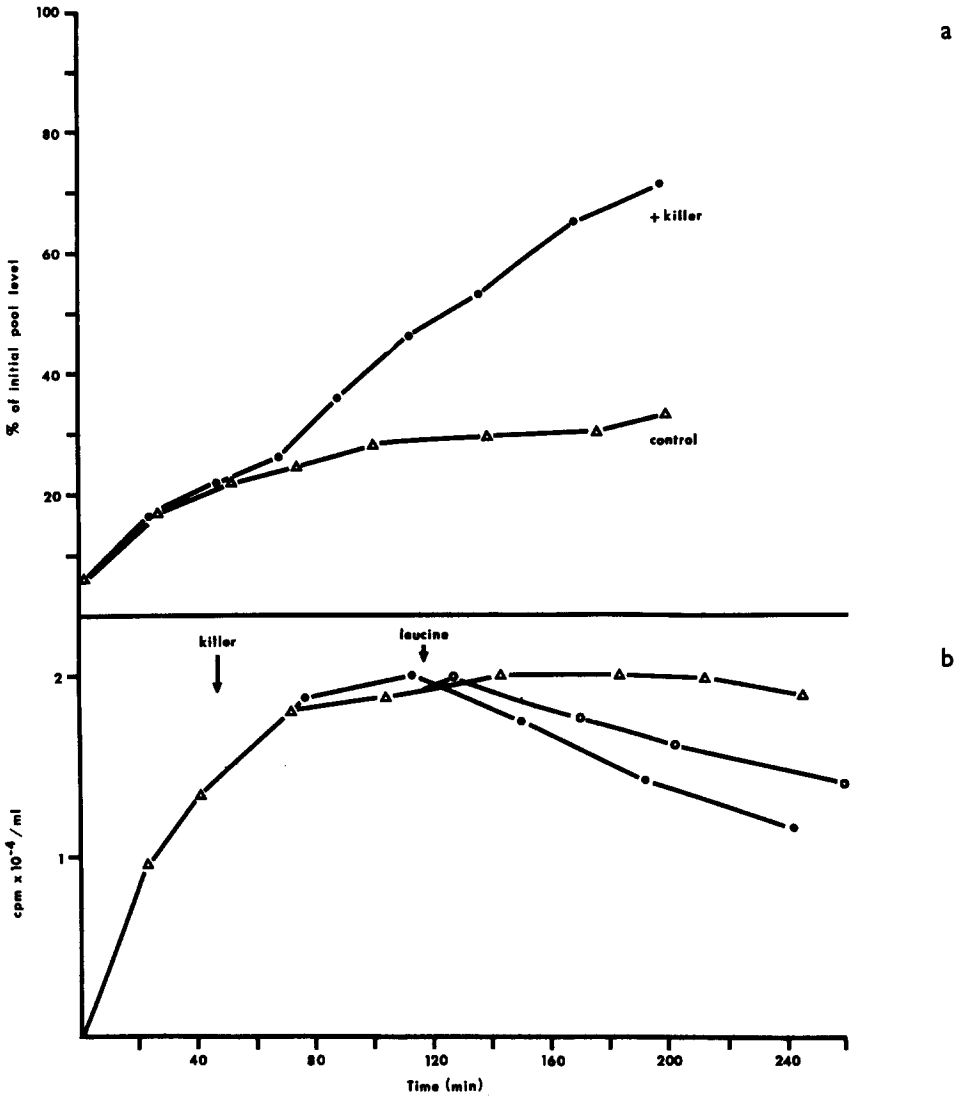

Fig. 4. Effects of killer on accumulated pools. (a) S_{14} grown on minimal medium + $5 \cdot 10^{-4}$ M adenine at 23–24 °C, D-[^{14}C]glucose ($5.5 \cdot 10^{-2}$ M, 0.014 Ci/mole) was added at a cell concentration of $8 \cdot 10^{6}$ cells/ml and growth continued for 4 h to a concentration of $1.7 \cdot 10^{7}$ cells/ml. Cells were filtered, washed and resuspended in unlabelled medium and killer at multiplicity 5.0 was added to one portion of the culture. At intervals cells were removed by filtration and the radioactivity in the medium estimated. Total pool was determined by extracting washed cells with 60% ethanol. Incorporated material was measured by counting cells on filter discs after pool extraction. △, control; ●, *plus* killer. (b) S_{14} grown as in Fig. 1a to $1.4 \cdot 10^{7}$ cells/ml, was treated with cycloheximide, 40 μg/ml, for 33 min, L-[^{14}C]leucine ($4 \cdot 10^{-7}$ M, 312 Ci/mole) was added and after 47 min, killer added to a portion of the culture. At 117 min, L-leucine ($5 \cdot 10^{-4}$ M) was added to another portion of the culture. Pools were determined as in a. At 200 min, incorporation into protein had ceased in the control culture and was 5% of the counts found in the pool. Survival in the control culture was 36% at 252 min, survival on killer treatment was 0.9% of the control value. △, control; ●, *plus* killer; ○, *plus* leucine.

An explanation for continued production of ATP by sensitive strains would be that killer treatment removes the control of ATP on its own synthesis, by a lowering of the energy charge of the cell[9]. The energy charge parameter is sensitive to changes in ATP concentration and so continued ATP production during pool depletion would be expected until some component became limiting.

The ATP level in killer-treated cells falls, but does not fall immediately to zero (difference between total and medium ATP in Fig. 3). ATP reduction *per se* may not then be directly responsible for inhibition of macromolecular synthesis, though ATP leakage does correspond well with the time of general macromolecular inhibition.

Leakage from labelled cells

Growing yeast leak very little ATP and it was important to find the extent of the killer-induced increase in membrane permeability. The data in Figs 1 and 2 indicate that no detectable loss of labelled macromolecules occurs over the ATP leakage period, and that the major leakage seen was from the adenine-derived pool. The continued production of ATP during leakage implies that there was no general loss of metabolites. To test for leakage from cells during killer treatment, a sensitive culture was grown on minimal medium with D-[^{14}C]glucose as sole carbon source: the culture was washed free of labelled glucose and resuspended in unlabelled glucose medium: killer was added and the efflux of radioactivity monitored. Loss of incorporated counts was small (less than 10%) and was not significantly different between killer treated and control (data not shown). Increased efflux occurred over the ATP leakage period on killer treatment (Fig. 4). The increase was chiefly pool material and the loss was not complete. Analysis of the effluxed material using Sephadex G-25 showed a small amount of macromolecular components (3% of total counts with killer, 1% of total in control). The remainder of the radioactivity (97% on killer treatment) co-chromatographed as a single peak with a glucose standard. D-Glucose 6-phosphate and ATP were resolvable from glucose on the column. Our finding suggests that while killer increases glucose efflux, not all components are lost from the cells.

Leucine pool depletion

The effect of killer on the leucine pool accumulated after cycloheximide inhibition was tested (Fig. 4), in an experiment similar to that performed on chloramphenicol-inhibited *E. coli* given colicin A[10]. With killer treatment the leucine pool fell slowly after a lag, and after the period of total ATP loss, the leucine pool had fallen by 25%. Addition of cold leucine also caused a slow reduction in the labelled pool. These results argue against a complete loss of membrane integrity and are in agreement with the data on the stability of the leucine pool (Fig. 2a).

DISCUSSION

These results are consistent with a killer-induced alteration in the yeast cell membrane. The coordinate inhibition of macromolecular synthesis could be explained by loss of essential metabolites over this period. The extent and nature of killer action remain unknown, though cell lysis could not have occurred, as there is little loss of cellular macromolecules during the period of general inhibition. ATP, glucose and leucine are lost from the cells to varying extents, so the defect is probably not specific

for pool components. Depletion of cellular ATP by leakage and the large ATP accumulation in the medium suggest a breakdown in ATP regulation, though it is uncertain if the rate of ATP synthesis increases with killer. In yeast, the alteration in ATP metabolism is quite different from that found in *E. coli* with colicin E_1 where ATP depletion occurs as a consequence of ATPase action[11].

ACKNOWLEDGEMENTS

We thank K. Al-Aidroos, R. G. E. Palfree and Dr J. M. Somers for help. This work was supported by a grant from the National Research Council of Canada.

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