THE MODE OF ACTION OF YEAST TOXINS

by

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ABSTRACT

The *Saccharomyces cerevisiae* killer toxin kills sensitive yeast strains *S. cerevisiae* K19.10 and *Torulopsis glabrata* ATCC 15126 by a mechanism involving inhibition of macromolecule synthesis, depletion of ATP and promotion of potassium efflux from cells to the medium. The toxin from *T. glabrata*, PEST, appears to kill *S. cerevisiae* by a similar mechanism.

Killer toxin action, but not PEST action, is inhibited by either energy-poisons or cycloheximide. Energy-poisons, particularly 2,4-dinitrophenol, appear to block events in killer toxin action that are subsequent to toxin binding.

A reliable and versatile spheroplasting procedure for yeasts has been developed. Spheroplasts from toxin-sensitive or toxin-producing (toxin-immune) cells retain the response of the cells to toxins, as measured by potassium efflux. Spheroplasts from some toxin-resistant mutants are toxin-sensitive.

The resemblance between the mode of action of yeast toxins and the membrane-acting colicins is discussed.

RESUME

La mort des levures de type *Sacchacomyces cerevisiae* et *Torulopsis glabrata* ATCC 15126 par la toxine ("Killer") produite par certaines souches de *Saccharomyces cerevisiae* semble procéder par l'entremise d'une inhibition de la synthèse des macromolécules, par une diminution progressive de la quantité d'ATP et par une perte de potassium. La toxine ("PEST") élaborée par les *Torulopsis glabrata* semble tuer les *Saccharomyces cerevisiae* par les mêmes mécanismes.

On peut prévenir les effets de la toxine "Killer" par l'addition des poisons métaboliques ou de cycloheximide, mais ces produits sont inopérants en présence de la toxine "PEST". C'est seulement après contact entre la toxine et la cellule que les poisons métaboliques, le 2,4-DNP en particulier, peuvent enrayer ses effets nocifs.

Une méthode sûre et versatile de préparation de sphéroplastes à partir de levures a été élaborée. L'étude du flux de K^+ a permis d'établir que les sphéroplastes des cellules sensibles à la toxine de même que ceux des cellules produisant la toxine (résistantes à la toxine) conservent les mêmes réactions que les cellules d'origine. Les sphéroplastes de certains mutants résistants sont cependant susceptibles à la toxine.

La resemblance entre le mode d'action des toxines des levures et celui des colicines sur les membranes est considérée.

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PREFACE

This thesis is organised into eight chapters and two appendices. Chapter 1 contains a general introduction, Chapter 2 the general materials and methods, Chapters 3 through 7 the results, and Chapter 8 a general discussion. For the most part, detailed description of experimental procedure is restricted to the legends to figures and tables. Appendix A contains some of the results of potassium-efflux measurements which were omitted from the text in the interest of brevity. Appendix B contains copies of the papers that have been written at least in part on the basis of the results described in the text.

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ABBREVIATIONS

A_x	absorbance (turbidity), where subscript denotes the wavelength of the light, in nanometres;
AMP	adenosine 5′-monophosphate;
ADP	adenosine 5′-diphosphate;
ATP	adenosine 5′-triphosphate;
ATPase	adenosine 5′-triphosphatase;
CCCP	carbonylcyanide m-chlorophenylhydrazone;
cpm	counts per minute;
DNP	2,4-dinitrophenol;
DTT	dithiothreitol;
EDTA	ethylenedinitrilotetraacetic acid;
IAA	iodoacetic acid;
KU	killing units (of toxin);
PEST	pool efflux-stimulating toxin;
pNPP	p-nitrophenylphosphate;
pNP	p-nitrophenol;
tris	tris-(hydroxymethyl) aminomethane;
g.	acceleration due to gravity;
v/v	volume per volume;
w/v	weight per volume.

UNITS

The "Guidelines concerning thesis preparation" (McGill University, Montreal; January 1978 revision), part 8 (i), states that "the use of SI units (international system of metric units) is mandatory. When for historical reasons, or reasons of clear reader preference, other units are used, the SI equivalent must be given in a footnote at the first opportunity."

For the reason of clear reader preference, the units in use currently in the microbiological literature have been used in this thesis. Given below are the units used and their SI equivalents.

UNITS

	Thesis[b]		SI equivalent[a,b]		
Quantity	name	symbol	name	symbol	definition[c]
Volume	litre	litre	litre	L	10^{-3} m^3
	millilitre	mL	-	mL	10^{-6} m^3
	microlitre	µL	-	µL	10^{-9} m^3
Length	centimetre	cm	-	cm	10^{-2} m
	millimetre	mm	-	mm	10^{-3} m
	nanometre	nm	-	nm	10^{-9} m
Mass	gram	g	-	-	10^{-3} kg
	milligram	mg	-	-	10^{-6} kg
	microgram	µg	-	-	10^{-9} kg
	mole	mole	mole	mol	base unit
	millimole	mmole	-	-	10^{-6} mol
	nanomole	nmole	-	nmol	10^{-9} mol
	picomole	pmole	-	pmol	10^{-12} mol
Time	hour	hour	hour	h	3600 s
	minute	minute (min)	minute	min	60 s
	second	second	second	s	base unit
Concentration	molar	M	-	-	mol dm^{-3}
	millimolar	mM	-	-	mol^{-3} dm^{-3}
	weight per volume	w/v	-	-	kg m^{-3}
	volume per volume	v/v	-	-	m^3 m^{-3}

Radioactivity	curie	Ci	-	-	s^{-1}
	millicurie	mCi	-	-	s^{-1}
	microcurie	μCi	-	-	s^{-1}
Pressure	pounds per square inch	p.s.i.	pascal	Pa	m^{-1} kg s^{-2}
Acceleration	acceleration due to gravity	g.	-	-	m s^{-2}
Temperature	degrees Celsius	°C	degrees Celsius	°C	0 °C = 273 °K

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[a]Taken from Morris, J.G. (1974), A Biologists Physical Chemistry. 2nd Ed., Edward Arnold, London, and Physics Bulletin (1973) 24: 672-673.

[b]Prefixes: d = deci (10^{-1}); c = centi (10^{-2}); m = milli (10^{-3}); μ = micro (10^{-6}); n = nano (10^{-9}); p = pico (10^{-12}).

[c]In terms of the SI base units. Base units include m (metre), kg (kilogram), s (second), mol (mole), and K (thermodynamic temperature).

Chapter 1

GENERAL INTRODUCTION

I. Occurence of Yeast Toxins

Killer, neutral and sensitive yeast strains of *Saccharomyces cerevisiae* were first described by Bevan and Makower (1963). Killer strains secreted into their culture medium an activity (killer toxin) which was lethal to other, closely related, strains (sensitive strains). Killer strains were immune to their own toxins. Cultures of the third phenotype, neutral, neither secreted toxin nor were sensitive to it.

It has since become clear that the ability to secrete toxins is widespread in yeasts. Phillipskirk and Young (1975) found that seven of twenty-five yeast genera produced activity toxic to *S. cerevisiae*. Activity toxic to yeasts has also been reported in cultures of the pathogenic yeast *Torulopsis glabrata* (Bussey and Skipper, 1975; this thesis), *Candida albicans* (Mitchell, 1974), brewery yeasts (Maule and Thomas, 1973; Rogers, 1976), yeasts isolated as contaminants in sake mash (Inamura, Kawamoto and Takaoka, 1974) and yeasts used in wine-making (Naumova and Naumov, 1973; Naumov, Tjurina and Buzjan, 1973). Many of the toxin-producing strains are sensitive to the toxins produced by others; Rogers and Bevan (1978) have classified a selection of toxin-producers into four groups, based on the sensitivity of each strain to the toxins produced by the others.

A system analogous to the *S. cerevisiae* killer toxin system has been described in the smut-fungus *Ustilago maydis* (Day and Anagnostakis,

1973) -- killer, neutral and sensitive phenotypes have been described.

II. Genetics and Molecular Biology of the Killer System

Considerable progress has been made towards an understanding of the genetic control of the killer system in *S. cerevisiae* (for reviews, see Wickner, 1976; Pietras and Bruenn, 1976).

Initially, Somers and Bevan (1969) established that the determinant of the killer character, i.e. the characteristics of toxin production and toxin-immunity, was inherited cytoplasmically. Crosses between killer, neutral and sensitive strains suggested that the killer and neutral phenotypes were determined by cytoplasmic factors (k, and n, respectively), whose maintenance required the presence of M, a nuclear allele. Killer strains were designated M(k), neutral strains M(n), and sensitive strains either M(o) or m(o), where o denoted the absence of the cytoplasmic determinant. Other genotype notations are also in use (see Wickner, 1976; Pietras and Bruenn, 1976).

It has since been established that killer strains contain two species of double-stranded RNA (dsRNA), one of which, M (or P2), of MW 1.4 x 10^6, is essential to the killer character (Bevan, Herring and Mitchell, 1973; Vodkin and Fink, 1973; Herring and Bevan, 1974; Vodkin, Katterman and Fink, 1974). Both M and the larger dsRNA species, L (or P1) (MW 2.5 x 10^6), are encapsidated in virus-like particles, or VLP's, (Herring and Bevan, 1974), referred to as V2 and V1, respectively. The L dsRNA may function as a "helper" genome to the M species: *in vitro* translation of L produces a protein which, while identical to the predominant coat-protein of V1, also cross-reacts antigenically with a

component of the V2 coat-proteins (Hopper *et al.*, 1977). *In vitro* translation of the M dsRNA produces a 36,000 MW protein containing peptides in common with purified killer toxin (Bostian, Hopper, Rogers, Rowe and Tipper, manuscript in preparation) and it seems likely that this molecule is the precursor of both the toxin and the toxin-immunity component(s). Neutral strains contain the same complement of dsRNA's as do killer strains (Bevan, Herring and Mitchell, 1973; Vodkin, Katterman and Fink, 1974) and are presumably defective at producing active toxin due to an altered M dsRNA genome.

The maintenance of the killer plasmid, originally shown to be dependent on the presence of the nuclear allele, M, (Somers and Bevan, 1969), is now known to be subject to the control of numerous nuclear genes. In addition to the M/m locus, there are at least nine nuclear genes whose mutation results in the cell's inability to maintain the plasmid (Fink and Styles, 1972; Wickner, 1974; and see Wickner, 1976). Nuclear mutations affecting toxin production or toxin immunity, as distinct from those affecting plasmid-maintenance, are also known (Wickner, 1974; Wickner and Liebowitz, 1976).

Toxin-sensitive cells lack the M dsRNA (Bevan, Herring and Mitchell, 1973; Vodkin, Katterman and Fink, 1974). Resistant mutants are readily isolated from mutagenised cultures of sensitive cells and three classes of mutation to resistance were defined by Al-Aidroos and Bussey (1978), on the basis of complementation tests. Group I and group II mutants were deficient at binding killer toxin; the group III mutant had wild-type toxin-binding properties and was therefore resistant presumably because of an alteration to some structure necessary

to events in toxin action subsequent to binding.

III The Mode of Action of Killer Toxin

Toxin binding

The first event in toxin action is presumably a binding reaction between toxin and the surface of the sensitive cell. Killer toxin is a large molecule. When isolated partially from the other components of killer-culture supernatant fluids the toxic activity is associated with glycoproteins of MW 2×10^5 to 1×10^6 (Bussey, 1972; Palfree and Bussey, unpublished results). When isolated to homogeneity the toxin is an 11,000 MW protein (Palfree and Bussey, manuscript in preparation). On the basis of its size there is no reason to suppose that toxin can penetrate the yeast cell envelope. More direct evidence that killer toxin binds initially to the cell wall was provided by the demonstration that glusulase, an enzyme mixture that degrades the wall, spared a fraction of sensitive cells that had bound a potentially lethal dose of toxin from suffering lethal damage; glusulase did not affect toxin activity *per se* (Bussey, 1972).

The nature of toxin binding to the yeast cell surface has yet to be established but some information has been obtained by measuring the ability of cells and spheroplasts to remove toxin activity from solution (the activity-removal assay). Whole cells of the toxin-producing strain K12 (i.e. toxin-immune) and its toxin-sensitive derivative, strain S14, removed toxin activity to an equal degree (Bussey, Sherman and Somers, 1973). That most of the toxin "bound" in this way is unnecessary to kill the sensitive cell was implied by the

finding that S14 spheroplasts, while fully toxin-sensitive, bound less than 1 percent of the amount bound by S14 cells (Bussey, Sherman and Somers, 1973).

It appears then that the yeast cell wall, more specifically the wall components altered by the enzymes used to prepare spheroplasts, is responsible for non-specific adsorption of killer toxin, non-specific in the sense that the great majority of molecules so bound do not function further in toxin action. Nevertheless, these cell wall binding sites (as defined by the activity-removal assay) may be necessary to toxin action in whole cells. Of the three classes of toxin-resistance mutation described by Al-Aidroos and Bussey (1978), two correlated with toxin binding deficiency of cells.

Effects on sensitive cells

Bussey and Sherman (1973) proposed that killer toxin acts on sensitive cells to damage the plasma membrane. Toxin-treated cells displayed coordinate inhibition of macromolecule synthesis, depletion of cellular ATP by efflux of pools to the growth-medium, and volume-reduction that correlated with an increase in culture turbidity (Bussey, 1972; Bussey and Sherman, 1973; Bussey, 1974). These changes were preceded by a delay of about 50 minutes following toxin addition to the cultures and were coincident; they seemed best explained as the consequence of some primary alteration to the permeability of the yeast plasma membrane.

The known effects of toxin on yeast cells resemble in some respects those of certain of the bacteriocins, the colicins K, E1, A

and I, on sensitive bacteria (see Luria, 1973, and Holland, 1975, for recent reviews on colicin action). In both instances, proteins secreted by certain strains kill other closely related strains by mechanisms that appear to involve primarily changes to plasma-membrane function. The rationale behind the experiments done to elucidate the mode of action of the membrane-acting colicins and the hypotheses put forward have certainly influenced the work on yeast toxin action. Of particular usefulness has been the scheme of Nomura (1964). Nomura proposed three essential elements to colicin action: (i) a *receptor* site on the cell surface, to which the toxin initially binds; (ii) a *biochemical target*, whose modification may itself be lethal to the cell or may lead to disruption of a "killing target"; (iii) a *transmission* mechanism which connects the toxin with its target. While the advent of more information on both the structure of colicins and their mechanism of action has made the concept of a transmission mechanism less necessary (see Holland, 1975), the scheme of Nomura still provides a helpful framework for thinking about the molecular mechanism by which an exogenously-supplied macromolecule may affect cell function.

AIM OF THE INVESTIGATION

My objective was to further our understanding of the mechanism of action of yeast toxins.

Chapter 2

GENERAL MATERIALS AND METHODS

Yeast Strains

Table 2.1 lists the strains, along with their genotype and source. Table 2.2 lists the strains in terms of their phenotype with respect to the toxins used in this work. Several terms used in Table 2.2 are discussed below.

I. Toxin-immune Strains

Both killer toxin-producing (killer) strains and neutral strains are immune to killer toxin. Neutral strains were not examined in this work. The immunity of killer strains is determined by the P2 (M) dsRNA genome (General Introduction); it is not known if the immunity of *T. glabrata* ATCC 15126 to its toxin, PEST, is similarly controlled by a cytoplasmic genome. Toxin-producing strains are sensitive to the toxins produced by strains of other genera (Al-Aidroos and Bussey, 1978; Rogers and Bevan, 1978); of the strains used here, K12 and K19 were PEST-sensitive and *T. glabrata* ATCC 15126 was killer toxin-sensitive.

II. Toxin-sensitive Strains

Three types were used: (a) those derived from toxin-producing strains, such as K19.10 from K19 and S14 from K12; (b) those that are toxin-producers but sensitive to toxins from other genera (see I); (c) strain S6, a "supersensitive" strain of unknown type.

Table 2.1 Yeast strains: genotype and source

Strain[a,b]	Genotype[c]	Source
K19	*a trp 5 leu 1 M(k)*	J.M. Somers
K19.10	*a trp 5 leu 1 M(o)*	J.M. Somers
K12 ATCC 28683	α *ade 2.5 M(k)*	H. Bussey
S14 ATCC 28684	α *ade 2.5 M(o)*	H. Bussey
S14.96 ATCC 28686	α *ade 2.5 M(o) kre 1*	K. Al-Aidroos
S14.94	α *ade 2.5 M(o) kre 1*	K. Al-Aidroos
S14.14 ATCC 28685	α *ade 2.5 M(o) kre 2*	K. Al-Aidroos
S14.MB6	α *ade 2.5 M(o) kre 3*	K. Al-Aidroos
S14.75	α *ade 2.5 M(o) kre*	K. Al-Aidroos
S14.93	α *ade 2.5 M(o) kre*	K. Al-Aidroos
R18	not known	H. Bussey
R23	not known	H. Bussey
R94	not known	H. Bussey
S30	*a trp 5 leu 1 M(o)*	J.M. Somers
S30.5	*a trp 5 leu 1 M(o) kre 1*	K. Al-Aidroos
S30.16	*a trp 5 leu 1 M(o) kre 1*	K. Al-Aidroos
S30.49	*a trp 5 leu 1 M(o) kre 2*	K. Al-Aidroos
S30.53	*a trp 5 leu 1 M(o) kre 2*	K. Al-Aidroos
S6	not known	D.T. Rogers
T. glabrata ATCC 15126	not known	ATCC

[a] *S. cerevisiae*, unless noted otherwise.

[b] ATCC = American Type Culture Collection (Rockville, Maryland).

[c] Gene symbols: a and α denote mating-type; *trp*, *leu* and *ade* denote requirement for tryptophan, leucine or adenine; M(k) is the killer genotype and M(o) sensitive genotype (Somers and Bevan, 1969); *kre* denotes killer toxin-resistance (these designations are provisional, see Al-Aidroos and Bussey, 1978).

Table 2.2 Yeast strains: phenotype.

Phenotype[a,b]	Strain	Comments[c]
Killer toxin immune	K19	-
	K12	Source of killer toxin
PEST immune	*T. glabrata*	Source of PEST
Killer toxin sensitive	K19.10	Standard sensitive strain in this work
	S14	-
	S30	-
	S6	Supersensitive, used in well-tests
	T. glabrata	
PEST sensitive	K19	
	K12	
	K19.10	
	S14	
	S14.75	Derived from S14
	S30.49	Derived from S30
	S30.53	Derived from S30
Killer toxin resistant	S14.94	Derived from S14
	S14.96	Derived from S14
	S14.14	Derived from S14
	S14.MB6	Derived from S14
	S14.75	

Killer toxin resistant (continued)	S14.93	Derived from S14
	R18	Derived from SS14
	R23	Derived from SS14
	R94	Derived from SS14
PEST resistant	S14.94	-
	S14.96	-
	S14.14	-
	S30.5	-
	S30.16	-
	R18	-
	R23	-
	R94	-

---

[a]Refers to the response of each strain to toxins when used as the seed-organism in the seeded-plate assay. Refer to text for distinction between immunity and resistance.

[b]Several of the resistant mutants are only partially resistant. Refer to Chapter 7 for an independent measure of the resistance of strains.

[c]SS14 is a supersensitive sterile derivative from the same parent as S14 (Al-Aidroos and Bussey, 1978).

III. Toxin-resistant Strains

These were mutants derived from toxin-sensitive cultures. Their resistance is of a distinct nature from that of the immune strains: they are derived from cells of a type known to lack the immunity determinant, the M dsRNA (General Introduction). Strains S14.14, S14.96, etc., and S30.5, S30.16, etc., were isolated from S14 and S30 respectively, by Al-Aidroos (1975). Strains R18, R23 and R94 were isolated from SS14 by Bussey (personal communication; for R18, see Bussey, Sherman and Somers, 1973). All the resistant mutants were isolated on the basis of resistance to the killer toxin.

Media

Table 2.3 lists the liquid and agar media. Given below are the recipes for preparing the media. In each case the components were dissolved in water and sterilised, then the medium completed by addition of the carbon/energy source (see subsequent section).

Liquid Media

i. YEP

960 ml contained ammonium sulphate, 4.0 g; dipotassium hydrogen phosphate, 8.7 g; succinic acid, 5.8 g; calcium chloride, 0.3 g; magnesium sulphate, 0.5 g; yeast extract, 5.0 g; peptone, 5.0 g; and ferrous sulphate, manganous sulphate, zinc sulphate and cuprous sulphate each 5 mg (these "minerals" were added from a stock solution containing 5 g of each per litre). pH was 4.6 to 4.8. The medium is based on the minimal medium described by Halvorson (1958).

Table 2.3 Media

Medium	Buffer	Carbon/energy source	pH	Purpose
<u>liquid media</u>				
YEPD	potassium phosphate/succinate	glucose	4.6-4.8	general; fermentative growth
NaYEPD	sodium phosphate/succinate	glucose	4.6-4.8	^{42}K loading; fermentative growth
NaYEPE	sodium phosphate/succinate	ethanol	4.6-4.8	^{42}K loading; aerobic growth
CB-YEPD	potassium phosphate/citrate	glucose	3-7	testing pH-dependence of toxin action
<u>agar media</u>				
YEPD-agar	see YEPD	glucose	4.6-4.8	viable counts
NaYEPD-agar	see NaYEPD	glucose	4.6-4.8	viable counts
NaYEPE-agar	see NaYEPE	ethanol	4.6-4.8	viable counts
CB-YEPD-agar	see CB-YEPD	glucose	3-7	viable counts
slant agar				
(a) glucose	none	glucose	-	stock cultures (fermentative cells)
(b) glycerol	none	glycerol	-	stock cultures (aerobic cells)
seeded-plate agar				
(a) unbuffered	none	glucose	-	well-test for toxins
(b) buffered	potassium phosphate/succinate	glucose	4.6-4.8	well-test for toxins

ii. NaYEP

960 ml contained the components of 960 ml YEP, except that potassium phosphate was replaced by disodium hydrogen phosphate (13.4 g) and 38 mg potassium chloride was included (5 mM KCl, in 1 litre complete medium). pH was 4.6 to 4.8.

iii. Pre-filtered YEP

The composition was that of YEP. The yeast extract and peptone was dissolved and filtered under 40 pounds per square inch pressure through an Amicon PM 30 membrane before being added to the remaining components of YEP.

iv. CB-YEP

The medium contained 5 g peptone, 5 g yeast extract, 760 ml water and 200 ml buffer. Buffers were as follows:

pH (final medium)	Citric acid (g)	Dipotassium hydrogen phosphate (g)	Volume (ml)
3.4	16.7	7.2	200
4.2	12.9	13.4	200
5.1	10.2	18.0	200
6.0	7.8	22.0	200
7.0	3.7	28.7	200

Agar media

i. Medium for viable counts

YEP-agar and NaYEP-agar was the liquid medium supplemented with 20 g agar per 960 ml. Medium was steamed, autoclaved, cooled to about 60°C, completed by addition of carbon/energy source and poured into petri-plates.

CB-YEP-agar was prepared from pH 3 to 7. It was necessary to autoclave the agar, peptone and yeast extract separately from the buffer component of CB-YEP medium. To prepare 960 ml, two sterile solutions were mixed: (1) 200 ml buffer (see CB-YEP liquid medium), (2) 760 ml water containing 20 g agar, 5 g peptone and 5 g yeast extract.

ii. Medium for seeded plates

For routine work, seeded-plate agar contained yeast extract, 10 g; peptone, 20 g; agar, 10 g, in 960 ml. For pH 4.7 seeded-plates, the medium included the succinate-phosphate buffer of YEP liquid medium. The agar was autoclaved separately from the remainder of the medium. A subsequent section describes the preparation of seeded-plates.

iii. Slant agar

960 ml contained 20 g peptone, 20 g yeast extract and 20 g agar. The medium was autoclaved, completed by addition of either glucose or glycerol (see below) and dispensed into sterile screw-capped bottles. The bottles were left at an angle while the medium set, then were stored in the cold room.

Energy/carbon sources

Unless specified otherwise yeasts were grown in media containing 2 percent w/v glucose. Glucose was autoclaved as a 50 percent w/v solution and added as necessary to liquid and agar media. Media containing glucose are suffixed with D, e.g. YEPD.

For aerobic yeast cultures, the cells were inoculated into medium containing either glycerol (suffix G) or ethanol (suffix E) as the carbon/energy source. Glycerol was autoclaved as a 50 percent v/v solution before use; final concentration in media was 4 percent v/v. Ethanol was used directly from a stock of absolute ethanol; final concentration in media was 2 percent v/v.

Sterilisation

All components of media were sterilised by being autoclaved at 15 pounds per square inch for 15 minutes.

Culture maintenance

Stock cultures were stored at 0-4°C as streaked-cultures on the surface of slant-agar. Periodically the stock cultures were removed by re-streaking them onto fresh slants and incubating them at 30°C for 2 to 3 days. Most stocks required renewal at only 3 to 4 month intervals; strain K19.10 was renewed fortnightly since it was in constant use as an inoculum. The K19.10 stock culture renewals were done from as few serially-subcultured slants as possible.

Culture growth

Unless noted otherwise, yeast cells were inoculated into growth medium so that overnight incubation (about 17 hours) gave an "active"

culture, i.e. a culture that resumed growth without appreciable lag when diluted with fresh medium. Cultures were grown to A_{600} 1.2 to 1.3 (see Figure 2.1) for most experiments with toxins.

Glucose-grown cultures, for use the following day, were inoculated from slant agar-glucose stocks and incubated with just enough mixing to maintain the cells in suspension; they were incubated either in 5 ml lots on the roller drum at room temperature or in larger volumes in flasks in the water bath at 30°C. Ethanol-grown cultures, for use the following day, were inoculated from slant agar-glycerol stocks and incubated with vigorous mixing in a shaking water bath; the cultures were either 50 ml in 250 ml baffled flasks or 500 ml in 2 litre baffled flasks.

Measurement of culture growth

i. Absorbance

Culture absorbance was the most convenient way to follow the growth of cells and was used routinely. Most measurements were made at 600 nm (A_{600}) in 1 cm path-length cuvettes in a Gilford model 240 spectrophotometer; very dense cultures, such as those used in the preparation of toxins, were measured in a Klett-Summerson colorimeter with the blue filter.

ii. Viable count

Viable counts were the basic measure of the biological activity of cultures. Yeast suspensions were diluted with growth medium, plated onto agar-plates (duplicate plates per sample) and incubated for at least a day and a half. The colony number was averaged and the result expressed as colony-forming units per ml of undiluted culture.

iii. Wet weight, dry weight

The procedures for preparing yeast spheroplasts and yeast cell-homogenates were established on the basis of the wet weight of cells to be processed. Wet weight was the weight of the washed cells: the culture was harvested at 2090 x g. for 3 minutes, the cells washed with water, re-pelleted and the weight of the drained pellet measured. Dry weightof cells, a useful measure of the absolute amount of cellular material, was obtained by filtering a known volume of culture over a glass-fibre disc, washing the filter with water and weighing the filter once it had been dried for 60 minutes at 200°C in an oven.

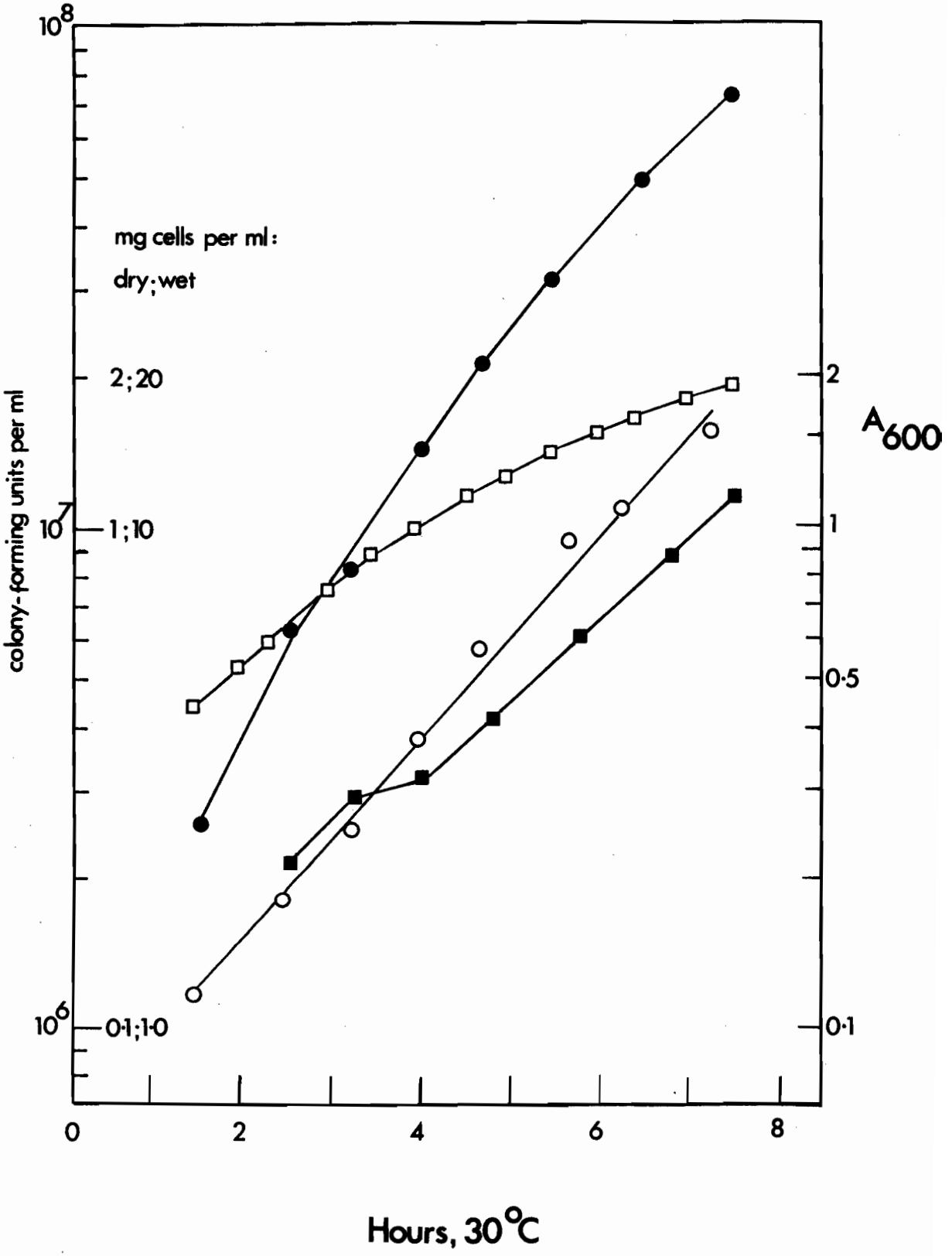
Figure 2.1 shows the growth of strain K19.10 at 30°C in NaYEPD medium, in terms of the various measures of growth. As expected, dry weight was the measure which most closely paralleled the viable count. Absorbance became a less sensitive measure as the culture increased in density beyond about 1.5×10^7 colony-forming units per ml. Wet weight was precise only beyond a certain mass of cells: about 140 mg wet cells per sample.

Toxins

Two toxins were used: the killer toxin produced by killer strains of *S. cerevisiae* and the PEST (pool efflux-stimulating toxin) produced by *T. glabrata* ATCC 15126.

The following procedures, based on protocols worked out by H. Bussey and R. Palfree in this laboratory, were used to prepare partially purified toxins for use with cells and spheroplasts.

To prepare killer toxin, 5 litres of prefiltered YEPD was


10^8
mg cells per ml:
dry;wet
2;20
1;10
10^6
0·1;1·0
colony-forming units per ml
2
1
0·5
0·1
A_{600}
0
2
4
6
8
Hours, 30°C

inoculated with a late-logarithmic phase culture of strain K12 to a density of 10-15 Klett units. The culture was incubated at 19°C in 2-litre flasks (1 litre per flask) and grown to Klett 450 or more. The cultures were then centrifuged at 2090 x g. for 3 min and the combined supernatant fluids concentrated to about 40 ml by filtration through an Amicon PM 30 membrane under 40 pounds per square inch of pressure. The concentrate was removed, the membrane washed several times with buffer (0.1 M sodium acetate, pH 4.7) and the combined concentrate and wash fluids clarified by centrifugation at 27,000 x g. for 15 min. One volume of 25 percent w/v polyethylene glycol 6000 in buffer was added slowly to the 27,000 x g. supernatant and the suspension stirred in the cold for at least 60 min. The suspension was centrifuged at 27,000 x g. for 15 min, the pellet (toxin) washed with 12.5 percent polyethylene glycol 6000 in buffer, and the washed pellet was then taken up in a small volume of buffer. The solution of killer toxin was clarified at 27,000 x g. for 15 min, then stored frozen until needed. Table 2.4 shows the progress of a typical purification.

PEST was prepared in a similar manner to killer toxin (Table 2.4). The inoculum was *T. glabrata* ATCC 15126 and the cultures were grown overnight at 30°C.

For the data given in Table 2.4, protein was measured with the Folin reagent (Lowry *et al.*, 1952) using serum albumin as standard, polysaccharide by the method of Badin *et al.* (1953), with D-glucose as standard and toxin potency (killing units) with strain S14 as the sensitive yeast (Bussey, 1972).

Table 2.4 Partial purification of killer toxin and pool efflux-stimulating toxin (PEST).

Purification step	KU[a]	Protein (mg)	Polysaccharide (mg)	Sp act (KU/mg protein)	Purification (fold)	Yield (%)
Killer						
Concentrated culture supernatant[b]	6.16×10^{10}	334.0	143	1.8×10^{8}	1	100
Polyethylene-glycol precipitate	1.27×10^{10}	5.8	37	2.19×10^{9}	12	21
PEST						
Concentrated culture supernatant[c]	2.56×10^{10}	356.0	160	7.19×10^{7}	1	100
Polyethylene-glycol precipitate	1.10×10^{10}	6.9	51	1.59×10^{9}	22	43

[a]Killing units.

[b]From 5 litres of *S. cerevisiae* K12, grown at 19°C to 450 Klett units (blue filter).

[c]From 5 litres of *T. glabrata*, grown at 30°C to 550 Klett units.

Assays of toxin activity

i. Viable counts

Cells were grown to A_{600} 1.2 to 1.3, treated with toxin and incubated for 3 hours. At intervals, samples of culture were diluted, then plated on the appropriate nutrient agar for colony-counts. Culture survival was expressed as the percentage of cells able to produce a colony, 100 percent being the viable count immediately before addition of toxin.

ii. Well tests (seeded-plate assays)

The method was that of Wilkins (1949), as modified by Woods and Bevan (1968). It is based on the ability of a toxin to generate, in nutrient agar seeded with sensitive cells, a zone in which the cells do not grow. Cells to be used as the seed were grown to late-logarithmic or stationary-phase, then added to seeded-plate agar (cooled to about 55°C) at 1 ml culture per 100 ml agar and poured into petri plates at 10 ml per plate. Seeded plates were stored at 0 to 4°C and remained useful for assays for a week. To assay toxins, 13 mm-diameter wells were punched in the agar and 0.1 ml toxin added to each well. Plates were incubated either overnight at 18°C and then for a few hours at 30°C (assay for killer toxin), or overnight at 30°C (assay for PEST). Toxin action is seen as a clear zone surrounding the well (Plate 6.1, photograph B) and the diameter of the zone is proportional to the logarithm of the dose of toxin (Woods and Bevan, 1968; for PEST, see Figure 4.6). The well-test was used routinely to check the phenotype of immune (or resistant) and sensitive strains and to test the potency

of toxin preparations (using strain S6 as the indicator organism).

Cell wall-degrading enzymes

Glusulase was purchased from Endo Laboratories, Inc., Garden City, N.Y. Zymolyase-5000 was from Kirin Brewery Co., Ltd., Takasaki, Gumma Prefecture, Japan.

Preparation of spheroplasts

Chapter 6 is concerned with the preparation of yeast spheroplasts. Given here is the glusulase procedure (Hutchison and Hartwell, 1967; Bussey, Sherman and Somers, 1973). Cells were grown at 30°C in YEPD medium to 2 x 10^7 colony-forming units per ml, harvested, washed with water, and resuspended in 0.8 M sorbitol to one-tenth of the original volume of culture. Glusulase was added to 1 percent v/v and the cells converted to spheroplasts by incubation for 120 min at 30°C, with very gentle shaking.

Harvesting and washing of cells and spheroplasts

Cells were harvested by centrifugation at 2090 x g. for 3 to 4 min in either the Sorvall HB-4 or GSA rotor. The pellets were washed by resuspension, centrifugation and resuspension: this "wash cycle" was repeated as necessary. Washed cells (or spheroplasts) were usually resuspended finally in a volume equal to the original volume of suspension.

Spheroplasts were harvested by centrifugation at 164 x g. for 5 to 10 min (zymolyase spheroplasts; see Chapter 6) or at 650 x g. for 15 min (glusulase spheroplasts; modified glusulase spheroplasts, Chapter 6)

in the HB-4 rotor. Spheroplasts, like cells, were washed by cycles of centrifugation and resuspension.

Radioactive labeling of cells: definition of wash, macromolecule and pool fractions

i. To measure the effect of toxins on the incorporation of [^{14}C] precursors into cellular components, cultures were treated at time zero with both the precursor and the toxin (Bussey and Sherman, 1973). The precursors were either L-[U-^{14}C] arginine (2.5×10^{-4} M, 0.08 Ci per mole), cytosine -[2-^{14}C] sulphate (2.5×10^{-4} M, 1.6 Ci per mole), D-[U-^{14}C] glucose (0.11 M, 3.6 Ci per mole), or L-[U-^{14}C] adenine (5×10^{-4} M, 0.08 Ci per mole), final concentration in the cell suspensions. To sample the cells, 0.5 ml culture was filtered over a glass-fibre disc, washed with 5 ml unlabeled growth medium, transferred to a fresh filtration assembly and washed with 5.0 ml 60 percent v/v ethanol. Radioactivity in the ethanol-wash was defined as that in the cellular pools, radioactivity retained by the filter as that in cellular macromolecules (Bussey and Sherman, 1973).

ii. To measure the effects of toxins on the fate of labeled cellular components, the cells were labeled with radioactive arginine (L-[U-^{14}C] arginine, 5×10^{-4} M), washed with unlabeled growth medium to reduce the cpm in the suspension fluids to background (3 wash-cycles was generally sufficient), and incubated at time zero with toxin. The effects of toxins on the cells was followed by measuring the radioactivity in each of three fractions: the wash, the macromolecules and the pools.

0.5 ml culture was filtered over a glass-fibre disc and washed with 5.0 ml unlabeled growth medium: radioactivity in these wash fluids was defined as that effluxed from cells. The filter was then transferred to a fresh filtration assembly and washed with 5.0 ml 60 percent v/v ethanol: radioactivity in this wash was cpm in cellular pools and that retained by the filter was cpm in macromolecules. By definition, the sum of the radioactivity in the wash, pools and macromolecules was equal to the total cell-bound radioactivity at time zero, provided ^{14}C counting efficiency was the same in each fraction. In all experiments shown this book-keeping was done; in no instance did the recovered cpm differ from the time-zero cpm by more than ten percent.

Radiochemicals; measurement of radioactivity

^{14}C-labeled chemicals were purchased from either New England Nuclear Corp., Boston, Massachusetts, or Amersham Corp., Oakville, Ontario, and the radioactivity measured in 5 to 10 ml Aquasol (New England Nuclear).

^{42}KCl was from New England Nuclear, as was $^{86}RbCl$. ^{42}K was measured by its Cerenkov radiation in 2.5 mM 7-amino-1,3-napthalene disulphonic acid, monosodium salt (Technical grade, Eastman Organic Chemicals, Eastman Kodak Co., Rochester, New York), as suggested by Lauchli (1969). ^{86}Rb was counted in Aquasol.

All counting of radiation was done with an Intertechnique ABAC SL 40 liquid scintillation spectrometer, using glass counting vials.

Measurement of ATP

In preliminary experiments to measure the effect of killer toxin on cellular ATP, the cultures were extracted for ATP by the method described by Foulds (1971), as used by Bussey and Sherman (1973). This protocol involves extraction of samples with boiling water. In subsequent experiments, nucleotides were extracted from samples by a hot-ethanol treatment (see Chapter 3). In either case, ATP was measured in extracts by the firefly-lantern luciferase-luciferin assay, as outlined by Chapman *et al.* (1971). To measure ATP, 0.1 ml of reconstituted firefly-extract (Sigma FLE-50; 50 mg dissolved in 5 ml water and clarified by centrifugation at 27,000 x g. for 15 min) was added to a glass scintillation vial containing 1.0 ml buffer (40 mM glycylglycine, 3 mM $MgCl_2$, pH 7.34) and 0.1 ml sample. The vial was then lowered into the counting chamber of a Beckman LS-100 scintillation counter (set at 100 percent "gain" and with the energy "window" open) and, exactly 7 seconds after the addition of firefly-extract, the light emission over a 6-second interval was measured. Light emission was recorded as "cpm" (see Chapter 3, Figure 3.7).

"Buffer"

Toxins were prepared in 0.1 M sodium acetate buffer, pH 4.7, and in experiments in which samples of cells or spheroplasts were treated with toxin a separate sample (the control) was treated with an equivalent volume of the acetate buffer.

Chemicals

Chemicals were purchased from Fisher Scientific Co., Montreal, Quebec, J.T. Baker Chemical Co., Phillipsburg, New Jersey, Sigma Chemical Co., St. Louis, Missouri and Eastman Organic Chemicals Division, Eastman Kodak Co., Rochester, New York.

Water-insoluble chemicals (DNP, CCCP and antimycin) were dissolved in absolute ethanol; when used in cell or spheroplast suspensions the final ethanol concentration was one percent v/v or less.

Filters

2.1 cm glass-fibre filters were purchased from Whatman Inc., Clifton, New Jersey (Reeve Angel brand). Diaflo PM 30 membranes were from Amicon Corp., Lexington, Massachusetts.

Microscopy; photomicroscopy

Cells and spheroplasts were examined with a Carl Zeiss photomicroscope, using phase-contrast optics and the green interference-filter. Total-counts of spheroplasts were done under bright-field optics in the photomicroscope using a hemacytometer. Photomicrographs were taken by Robert Lamarche of the McGill Biology Department's Photographic Centre.

Chapter 3

EFFECTS OF *S. cerevisiae* KILLER TOXIN ON YEAST CELLS

Introduction

Woods was the first to examine the nature of toxin action on sensitive cultures (Woods, 1966; Woods and Bevan, 1968). He established that cultures were most sensitive to toxin when growing actively at pH 4.6 to 4.8 and attributed the pH-dependence to the properties of the toxin. Woods' results also suggested that the binding of toxin to cells and the damaging events in the cells were distinct processes, since the post-adsorption conditions affected the degree of killing of the cultures.

Bussey confirmed the observations of Woods, and demonstrated that killer toxin treatment resulted in changes to the cells that seemed best accounted for in terms of a toxin-induced alteration to the plasma membrane. Following a lag-period of about 50 min, toxin treated S14 cultures showed a transient increase in turbidity, depletion of the cellular pools of ATP, and coordinate inhibition of the synthesis of cellular macromolecules derived from ^{14}C-labeled leucine, adenine and glucose supplied in the growth medium. There was no loss from the cells of labeled macromolecules. The uptake of labeled precursors from the medium was shut down in the cells coincidentally with the time of onset of the other events but there was only limited efflux of the accumulated pools of low MW cellular components (Bussey, 1972; Bussey and Sherman, 1973). This evidence suggested that all of the effects

of toxin were secondary to some initial damage to plasma-membrane permeability.

The most intriguing result of these experiments was that, in the toxin-treated cultures, cellular ATP appeared to be not only effluxed to the medium but effluxed to a level 4 to 20-fold in excess of the total amount found in untreated cultures (Bussey and Sherman, 1973). It seemed that the toxin dislocated the mechanism for the control of ATP synthesis in the cells.

My initial objective was to develop a sensitive assay for toxin action, an assay based on the measurement of some "real-time" event in the toxin-treated cultures. Preliminary to this, however, it was decided to check out the characteristic effects of killer toxin on sensitive cultures, in particular the ATP over-production. Sensitive strain *S. cerevisiae* K19.10, a derivative of the killer toxin-producing K19, was used in most experiments. Also examined was the effect of killer toxin on the pathogen *T. glabrata*.

Results

i. Effects of killer toxin on *S. cerevisiae*

Culture death and culture turbidity increase.

Figure 3.1 illustrates the phenomenon being examined in this thesis, the toxin-induced killing of a population of sensitive cells. Survival curves for strains K19.10 and S14 have been given. Figure 3.2 illustrates the other readily-measured effect of toxin -- an increase in culture turbidity. Figure 3.2 also demonstrates the resistance to killer toxin of strain K19, the toxin-producing

Figure 3.1 Survival of sensitive strains in the presence of killer toxin.

YEPD cultures of strains S14 and K19.10 were grown at 22 to 24°C to A_{600} 1.0 to 1.2, treated at time zero with killer toxin and sampled at intervals for their viable count on YEPD-agar. Symbols: (○), S14; (●), K19.10.

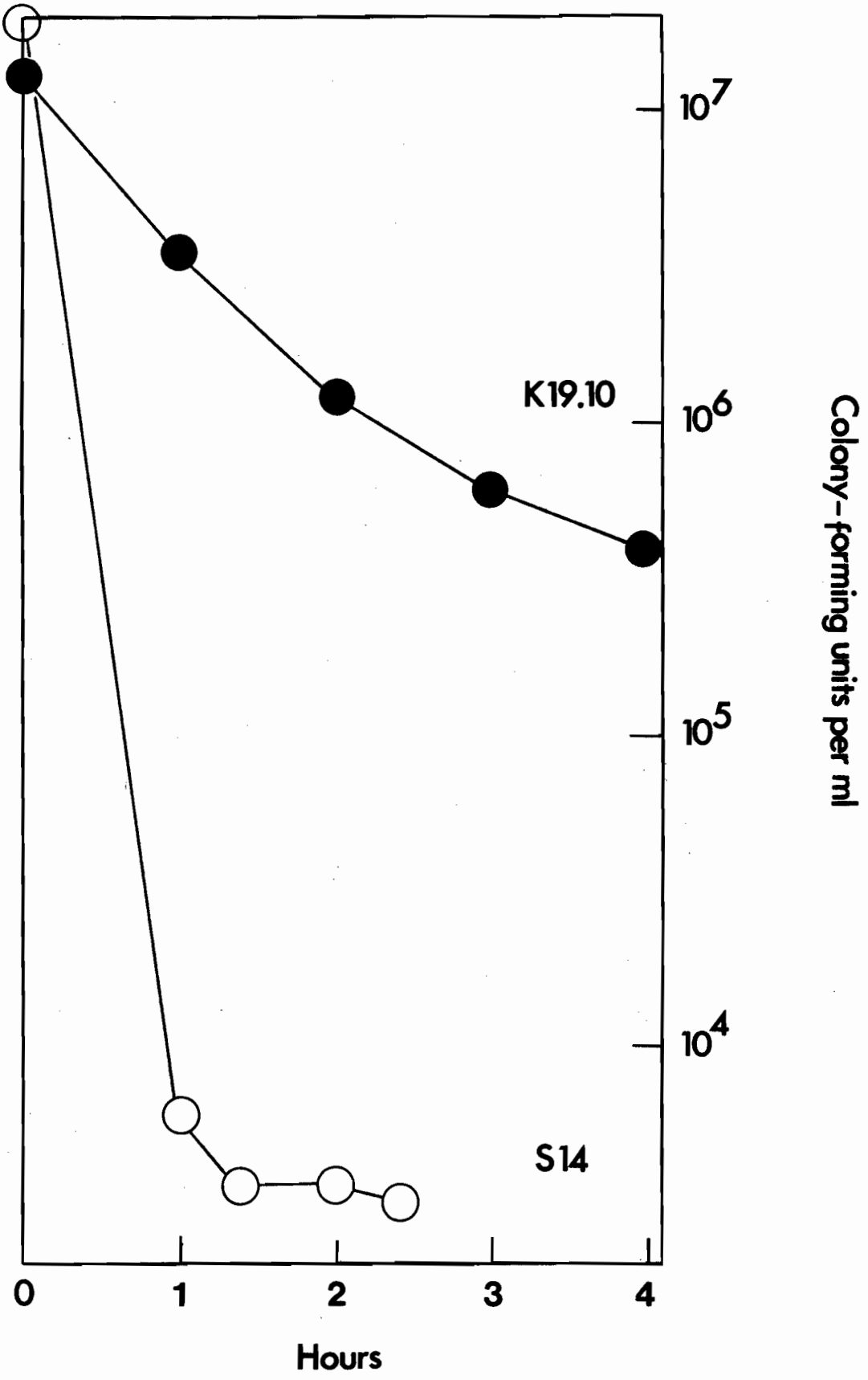
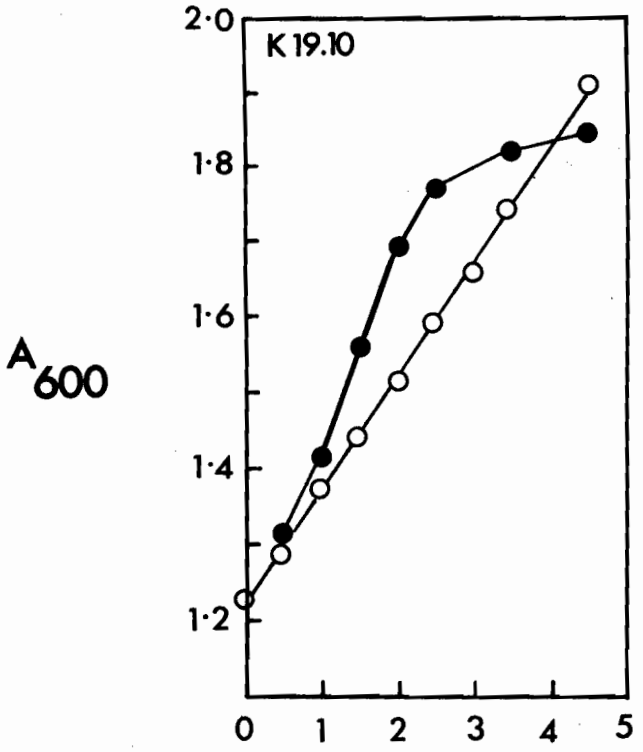
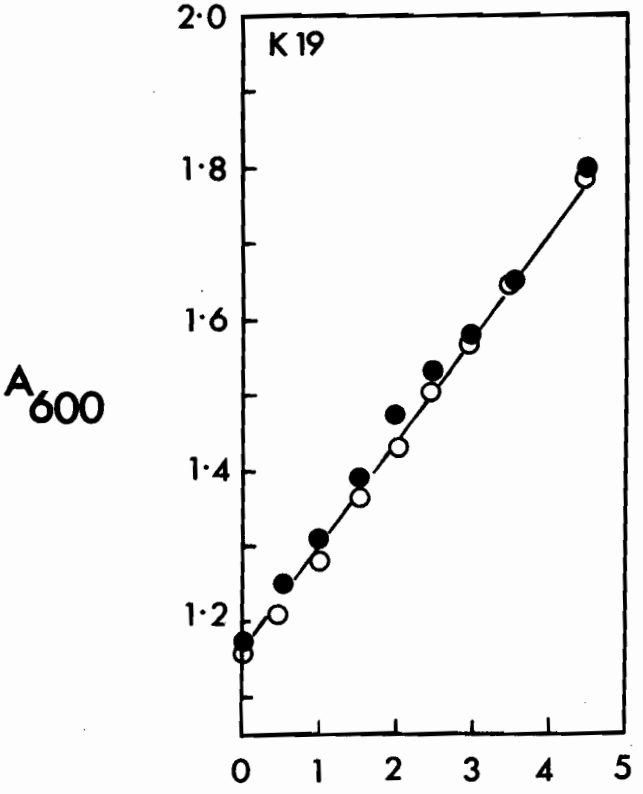

K19.10
S14
10^7
10^6
10^5
10^4
0
1
2
3
4
Hours
Colony-forming units per ml

Figure 3.2 Response of culture absorbance to killer toxin.

Cultures of strains K19 (killer toxin-producing) and K19.10 (toxin-sensitive) were diluted to A_{600} 0.5 and incubated at 30°C to A_{600} 1.1 to 1.2. The cultures were then transferred to 22 to 24°C and treated with either killer toxin or with an equal volume of growth medium. Culture absorbance was measured, at intervals. Percent survival of toxin-treated cultures at plus-270 min was 230 percent (K19) and 5 percent (K19.10). Symbols: (●), plus toxin; (O), plus medium.


K 19.10
2·0
1·8
1·6
1·4
1·2
A600
0
1
2
3
4
5


K 19
2·0
1·8
1·6
1·4
1·2
A600
0
1
2
3
4
5
Hours

parent strain of K19.10. These experiments and all others with killer toxin were done in pH 4.6 to 4.8 medium and at room temperature (22 to 24°C, unless noted otherwise).

The linear relationship between the time of exposure of cells to toxin and the fraction of the population surviving (shown for strain K19.10 in Figure 3.1) implies that the killing is a statistical phenomenon, the cells having an approximately constant probability of receiving lethal damage within any given interval. The eventual levelling-off in the survival-curve (strain S14, Figure 3.1) presumably reflects an equilibrium between the rate at which the population is being damaged, the growth of toxin-resistant variants in the population and the rate of toxin-inactivation under these conditions.

In terms of the survival-curve, strain S14 is clearly more sensitive to toxin than K19.10. This difference may reflect strain-dependent characteristics such as the efficiency of toxin-binding, the sensitivity of the toxin's cellular target to a given amount of bound toxin and the capacity of the cells to repair damage. In terms of "real-time" events such as toxin-induced potassium leakage from cells (see below), however, there is no evidence that the damaging events in strains of different toxin-sensitivity are distinct. The probability that cells of any one sensitive strain will be damaged by a given dose of toxin is a characteristic of that strain, but the damaging events appear to be common to all toxin-treated sensitive strains (see below).

Inhibition of macromolecule synthesis.

The addition of killer toxin to growing cultures of strain

K19.10 inhibited coordinately the incorporation into ethanol-insoluble cellular components of radioactivity from [^{14}C] glucose, adenine, cytosine (Figure 3.3), leucine and adenine (data not shown). There was no evidence in these results for loss from the cells of the labeled macromolecules.

Interference with accumulation of precursor pools

In the experiments illustrated in Figure 3.3, the cultures were also examined for the cellular accumulation of pools of low MW components derived from the labeled precursors supplied in the growth medium. As was the case with killer toxin-treated S14 cultures (Bussey and Sherman, 1973), toxin-treated K19.10 cultures stopped accumulating label at the same time that they stopped incorporating the label into macromolecules (Figure 3.4; compare with Figure 3.3).

Leakage of arginine-derived pools

In the trials shown in Figures 3.3 and 3.4, no attempt was made to assess whether killer toxin caused a leakage from cells of the labeled cellular components. The experiments illustrated in Figure 3.5 were done partially to answer this question (the effect of cycloheximide on the toxins' effects on efflux of cellular components, also shown in Figure 3.5, is discussed in Chapter 5). A 25 ml culture of strain K19.10 growing in YEPD was supplemented with [^{14}C] arginine and a portion (suspension 1) allowed to grow for four hours to load the cells with radioactivity in the ethanol-extractable and resistant fractions (General Materials and Methods). The cells were washed and resuspended in

Figure 3.3 Effect of killer toxin on synthesis of macromolecules.

Killer toxin or buffer (control) was added, together with labeled precursor, at time zero to cultures of K19.10 growing in YEPD and the cells then sampled at intervals for ethanol-insoluble radioactivity. *A*, [^{14}C] adenine; *B*, [^{14}C] glucose; *C*, [^{14}C] cytosine. Culture survival in toxin-treated samples at plus-200 min was 0.7 percent (*A*), 1.5 percent (*B*) and 1.0 percent (*C*). Symbols: (■), plus toxin; (□), plus buffer.

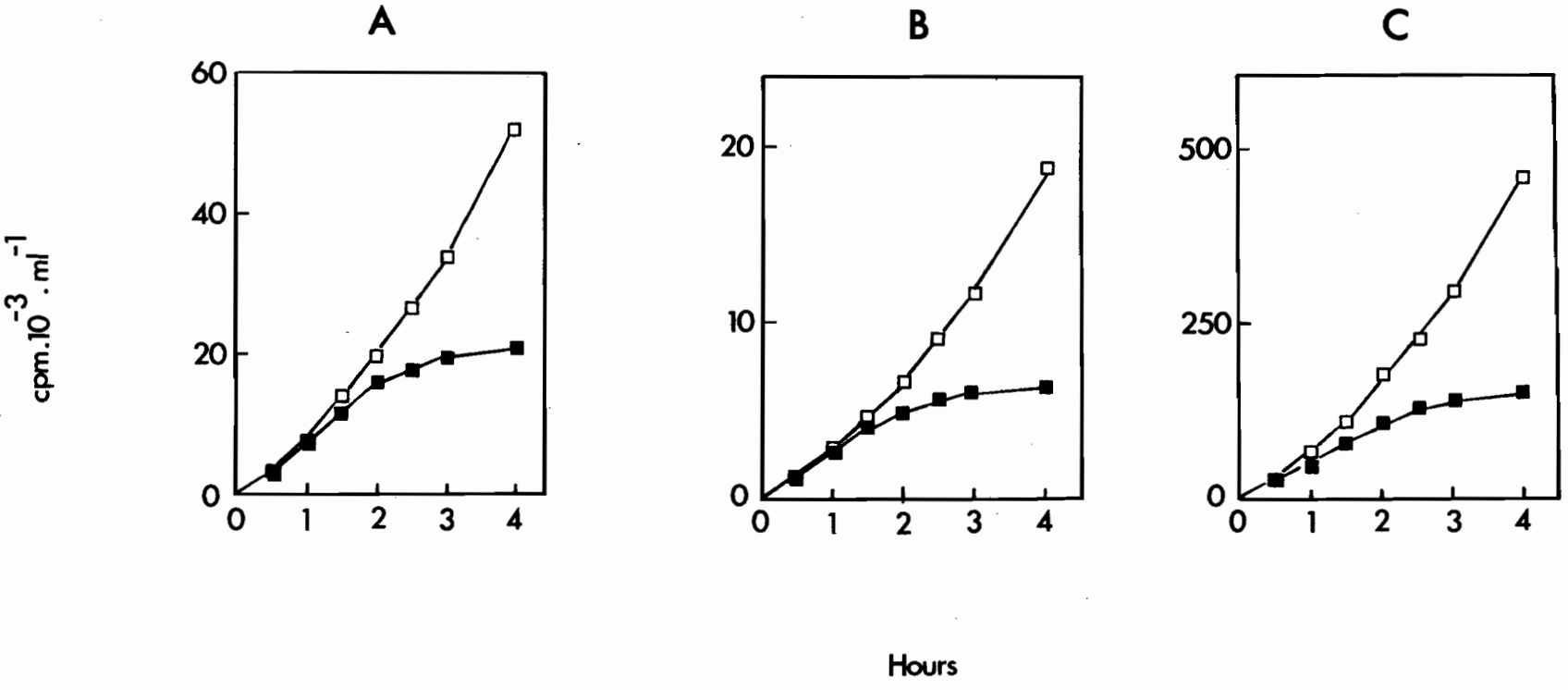

A
B
C
60
40
20
0
20
10
0
500
250
0
0 1 2 3 4
cpm.10^-3 . ml^-1
Hours

Figure 3.4 Effect of killer toxin on yeast pools.

In the experiments described in Figure 3.3, the cultures were sampled atintervals for the ethanol-soluble radioactivity in cells. *A*, [^{14}C] adenine-derived pools; *B*, [^{14}C] glucose-derived pools; *C*, [^{14}C] cytosine-derived pools. Symbols: (■), plus toxin; (□), plus buffer.

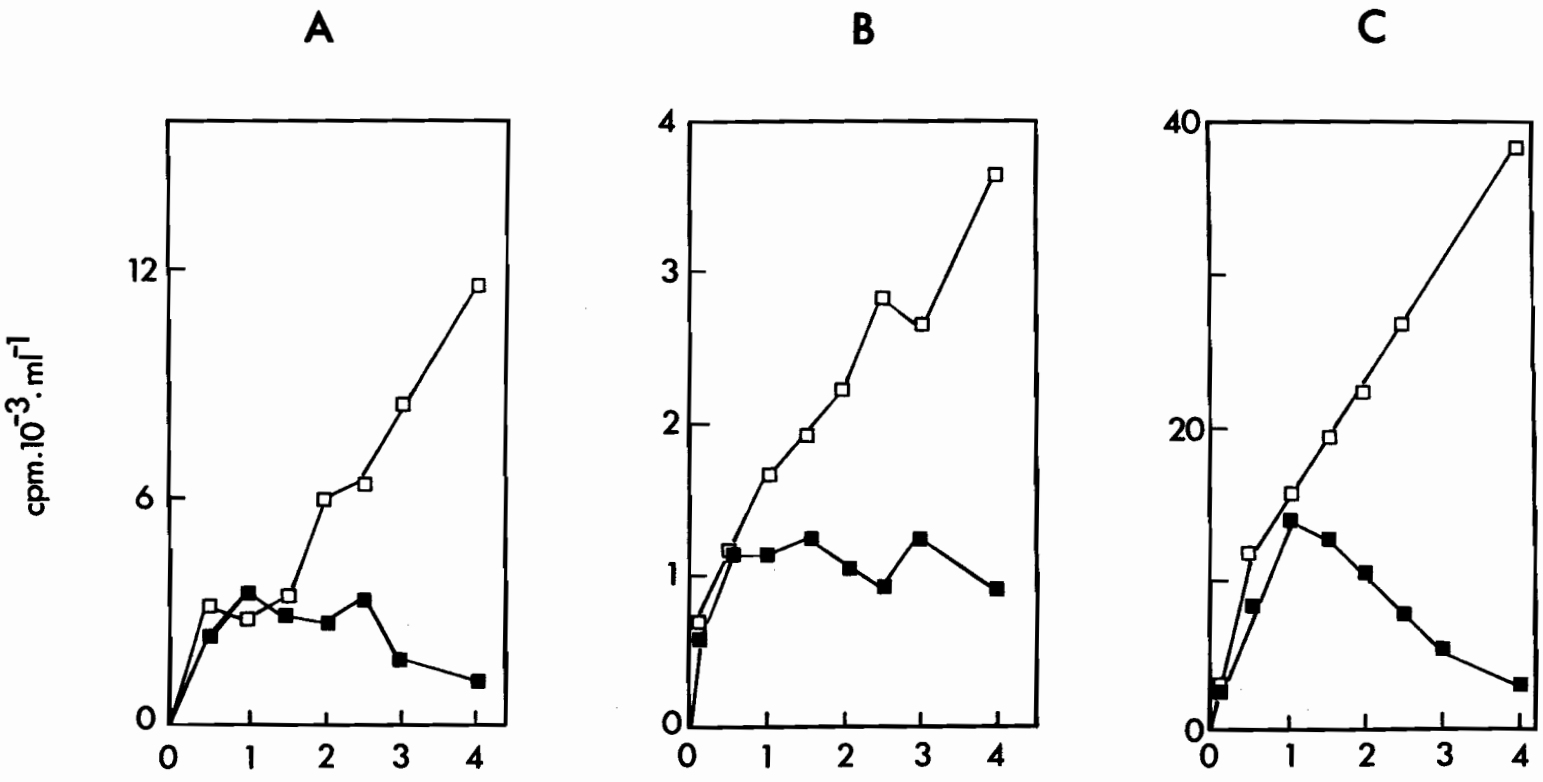
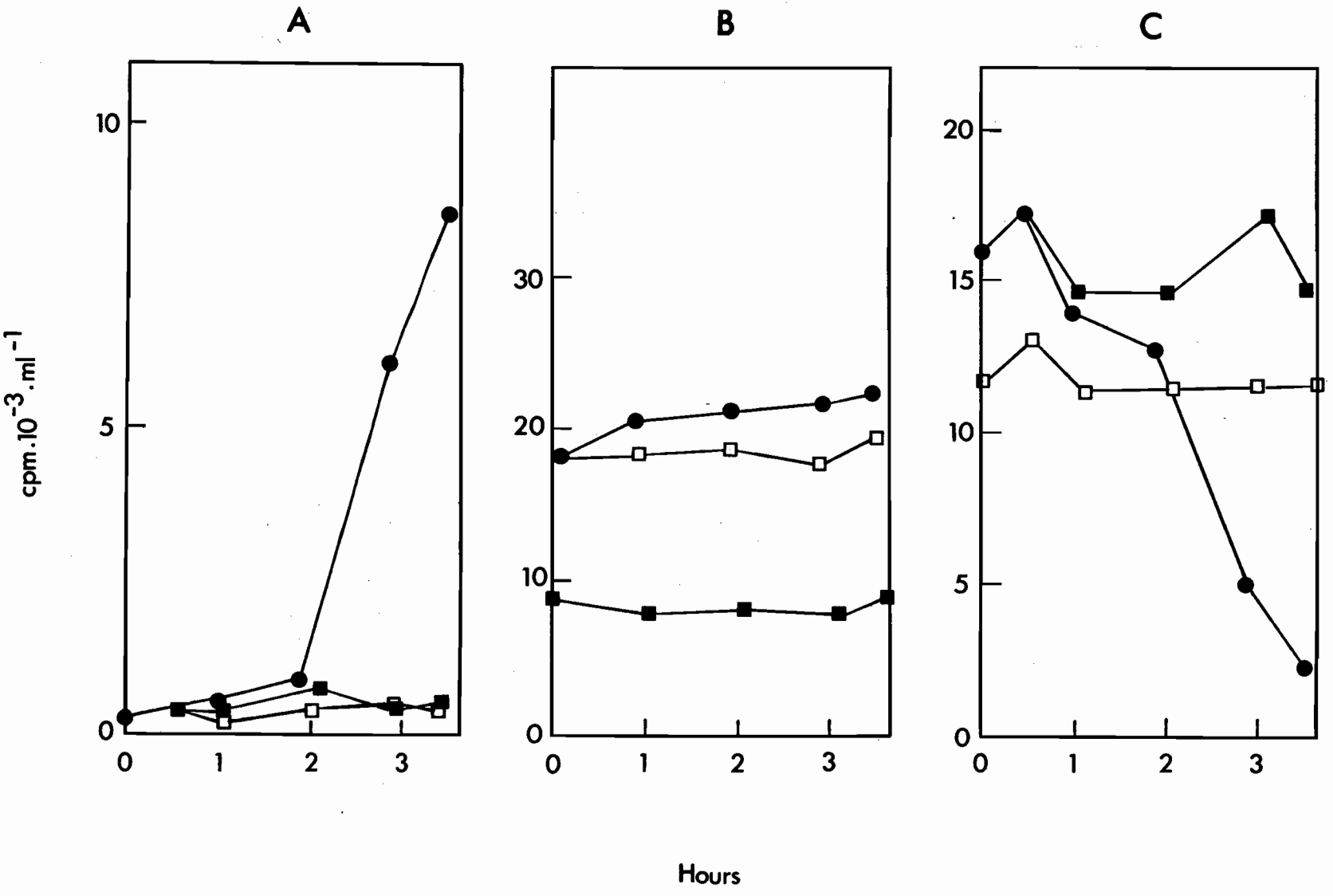

A
B
C
cpm.10^{-3}.ml^{-1}
12
6
0
4
3
2
1
0
40
20
0
0 1 2 3 4
Hours

Figure 3.5 Effect of killer toxin on cellular components derived from [^{14}C] arginine.

A 25 ml YEPD culture of strain K19.10 (A_{600} 0.9) was supplemented with [^{14}C] arginine (10 μ Ci) and incubated at room temperature. After about 2 hours the culture was split into two portions, cells 1 and cells 2, and cells 2 was treated with cycloheximide at 40 μg/ml. After a further 2 hours the cells were harvested and then washed with either YEPD (cells 1) or with YEPD plus cycloheximide (cells 2). The washed cells were incubated at time zero as follows: suspension 1, cells 1 plus killer toxin; suspension 2, cells 1 plus killer toxin and cycloheximide (40 μg/ml); suspension 3, cells 2 plus killer toxin. The three suspensions were sampled at intervals for radioactivity in the wash (plot *A*), macromolecule (plot *B*) and pool (plot *C*) fractions (see General Materials and Methods). Symbols: (●), suspension 1; (□), suspension 2; (■), suspension 3.


A
B
C
cpm.10^{-3}.ml^{-1}
Hours
0
1
2
3
5
10
20
30
15

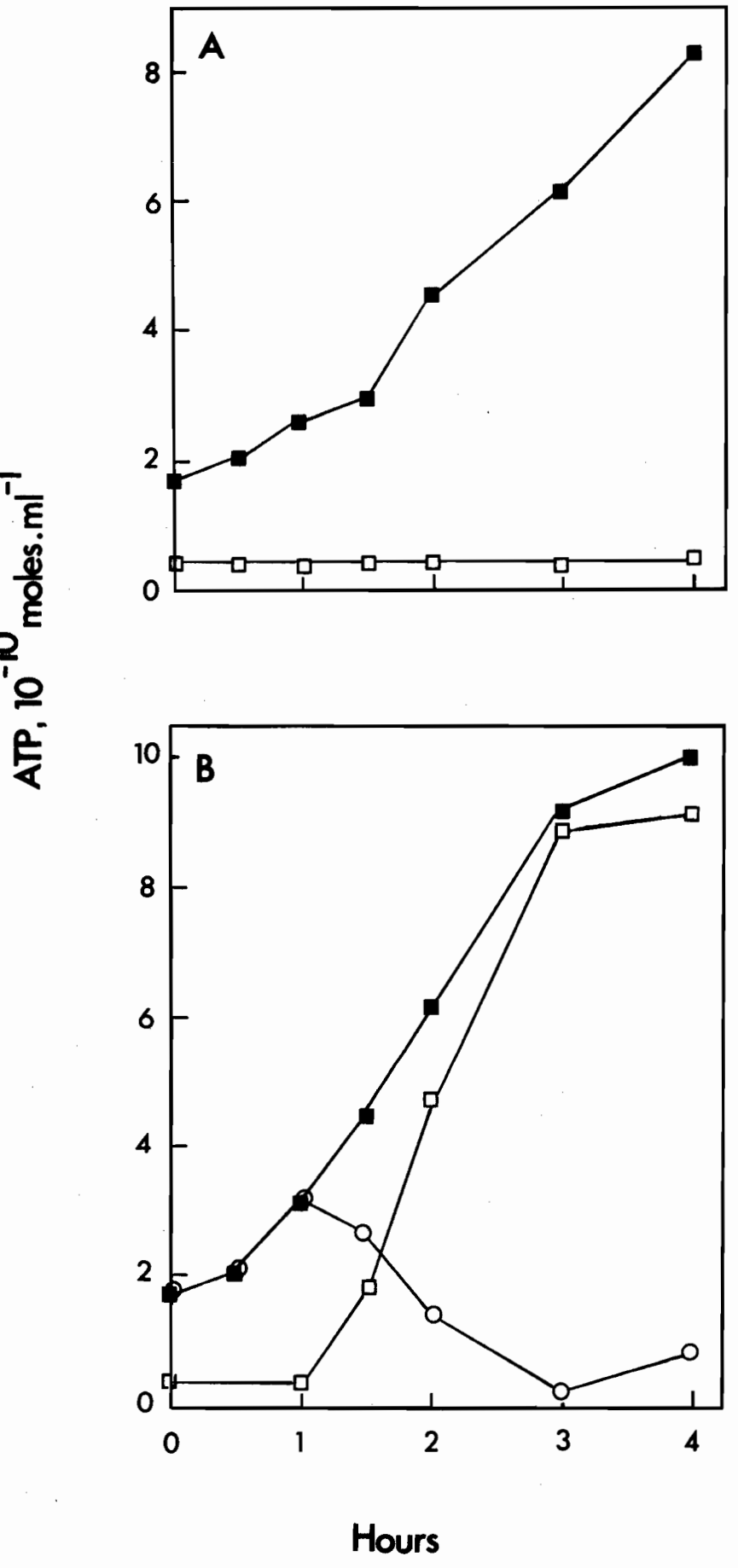
unlabeled medium and treated with killer toxin. Figure 3.5 (suspension 1) shows the distribution of radioactivity following addition of toxin. Virtually all of the cellular pools of radioactivity were lost from the cells to the medium (plots *C* and *A*), whereas cellular macromolecules were retained (plot *B*) (labeled cells incubated without toxin retain their pools of radioactivity tightly; data not shown). This efflux of arginine-derived radioactivity was an event quite distinct in time from the other events due to toxin action: the cells started to lose arginine-derived cpm at about plus-120 min; efflux of cellular ATP and ^{42}K (see below) began at about plus-60 minutes. On this basis, arginine-efflux was clearly secondary to the other events.

Discharge of cellular ATP and leakage of ATP to medium

A killer toxin-induced depletion of cellular ATP, with the appearance of an excessive amount of adenine nucleotide in the growth medium, reported for toxin-treated S14 cells by Bussey and Sherman (1973), was also seen in K19.10 cultures when the protocol used by Bussey and Sherman was used to extract the ATP from the cultures (Figure 3.6). It can be seen that not only did most of the ATP remaining after toxin treatment appear in the medium but also that the total amount in the culture after four hours (about 10×10^{-10} moles per ml) exceeded four-fold the amount present in an untreated culture at plus-60 minutes (2.6×10^{-10} moles per ml). The plus-60 min control ATP value is used for this comparison since it was the time after toxin addition when K19.10 cultures started to show metabolic alteration.

Figure 3.6 Effect of killer toxin on ATP in a sensitive culture.

A culture of strain K19.10 was treated at time zero with either buffer (plot *A*) or killer toxin (plot *B*) and sampled at intervals for ATP in the total culture and in the cell-free medium (General Materials and Methods). ATP in cells was the difference between that in the total culture and that in the medium. Survival of the toxin-treated suspension at plus-4 hours was 0.5 percent. Symbols: (■), total culture; (○), cells; (□), cell-free medium.


A
B
8
6
4
2
0
10
8
6
4
2
0
0
1
2
3
4
ATP, 10^{-10} moles.ml^{-1}
Hours

Since this overproduction of ATP was of great interest in terms of any hypothesis to account for toxin action, it was decided to use an alternative procedure for sampling nucleotides and further to measure AMP and ADP in toxin-treated cultures. Several sampling procedures for *E. coli* nucleotides had been described by Chapman *et al.* (1971). Their method based on an extraction of cells with hot ethanol was found, when suitably modified, to be useful for our purposes. The procedure finally adopted to measure adenine nucleotides is described in the following section.

Response of yeast adenine nucleotides to toxin

(a) Measurement of nucleotides

The following procedure was completed on the day of the experiment. Growing cultures at A_{600} 1.2 to 1.3 were treated with either toxin or buffer (control) and sampled at intervals for two fractions: the total culture and the cell-free medium. Total culture adenylates were sampled by adding 0.4 ml culture to 0.6 ml absolute ethanol at 80°C; after 10 min at 80°C the tube was transferred to ice and the contents diluted with 9.0 ml ice-cold water. The suspension was then filtered over a glass-fibre disc and the filtrate taken as the sample. To sample the cell-free medium, about 0.9 ml culture was filtered rapidly over glass fibre and 0.4 ml of the filtrate processed with ethanol as described for total adenylates.

Once all samples had been taken they were incubated essentially as described by Chapman *et al.* (1971) to convert AMP and ADP to ATP. All solutions were then assayed for ATP by the firefly lantern luciferase-

luciferin procedure (see General Materials and Methods).

Figure 3.7 shows calibration of known amounts of AMP, ADP and ATP, processed through the entire procedure used for measuring yeast adenylates. In principle the three sets of data should fall on the same line; it was clear, however, from repeated trials that this was not the case, particularly at low nucleotide concentrations. Known combinations of AMP, ADP and ATP were also processed through the procedure. The results, shown in Table 3.1, justified our confidence in using the procedure to measure mixtures of adenylates. Table 3.2 shows that the luciferase-luciferin assay was specific for ATP.

(b) Effects of toxin

Figures 3.8 and 3.9 show the effect of killer toxin on AMP, ADP and ATP in cultures of strain K19.10; Figure 3.10 shows the results of measuring the nucleotides in an untreated culture of this strain. The data shown have been corrected for adenylates measured in sterile YEP medium (Figures 3.8 and 3.9; 0.45 nmole ATP per ml, 0.31 nmole ADP per ml, 1.8 nmole AMP per ml; Figure 3.10: 0.49, 0.96 and 4.7 nmole per ml). As was done for the calculation of ATP in cells (Figure 3.6), the level of each nucleotide in cells in these trials was derived by subtracting nucleotide medium from nucleotide total culture. Some inconsistency was evident in the AMP data: in Figure 3.9, AMP in the medium exceeded that in the total-culture sample over the final sample points. In general, however, the results were consistent.

As was found in ATP measurement based on the water extraction

Figure 3.7 Standard curves for ATP, ADP and AMP.

Known amounts of ATP, ADP and AMP were extracted with ethanol and processed for the measurement of ATP as described in the text. *Cpm* refers to the light emmission from the luciferase-luciferin assay as measured in a scintillation counter. Symbols: (■), ATP; (□), ADP; (○), AMP.

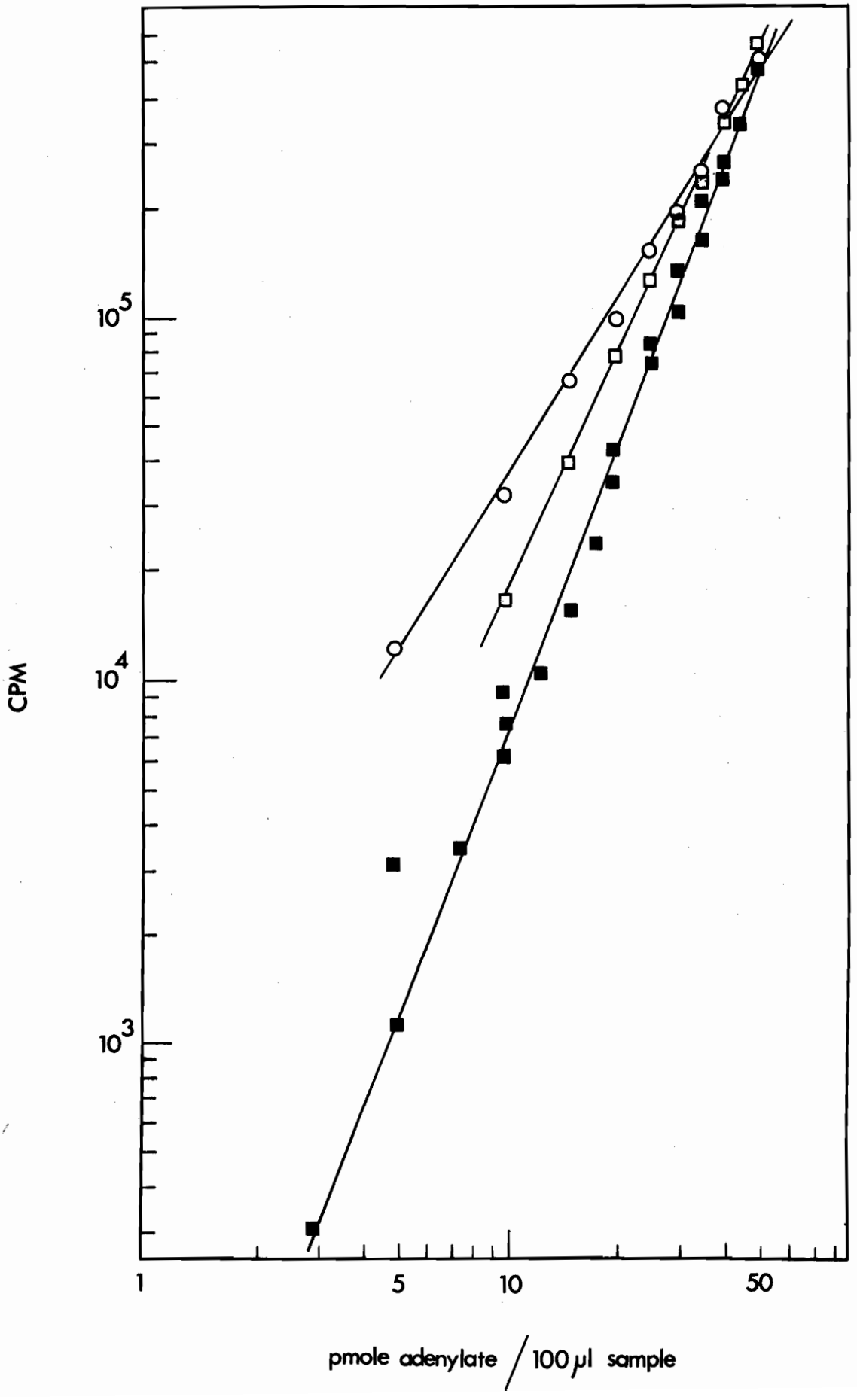

CPM
10^5
10^4
10^3
1
5
10
50
pmole adenylate / 100 µl sample

Table 3.1 Recovery of adenine nucleotides in standard mixtures.

Mixture	pmole adenylate added			pmole adenylate recovered[a]		
	ATP	ADP	AMP	ATP	ADP	AMP
1	5.0	5.0	5.0	5.0	6.0	5.5
2	10.0	10.0	10.0	10.0	10.5	10.5
3	15.0	15.0	15.0	16.0	16.0	14.0

[a]Each mixture was processed through the ethanol-extraction protocol described in the text, incubated with enzymes to convert AMP and ADP to ATP (Chapman *et al.*, 1971) and the samples then assayed for ATP as described in General Materials and Methods.

Table 3.2 Specificity of luciferin-luciferase assay.

pmole adenylate added[a]		pmole ATP measured
AMP	ADP	
25	-	2 - 3
50	-	3 - 4
-	25	less than 2
-	50	less than 2

[a]The samples were assayed directly for ATP by the firefly lantern luciferase-luciferin assay described in General Materials and Methods.

Figure 3.8 Effect of killer toxin on adenylates in a sensitive culture. 1. Sum of adenylates; ATP.

A YEPD culture of strain K19.10 (A_{600} 1.18) was incubated at time zero with killer toxin and sampled at intervals for adenine nucleotides in the total-culture and in the cell-free medium. Nucleotides in cells were calculated as described in the text. Plot *A*, sum of ATP, ADP and AMP in the total culture (■) and in the cell-free medium (□); plot *B*, sum of ATP, ADP and AMP in the cells. Plot *C*, ATP in the total culture (■) and in the cell-free medium (□); plot *D*, ATP in the cells. Culture survival at plus-320 min was 1.7 percent.

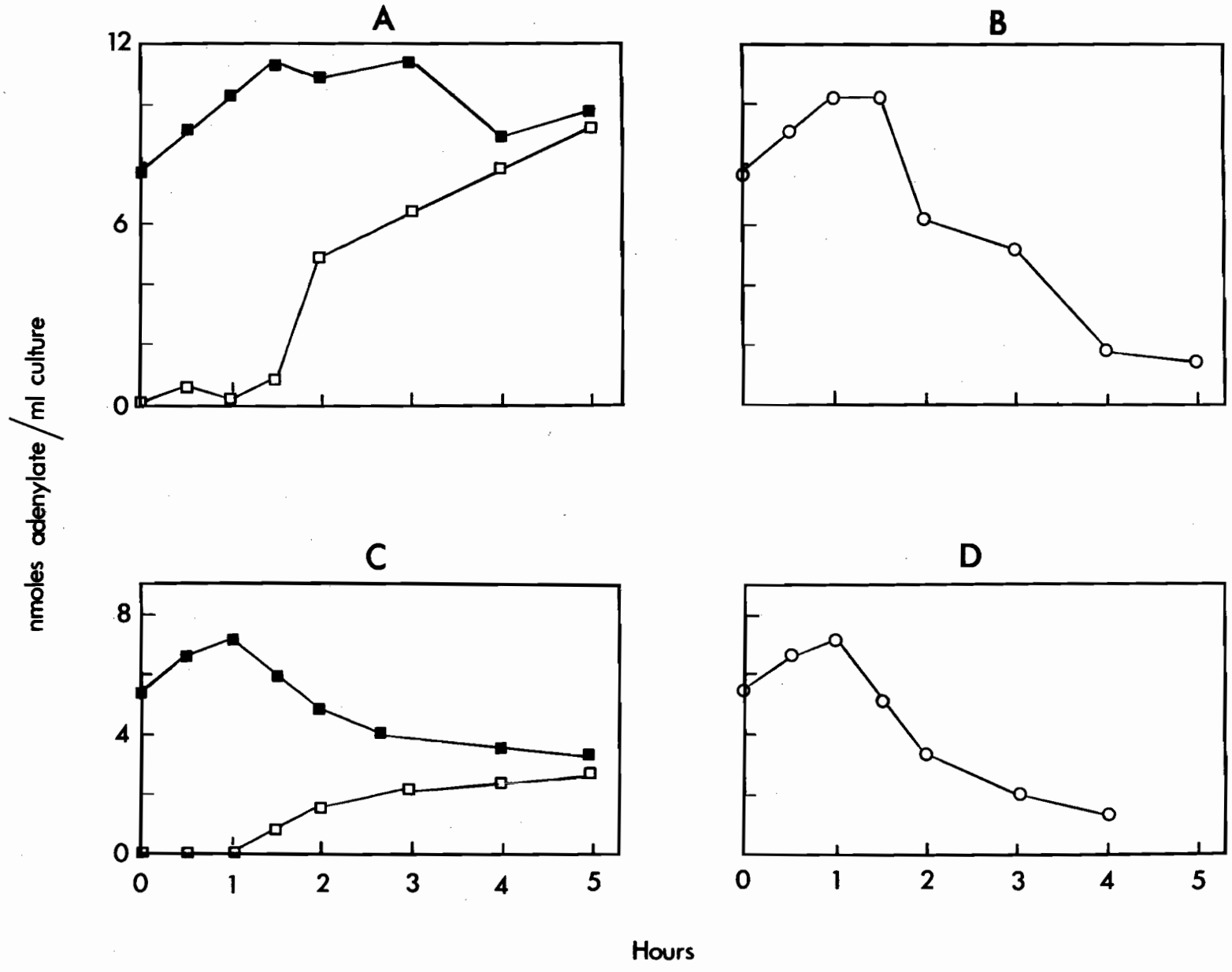

A
B
C
D
12
6
0
8
4
0
0
1
2
3
4
5
nmoles adenylate /ml culture
Hours

Figure 3.9 Effect of killer toxin on adenylates in a sensitive culture. II. ADP; AMP.

The experiment is described in the legend to Figure 3.8. Plot *A*, ADP in total culture (■) and in the cell-free medium (□); plot *B*, ADP in cells. Plot *C*, AMP in total culture (■) and in the cell-free medium (□); plot *D*, AMP in cells.

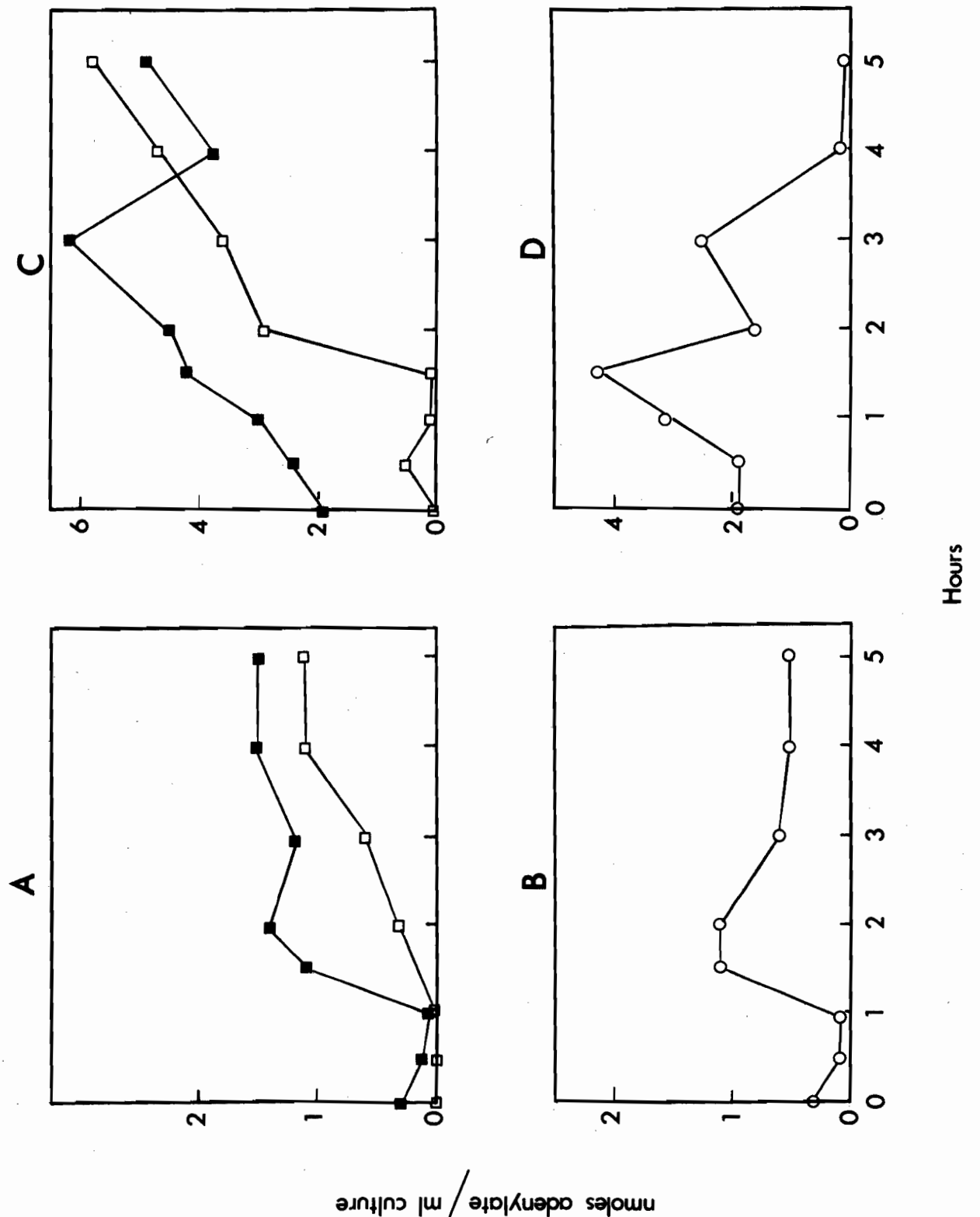
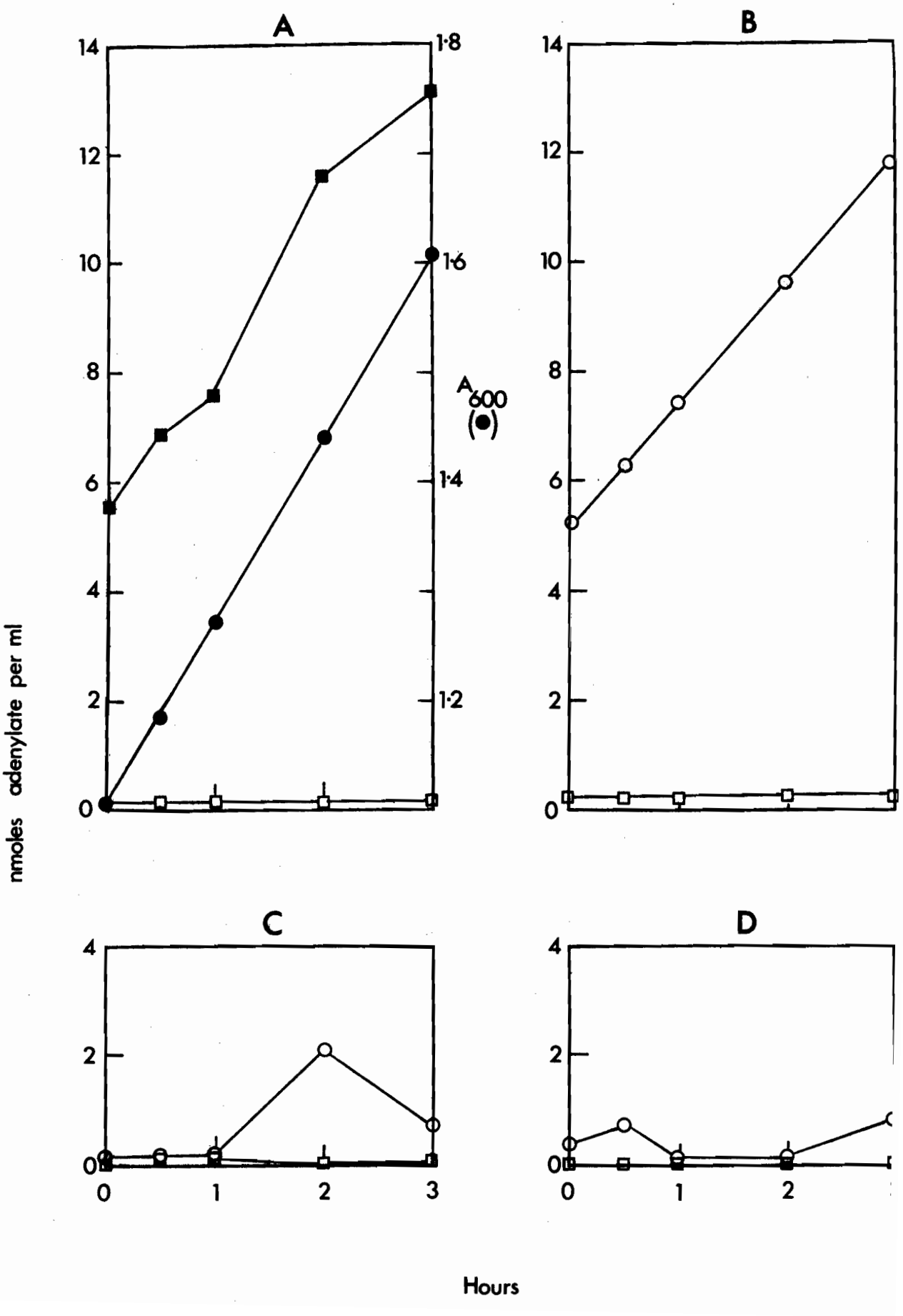

A
B
C
D
nmoles adenylate / ml culture
Hours
0
1
2
3
4
5
6

Figure 3.10 Adenylates in an untreated culture.

A YEPD culture of strain K19.10 was incubated at room temperature and sampled at intervals for adenine nucleotides as described in the legend to Figure 3.8. Culture absorbance was also measured. Plot *A*, absorbance at 600 nm (●); sum of ATP, ADP and AMP in total culture (■), and in cell-free medium (□). Plot *B*, ATP in total culture, ATP in cells (○); ATP in cell-free medium (□). Plot *C*, ADP in total culture, ADP in cells (○); ADP in cell-free medium (□). Plot *D*, AMP in total culture, AMP in cells (○), AMP in cell-free medium (□).


A
B
C
D
14
12
10
8
6
4
2
0
1·8
1·6
1·4
1·2
A600
(●)
nmoles adenylate per ml
0
1
2
3
Hours

protocol used by Bussey and Sherman (Figure 3.6) the measurement based on an ethanol extraction showed that toxin-treated sensitive cells discharged much of their ATP to the medium (Figure 3.8, plots *C* and *D*). There was no indication, however, of an overproduction of the nucleotide. Total-culture ATP in toxin-treated suspensions changed from 7 nmole per ml at plus-60 min after toxin addition to about 3.7 nmole per ml at plus-240 min (Figure 3.8, plot *C*); the decrease, about 3.3 nmole per ml, was largely accounted for by increases in total ADP and AMP (1.5 nmole per ml and 1.0 nmole per ml, respectively, between plus-60 and plus-240 min, see Figure 3.9, plots *A* and *C*).

This discrepancy between the ATP data obtained in these measurements and in the experiments of Bussey and Sherman (1973) and Figure 3.6 may be due to an underestimation of cell-bound ATP when the cells are extracted with hot water by the procedure of Foulds (1971) (see General Materials and Methods). In the measurement illustrated in Figure 3.6, total-culture ATP at plus-60 min was 0.17 nmole per ml; in the results shown in Figure 3.8, 5.2 nmole per ml. If hot water extracts cell-bound nucleotides inefficiently, relative to nucleotides free in the medium, then a toxin-induced leakage of ATP to the medium will result in an apparent overproduction of the molecule since the recovery will increase with the degree of leakage to the culture fluids. This explanation is only adequate if it is also supposed that the water-extraction procedure, regardless of the nature of the substrate to be extracted, results in relatively low recovery of ATP in comparison to the procedure using an extraction with ethanol: in the data shown in

Figure 3.6, total-culture ATP at plus-240 min was 1 nmole per ml, an amount still less than the 3.7 nmole per ml found at this time in the toxin-treated suspension treated with ethanol (Figure 3.8, plot *C*).

Apart from providing evidence for apparently interrelated changes in the levels of ATP, ADP and AMP in toxin-treated cultures (the sum of adenine nucleotides in the total culture was roughly constant from plus-60 to plus-240 minutes: Figure 3.8, plot *A*), these results also showed that the yeast envelope became permeable to these low MW cellular components. Between plus-90 and plus-120 minutes after addition of toxin the adenylates were each found in the cell-free medium (Figure 3.8, plot *C*; 3.9, plots *A* and *C*); in control (untreated) cultures these molecules were retained by cells (Figure 3.10).

Measurement of AMP, ADP and ATP in killer toxin-treated S14 cultures showed a result very similar to that described for strain K19.10: total culture ATP decreased, with the appearance of extra AMP and ADP, and the three nucleotides were leaked to the medium from approximately plus-50 minutes after toxin addition (data not shown).

Potassium efflux

Many species of single-celled organism maintain a gradient of potassium ions across their plasma membrane. In yeasts, the intracellular K^+ concentration has been estimated to be 200 to 250 mM (Jones *et al.*, 1965). In view of the known effects of killer toxin on membrane permeability to adenine nucleotides and the fact that the membrane-acting colicins promote K^+ efflux from sensitive bacterial cells (Hirata *et al.*, 1969; Dandeau, Billault and Barbu, 1969; Wendt, 1970; Feingold, 1970)

it was decided to test for the effect of killer toxin on the yeast potassium pools.

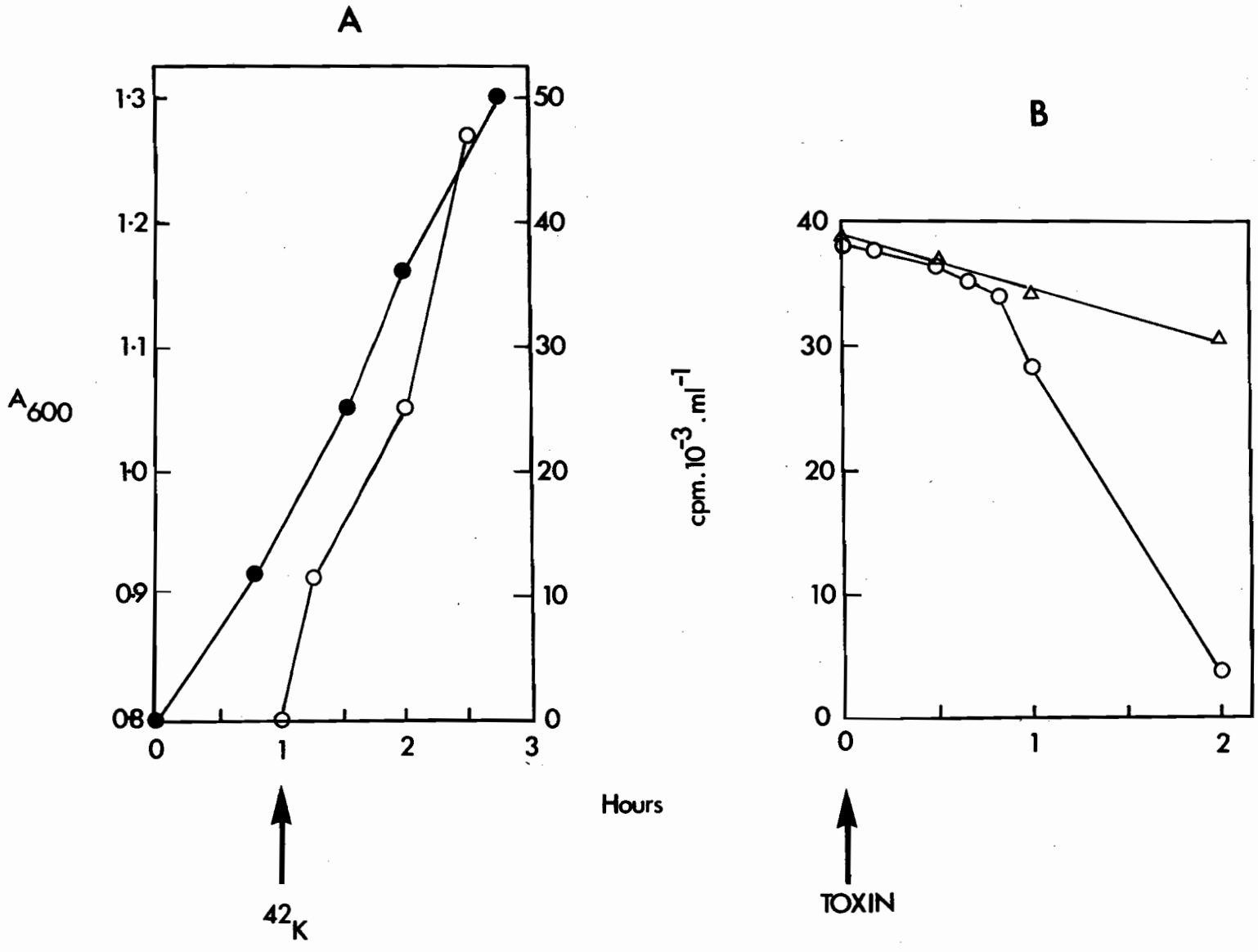
Initially, I attempted to use radioactive rubidium, ^{86}Rb, as a tracer for the potassium pools, since the isotope has a more convenient half-life than ^{42}K (^{86}Rb, $t_{\frac{1}{2}}$ = 19.5 days; ^{42}K, $t_{\frac{1}{2}}$ = 12.4 hours) and rubidium has often been used as an analogue for potassium. Unfortunately conditions could not be found such that the cells would grow normally in a medium containing Rb^+ in place of K^+. Subsequent trials with a medium containing $^{42}K^+$ and 5 mM KCl (NaYEP media, General Materials and Methods) were successful: all the strains examined grew normally in the "low" potassium medium, the cells took up the radioactive potassium and toxin-sensitive but not toxin-resistant or immune cells responded to toxin by discharging the radioactivity into the medium.

Figure 3.11 illustrates the loading of sensitive cells with ^{42}K and the effect of killer toxin on the accumulated ion. Cells were loaded by simply adding ^{42}KCl to NaYEPD cultures and incubating the cultures to the required cell density. Loading was measured by sampling the cultures at intervals for cell-bound radioactivity: 0.1 to 0.5 ml of culture was filtered over a glass-fibre disc, washed with 2 to 5 ml of unlabeled growth medium and the filter counted for ^{42}K. The cells appeared to load rapidly and then maintain pools of ^{42}K in proportion to their density (Figure 3.11, plot A). To measure ^{42}K efflux in the presence of toxin or other additives, the radioactive cultures were washed twice at the centrifuge with unlabeled growth medium, resuspended in medium to the original culture-volume and incubated with the appropriate additions; the

Figure 3.11 Potassium release from cells as a measure of killer toxin action. I. Loading of cells with ^{42}K; response of ^{42}K pools to toxin in a sensitive strain.

A NaYEPD culture of strain K19.10 was incubated at room temperature and supplemented with ^{42}KCl at the time marked by the arrow (plot *A*). Radioactivity in the cells and the culture density was measured at intervals. Symbols (plot *A*): (●), A_{600}; (○), radioactivity in cells.

The culture was harvested and the cells washed twice and resuspended in NaYEPD and then incubated at time zero with either killer toxin or with buffer (control) (plot *B*); radioactivity in cells was then measured at intervals. Symbols (plot *B*): (○), plus toxin; (△), control. Culture survival in presence of toxin was 1.6 percent at plus-120 min.


A
B
A_{600}
1·3
1·2
1·1
1·0
0·9
0·8
50
40
30
20
10
0
0
1
2
3
Hours
^{42}K
$cpm.10^{-3}.ml^{-1}$
40
30
20
10
0
0
1
2
TOXIN

radioactivity retained by the cells was then measured, as described for measurement of loading. The response of cellular ^{42}K to killer toxin in a culture of strain K19.10 is shown in Figure 3.11, plot *B*: following a lag-period of about 60 min, the cells lost their ^{42}K content. In the trial illustrated in Figure 3.11 the radioactivity in the cell-free medium of the toxin-treated suspension was also measured -- the ^{42}K lost from cells was recovered in the medium (Figure 3.12, plot *A*). There was clearly no problem with the counting efficiency of ^{42}K (Figure 3.12, plot *B*).

The depletion of cellular ^{42}K seen in killer toxin-treated suspensions of K19.10 was a specific response of sensitive strains to toxin. Sensitive strains S30 (Appendix A) and S14 (data not shown) responded to killer toxin in a similar manner to K19.10. Neither the toxin-producing strains K12 and K19 nor resistant mutants such as S14.96 lost ^{42}K in a way indicative of alteration to membrane permeability (Figure 3.13). In sensitive cultures incubated without toxin, and in toxin-producing or resistant cultures treated with toxin, the cells displayed a slow release of ^{42}K indicative of a $^{42}K/^{39}K$ exchange process across the plasma membrane (e.g. Figure 3.11, plot *B*, control).

In Figure 3.13 and in all further illustrations of ^{42}K release from cells (or from spheroplasts, see Chapter 7) the data is expressed as the percent ^{42}K remaining, where 100 percent is the radioactivity present in the cells immediately before incubation.

Potassium release due to toxin action was an event coincident with the other known events in toxin-treated sensitive cells (with the

Figure 3.12 Potassium release from cells as a measure of killer toxin action. II. Recovery of potassium in cell-free medium; recovery of potassium in system.

In the experiment described in the legend to Figure 3.11, plot *B*, the radioactivity lost from cells was measured by counting samples of the filtrates from the sampled cell suspensions. Plot *A*: radioactivity in filtrates of toxin-treated culture (O), radioactivity in filtrates of untreated culture (Δ). Plot *B*: recovery of radioactivity in system (the sum of cpm in cells and in cell-free filtrate). Symbols: (O), toxin-treated culture; (Δ), untreated culture.

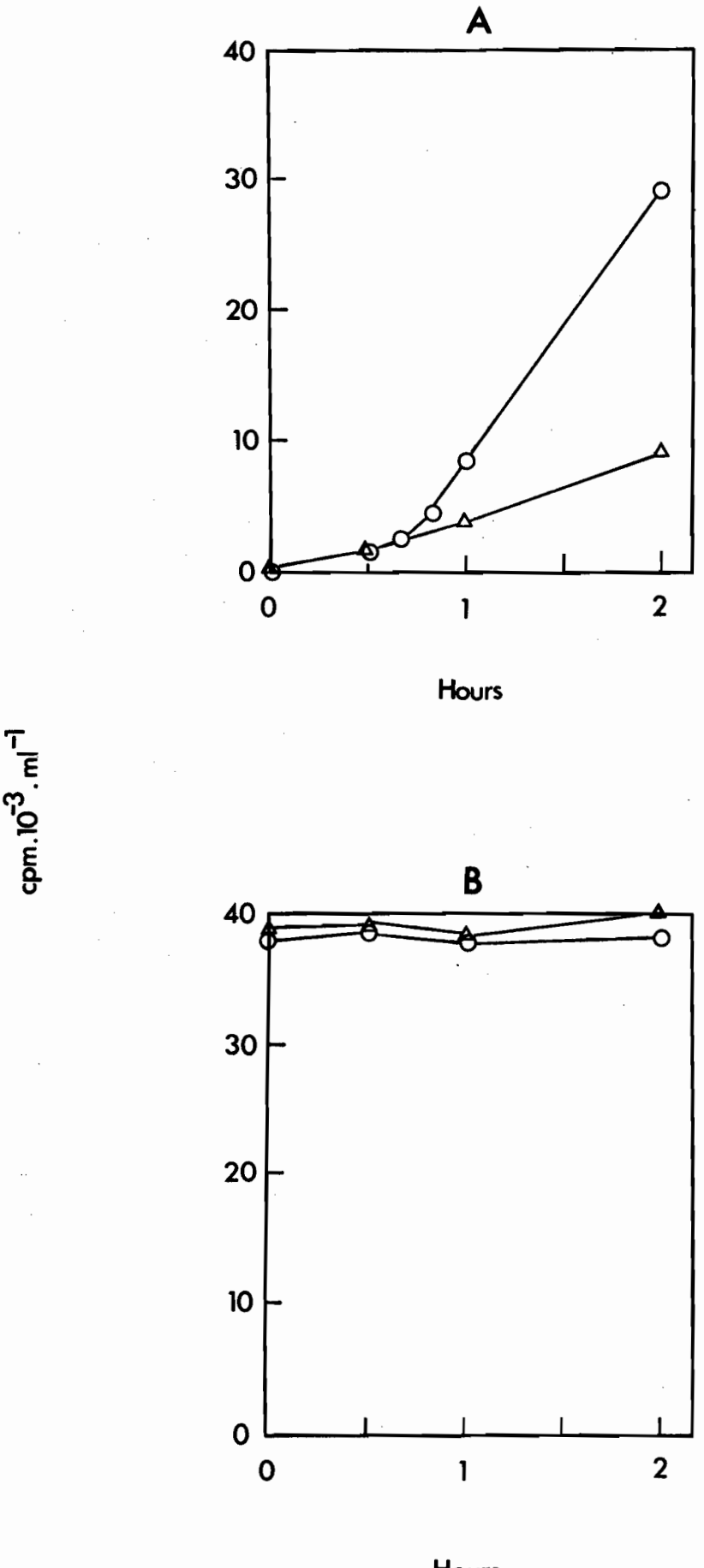
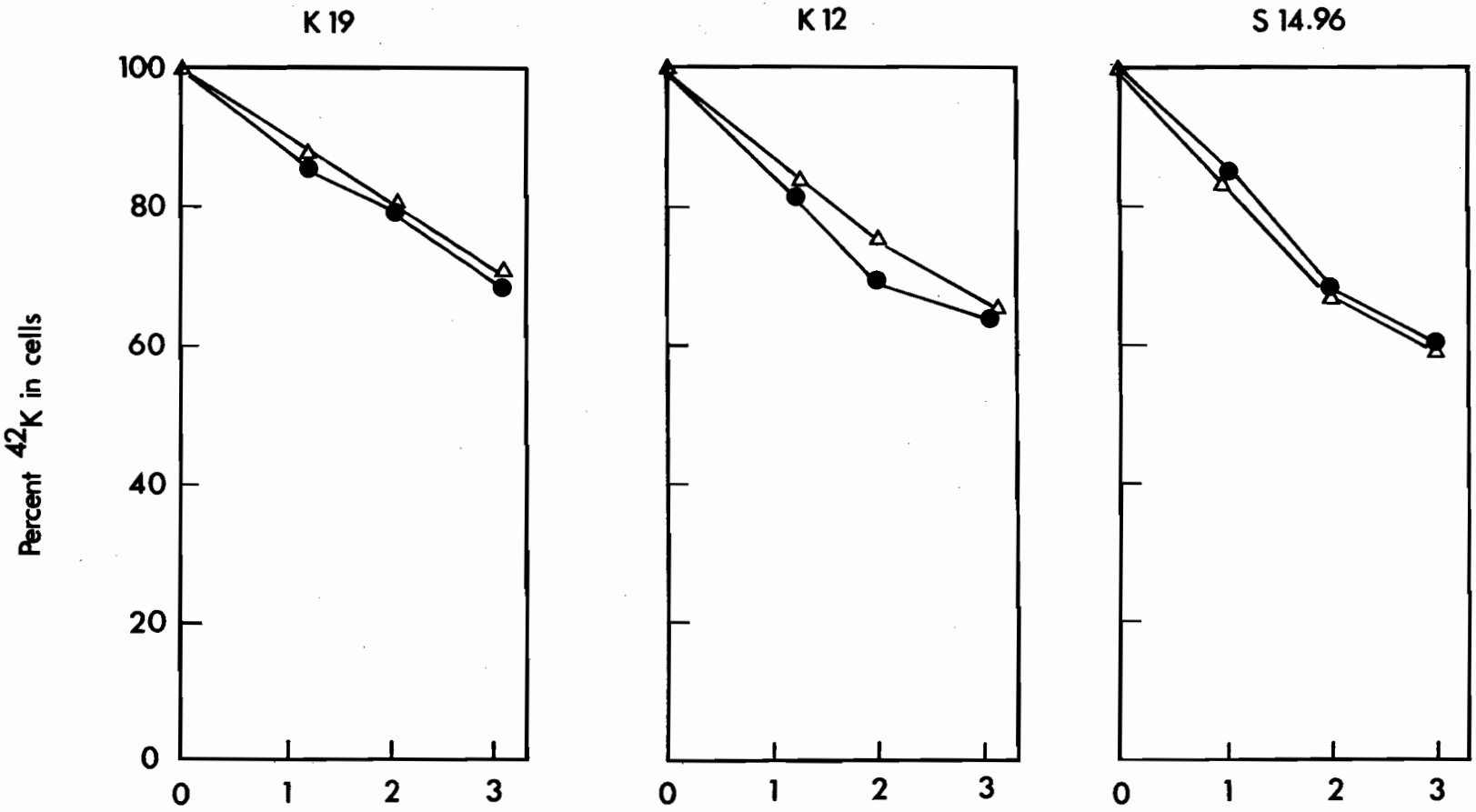

A
40
30
20
10
0
0
1
2
Hours
cpm.10^{-3}.ml^{-1}
B
40
30
20
10
0
0
1
2
Hours

Figure 3.13 Potassium release from cells as a measure of killer toxin action. III. Resistance of potassium pool to toxin in immune and resistant strains.

NaYEPD cultures of strains K12 and K19 (killer toxin immune) and S14.96 (toxin resistant mutant) were loaded with ^{42}K and treated at time zero with either killer toxin or with buffer (control). Radioactivity remaining in cells was measured, at intervals. The dose of toxin was sufficient to kill more than 98 percent of K19.10 cells by plus-180 min under these conditions. Symbols: (●), plus toxin; (Δ), control.


K 19
K 12
S 14.96
100
80
60
40
20
0
0
1
2
3
Percent ^{42}K in cells
Hours

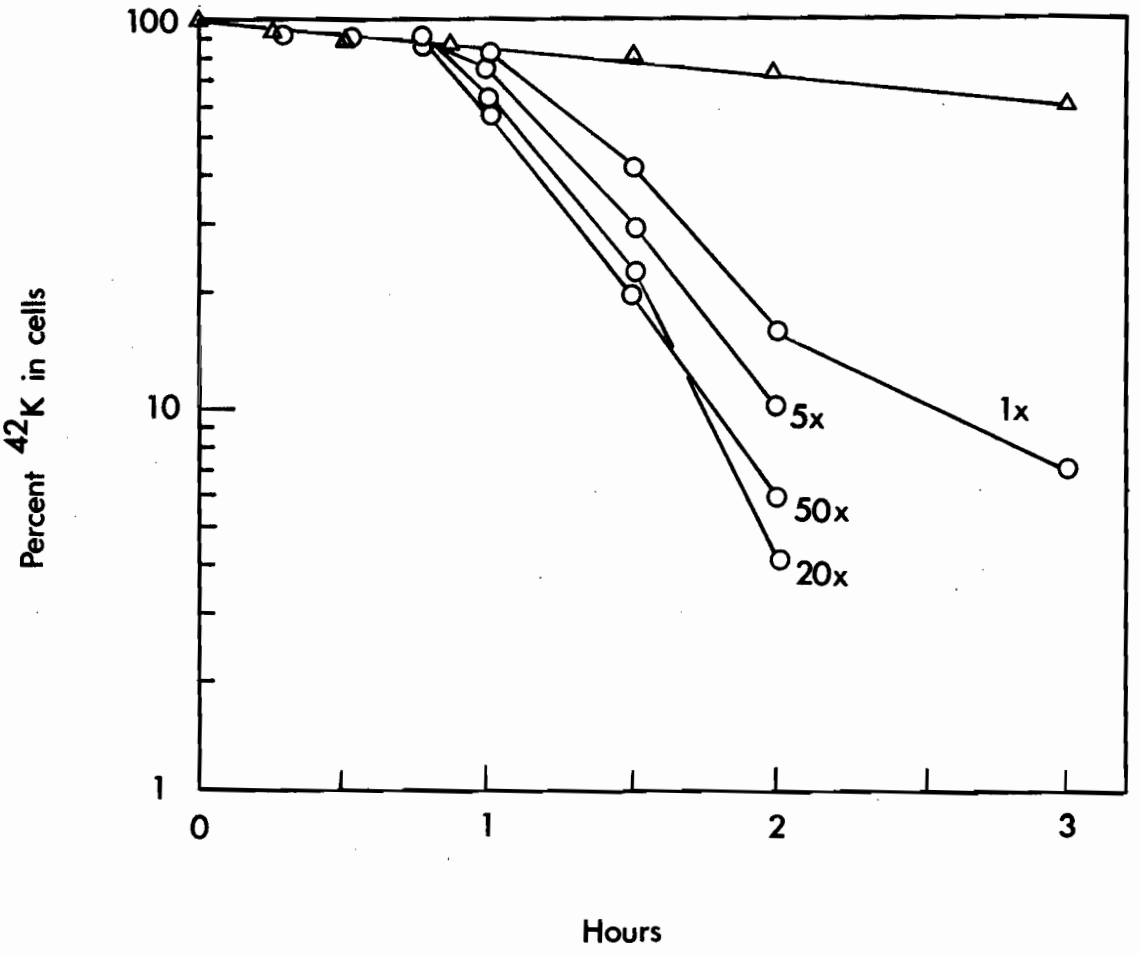
exception of arginine-pool release) and so was, like the other events, either an alternative measure of the primary toxin-induced alteration or an event secondary to the initial alteration. The potassium-release assay was a sensitive method for measuring the onset of damage in toxin-treated cultures and proved to be very useful in subsequent experiments.

Dose-independence of the lag-period

Increasing the amount of killer toxin in a sensitive culture results in a decrease in the surviving fraction of the population but apparently not in any change in the lag-period characteristic of the onset of metabolic alteration in the cells (Bussey, 1972; Bussey and Sherman, 1973; Bussey, 1974). The independence of the duration of the lag-period from the dose of toxin was confirmed here with strain K19.10, using ^{42}K-release as the measure of toxin action. Over a 50-fold range of killer toxin dose the lag before the onset of ^{42}K efflux from cells was constant at approximately 50 minutes (Figure 3.14). The rate of loss of ^{42}K from the population of cells was, however, dose-dependent, up to a limit (Figure 3.14). A reasonable explanation for these results is that increasing the dose increases the proportion of the population that binds a potentially-damaging amount of toxin within a given time-interval (up to a limit where the binding-sites are saturated), without increasing the rate at which the event subsequent to such toxin-binding occurs (the damaging event, here measured as potassium-release). In these terms, the dose-dependence of the rate of toxin-specific ^{42}K release in the population of cells shows

Figure 3.14 The lag-period of killer toxin action on the yeast potassium pool: independence from dose of toxin.

K19.10 cells growing in NaYEPD were loaded with ^{42}K, washed, resuspended in NaYEPD, and treated with a range of killer toxin concentrations. At intervals, radioactivity remaining in cells was measured. Symbols: (△), no toxin (control); (○), toxin, at dose indicated in Figure. Toxin at 1X was sufficient to kill 98 percent of the cells by plus-180 min.


100
10
1
0
1
2
3
Percent ^{42}K in cells
Hours
1x
5x
50x
20x

the dose-dependence of the proportion of the population affected and not the rate at which any one cell becomes affected (see Chapter 5, Results, for additional discussion).

II. Effects of killer toxin on *T. glabrata*

Torulopsis glabrata ATCC 15126, a yeast associated with vaginal infections, was one of a number of cultures obtained from the American Type Culture Collection in order to screen for the distribution of killer toxin-sensitivity among yeast genera (Bussey, personal communication; Al-Aidroos and Bussey, 1978). *T. glabrata* was found both to be sensitive to the *S. cerevisiae* toxin and to produce its own toxin, toxin active against both killer toxin-producing and sensitive strains (Chapter 4). The nature of the sensitivity of *T. glabrata* to killer toxin was examined, to test if the toxin acted similarly in both genera.

By the tests already described for assessment of damage in toxin-treated *S. cerevisiae* cultures, the killer toxin damaged *T. glabrata* suspensions by the same mechanism as it did *Saccharomyces* suspensions. Toxin-treated cells displayed a transient turbidity-increase, coordinate inhibition of macromolecule synthesis, inhibition of the accumulation of precursor pools, ATP depletion by ATP hydrolysis and by ATP leakage to the culture medium and release of ^{42}K (Bussey and Skipper, 1976, see Appendix B in this thesis; and unpublished results). All of the killer toxin-induced alterations were, as was the case in *S. cerevisiae* cultures, only obvious after a lag-period of about 60 minutes.

Discussion

These experiments show that the addition of killer toxin to

populations of growing sensitive cells results in a number of metabolic changes in the cultures. The accumulation of precursors from the growth medium is altered, the incorporation of these precursors into cellular macromolecules is inhibited, the cells lose their capacity to retain ^{42}K and adenine nucleotides, and the suspensions show a transient increase in turbidity. In agreement with the results obtained for the action of toxin on S14 cells (Bussey and Sherman, 1973), the effects of toxin on either *S. cerevisiae* K19.10 or *T. glabrata* ATCC 15126 were not distinguishable in terms of their time of onset. All the toxin-induced alterations, with the exception of the leakage from K19.10 cells of the arginine-derived pools, were coincident and preceded by a considerable lag-period following toxin addition. It would appear then that the coincident events are either different measures of the same effect or the consequences of some more primary event.

What is the damaging event due to killer toxin action? Bussey and Sherman (1973) suggested that the toxin alters plasma membrane permeability and the results shown here are consistent with such a hypothesis. Clear evidence for altered membrane permeability is provided by the results of challenging ^{42}K-loaded cells with toxin. In toxin sensitive, but not in toxin-producing or resistant cells, the ^{42}K is lost to the culture fluids. It is possible that the ^{42}K-release reflects a toxin-induced acceleration of a $^{42}K/^{39}K$ exchange reaction and not a net potassium efflux; if this were true then the addition of toxin to ^{42}K-loaded sensitive cells suspended in ^{42}K-containing medium should abolish the ^{42}K-efflux, since exchange would then not alter the ^{42}K

content of the cells. This test has not been applied but it seems likely, in view of the known toxin-induced breakdown in cellular impermeability to adenine nucleotides and arginine-derived pools, that the ion is lost from the cells. Consistent with the release from cells of K^+ is the finding of Bussey (1974) that toxin treated S14 cells decrease in volume. A volume decrease would be expected to result from the loss by cells of a major fraction of their osmotically-active components, provided the cell surface is flexible (i.e. the wall must invaginate, as well as the plasma membrane), since the decrease in internal osmotic potential should promote the efflux of cell water.

The toxin-induced coordinate inhibition of macromolecule synthesis, while an event approximately coincident with ^{42}K efflux, may well be secondary to an initial alteration to plasma membrane function. Since macromolecule synthesis was measured in terms of the incorporation of labeled compounds supplied in the growth medium, and since the uptake of these precursors appeared to be altered by toxin action, it is not surprising that the toxin inhibited biosynthesis. In retrospect it may have been more informative to have assessed the effect of toxin on synthesis by measuring the rate of synthesis from precursor pools of known specific activity.

The toxin-induced ATP hydrolysis to ADP and AMP cannot be readily explained in terms of altered membrane permeability. The event may reflect the rôle of ion gradients across the plasma membrane in the metabolism of cellular ATP; it is considered in these terms in the General Discussion.

While the results provide evidence of toxin-induced alteration to plasma membrane permeability they do not provide information on the mechanism of this alteration. Two distinct possibilities can be considered, however. (I) The interaction of toxin with components of the yeast surface may perforate the fabric of the membrane, resulting in a relatively non-specific permeability of the cell to all substances, up to a certain size limit. (II) The toxin may, more specifically, interfere with the uptake and retention by cells of only certain classes of low MW solutes (see below).

In the hypothesis of non-specific damage, the toxin's effects are specific only with respect to the size of the "hole". It is clear from the results that labeled macromolecules are retained by toxin-treated cells; there is no evidence, however, that the leakiness of the damaged cells is restricted to potassium ions and adenine nucleotides. The polyene antibiotics, known to interact specifically with sterols in the plasma membrane of eukaryotic cells, appear to kill yeasts by perforating the envelope, the size of cellular constituents being lost from cells being determined by the particular polyene (Lampen, 1966). Killer toxin, while requiring a certain cell wall structure(s) to be able to affect the cells (see Chapter 7), may, subsequent to this interaction with the wall, damage the membrane in a similar way to the polyenes, by making holes.

In the other hypothesis, toxin action is specific in that it affects the transport of solutes across the plasma membrane. One possibility is that the toxin protein binds to membrane components so as

to uncouple endergonic transport processes from their source of metabolic energy, by inactivating the energy-coupling components. The promotion of K^+ efflux from *E. coli* cells by the membrane-acting K and E1 colicins is envisaged as resulting from specific alteration by the proteins of the energised-state of the bacterial plasma.membrane (Holland, 1975) and killer toxin may affect the retention of solutes in a similar way. Toxin-induced ^{42}K release is consistent with an energy-uncoupling mechanism in that an approximately 0.2 M internal K^+ concentration (Jones *et al.*, 1965) represents considerable metabolic work on the part of the cell. It remains to be demonstrated, however, that killer toxin affects the retention by cells only of the solutes whose flux across the plasma membrane is energetically active. In the absence of evidence for an adenine-nucleotide transport system at the yeast cell membrane it is also difficult to account for toxin-induced adenylate leakage in terms of an energy-uncoupling mechanism.

Chapter 4

EFFECTS OF *T. glabrata* TOXIN (PEST) ON *S. cerevisiae*

Introduction

Cultures of the pathogenic yeast *T. glabrata* ATCC 15126 were found to contain activity toxic to both killer toxin-producing (immune) and sensitive strains of *S. cervisiae; T. glabrata* was resistant to its own toxic secretions (Bussey, personal communication). It was of considerable interest to see if the mechanism of action of the *T. glabrata* toxin(s) resembled that of the killer toxin, since it clearly bypassed the killer toxin-immunity mechanism possessed by killer-producing strains.

The toxic activity (PEST) was partially purified by Dr. Bussey from the supernatant fluids of *T. glabrata* cultures and shown to be associated with at least three distinct glycoprotein fractions of high MW (Bussey and Skipper, 1975). In view of the fact that killer toxin, thought initially to be associated with glycoproteins of high MW, is now known to be a relatively small protein when isolated to homogeneity (MW about 11,000; Palfree and Bussey, manuscript in preparation), it seems likely that the PEST is similarly a much smaller molecule than is apparent from its preliminary characterisation. For my experiments the toxin was the fraction precipitated from concentrates of *T. glabrata* culture fluids by polyethylene glycol (General Materials and Methods, Table 2.4), i.e. the high MW form(s).

Results

I. Effects on cells at 22 to 24°C and at pH 4.6 to 4.8

At 22 to 24°C and in pH 4.6 to 4.8 YEPD and NaYEPD media, the standard conditions for growing and treating cells with killer toxin, PEST-sensitive cells displayed metabolic alterations similar in most respects to the alterations shown by killer toxin-treated sensitive cells. PEST-treated cultures lost viability at an approximately constant rate, up to a limit (Figure 4.1, 22 to 24°C), and the cells showed a transient increase in turbidity, coordinate inhibition of macromolecule synthesis and precursor uptake, ATP depletion and ^{42}K discharge (Figures 4.8 plot *B*, 4.3, 4.4, 4.5). Included in Figure 4.5 is the result of treating strain K12 (killer toxin-producer) with PEST: in terms of the ^{42}K-release assay, its response to PEST was very similar to that of the killer toxin-sensitive strains K19.10 and S14.

In contrast to killer toxin, PEST did not appear to alter the plasma membrane's impermeability to ATP. The PEST-induced depletion of cellular ATP was accounted for by an increase in AMP and the AMP was found in the medium (Figure 4.4, plots *A* and *B*). As was the case in killer toxin-treated cultures, the sum of total culture adenylates (ATP plus ADP plus AMP) remained constant once the PEST's effects on cells were initiated. In cultures of strain K19.10, the sum of adenylates (Figure 4.4, plot *A*) was 9.5 nmoles per ml $\pm$ 0.5 nmoles per ml from plus-30 min after PEST addition to the end of the measurements; very similar results were obtained with PEST-treated cultures of strain S14.

Like the alterations to cellular processes elicited by killer

Figure 4.1 Survival of sensitive cells in the presence of PEST.

Cultures of strain K19.10 were grown* in YEPD to A_{600} 1.1 to 1.2, treated with PEST and sampled at intervals for viability. Symbols: (□), plus toxin (22 to 24°C); (■), plus toxin (30°C).

*The cultures were grown at the temperature at which they were to be treated with toxin.

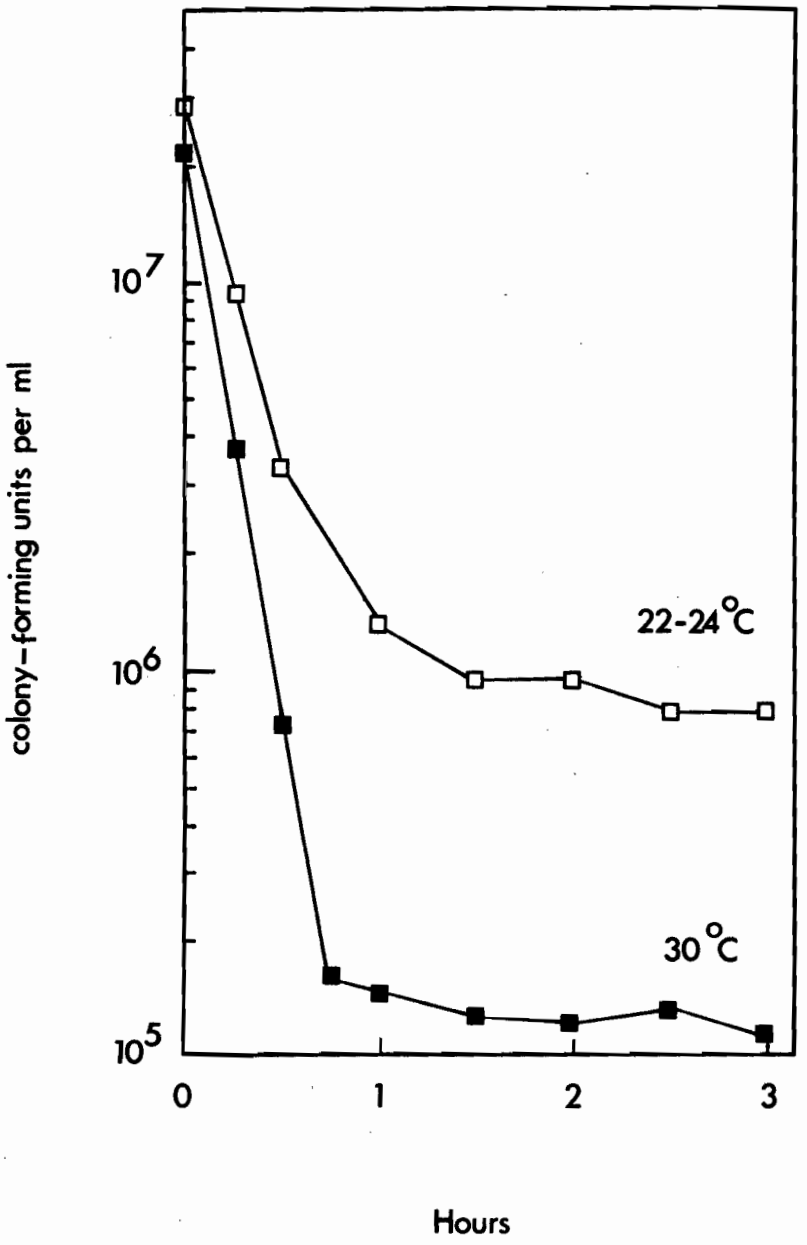

colony-forming units per ml
10^7
10^6
10^5
22-24°C
30°C
0
1
2
3
Hours

Figure 4.2 Effect of PEST on macromolecule synthesis.

PEST or buffer was added, together with the labeled precursor, at time zero to cultures of strain S14 (density A_{600} 1.0 to 1.1) and the cultures subsequently sampled at intervals for ethanol-insoluble radioactivity in the cells.

Plot *A*, [^{14}C] glucose-derived macromolecules;

plot *B*, [^{14}C] arginine-derived macromolecules;

plot *C*, [^{14}C] cytosine-derived macromolecules.

Symbols: (■), plus toxin; (△), plus buffer (control).

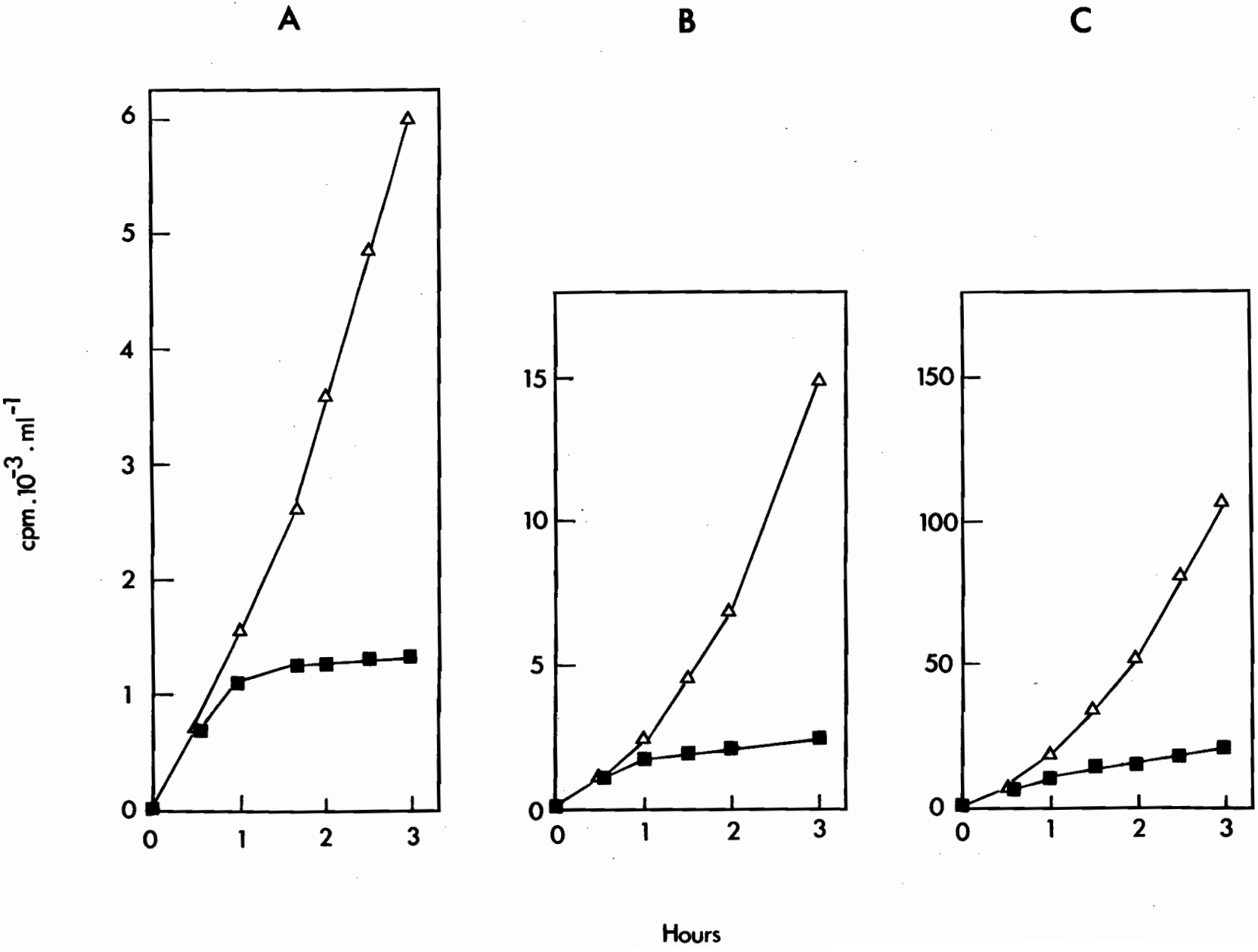

A
B
C
cpm.10^{-3}.ml^{-1}
Hours
0
1
2
3
4
5
6
10
15
50
100
150

Figure 4.3 Effect of PEST on yeast pools.

In the experiments illustrated in Figure 4.2, the cultures were sampled at intervals for ethanol-soluble radioactivity in cells.

Plot *A*, [^{14}C] glucose-derived pools;

plot *B*, [^{14}C] arginine-derived pools;

plot *C*, [^{14}C] cytosine-derived pools.

Symbols: (■), plus toxin; (□), plus buffer (control).

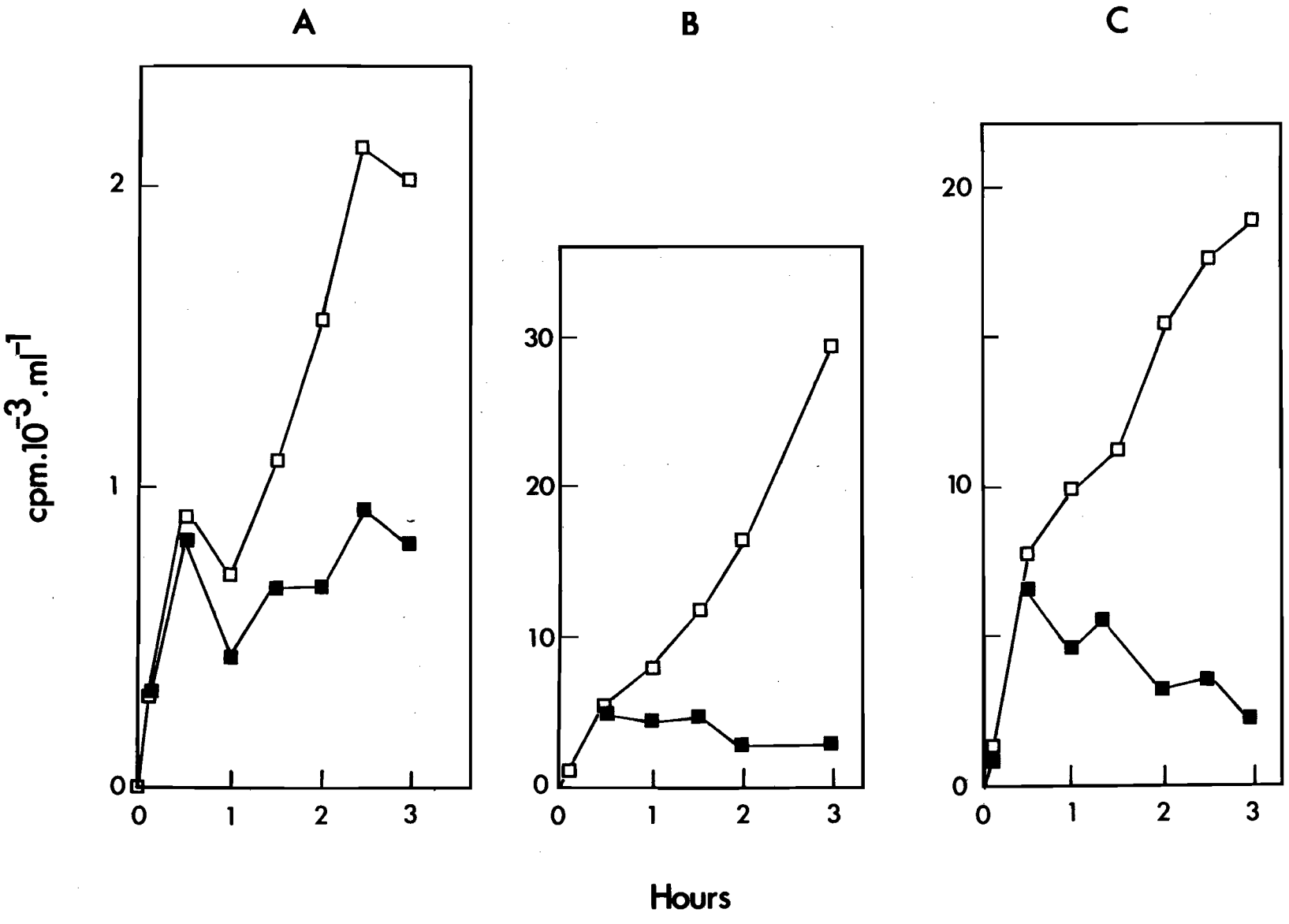

A
B
C
cpm.10^{-3}.ml^{-1}
Hours
0
1
2
3
10
20
30

Figure 4.4 Effect of PEST on yeast adenylates.

K19.10 cells in YEPD were grown at room temperature to A_{600} 1.1 and treated with PEST. At intervals, the culture was sampled for adenylates in the total-culture and the cell-free medium; adenylates in cells were calculated as described in Chapter 3. The concentration of each adenine nucleotide in the sterile growth medium is indicated by the arrows in plot *A*. Symbols: (●), ATP; (○), ADP; (□), AMP; (▲), sum of ATP, ADP and AMP. Plot *A*, adenylates in total culture; plot *B*, adenylates in cell-free medium; plot *C*, adenylates in cells. Survival of culture was 2.6 percent at plus-320 minutes.

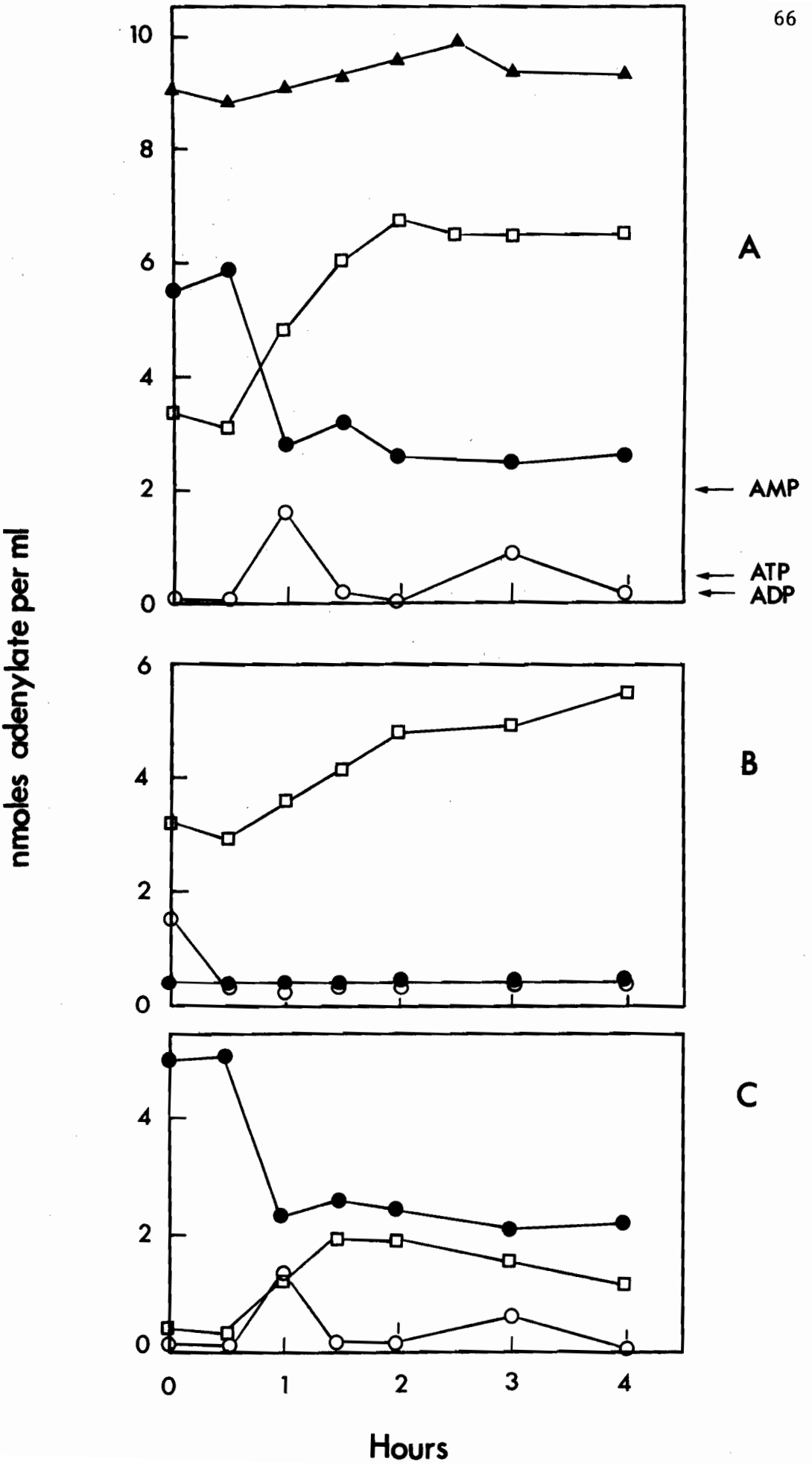
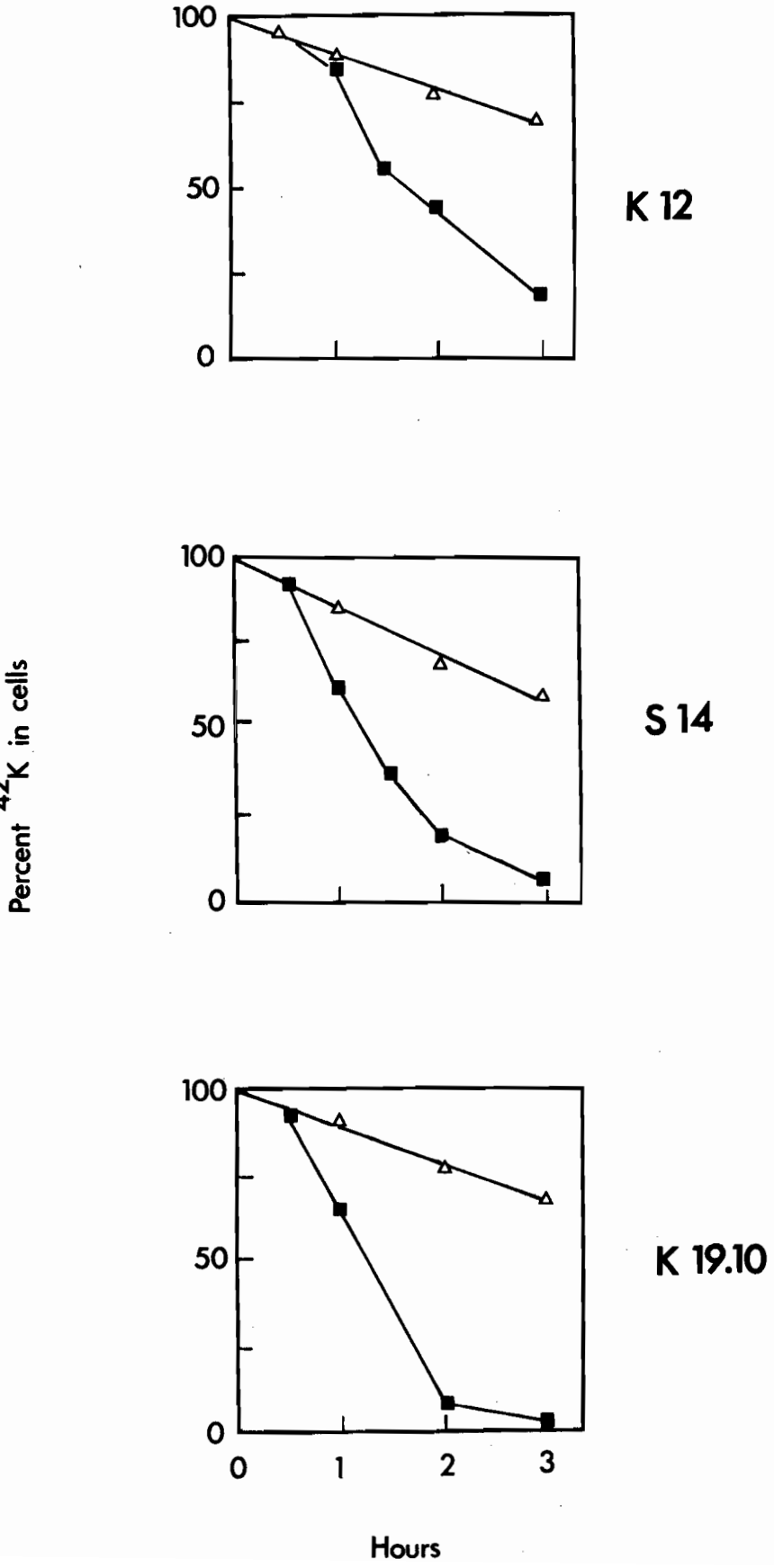

nmoles adenylate per ml
10
8
6
4
2
0
A
AMP
ATP
ADP
6
4
2
0
B
4
2
0
C
0
1
2
3
4
Hours

Figure 4.5 PEST induced potassium efflux.

NaYEPD cultures of *S. cerevisiae* K12, K19.10 and S14 were loaded with ^{42}K and treated at time zero with either PEST or with buffer (control); radioactivity remaining in cells was measured at intervals. Symbols: (■), plus PEST; (△), control.


100
50
0
K 12
100
50
0
S 14
Percent ^{42}K in cells
100
50
0
K 19.10
0
1
2
3
Hours

toxin, the alterations due to PEST were coincident and preceded by a delay after toxin addition (approximately 30 min; *cf.* about 60 min for killer toxin).

II. Effect of pH on PEST action

It was evident from preliminary trials that the *T. glabrata* toxin was biologically active over a considerable range of hydrogen ion concentration. Killer toxin is inactivated rapidly when incubated outside of the range pH 4.6 to 4.8 (Woods and Bevan, 1968).

PEST was stable at room temperature from pH 4 to 6; at pH 3, and to a lesser degree at pH 7, the toxin lost activity. These results are shown in Table 4.1: the toxin was incubated in the buffer components of CB-YEP medium (General Materials and Methods) at pH values from about 3 to 7 and at intervals samples were adjusted to pH 4.7 and assayed for toxic activity on pH 4.7-buffered seeded-plates. The validity of using seeded plates to measure the concentration of biologically-active PEST is illustrated in Figure 4.6.

When cells of strain K19.10 were grown in CB-YEPD media at pH values from about 3 to 7 and then challenged with PEST at the pH at which they had been grown, they were sensitive over the range 3 to 5.5. Maximum sensitivity was seen at pH 4.2, and the cells were resistant at pH 6 to 7 (Figure 4.7, clear circles). To check for the possibility that the pH-dependence of sensitivity to PEST reflected a pH-dependence of the ceullular synthesis of receptors and/or the toxin-target, strain K19.10 was grown at pH 4.7 and then treated with PEST over the range pH 3 to 7. The toxin sensitivity of cultures was dependent on the pH

Table 4.1 Effect of pH on PEST stability.[a]

pH	3.4	4.2	4.7	5.2	5.7	7.0
time (hours)						
0	100[b]	100	100	100	100	100
1	20	100	100	100	100	100
2	10	100	100	100	100	60
3	10	100	100	100	100	60

[a]PEST was diluted at time zero with citrate-phosphate buffer to the appropriate pH; at the times indicated, a portion of each solution was diluted with pH 4.7 buffer and assayed, in duplicate, for activity by the seeded-plate assay (General Materials and Methods).

[b]Denotes the percent activity remaining; time zero samples (PEST diluted to the appropriate pH, then immediately diluted to pH 4.7) contained equal toxic activity.

Figure 4.6 The seeded-plate assay for PEST activity: relation between dose of toxin and zone of inhibition.

A sample of PEST was diluted serially with 0.1 M sodium acetate buffer, pH 4.7, and the toxic activity at each dilution measured by the well test on agar plates seeded with strain S6 (General Materials and Methods). The data represent the averages of duplicate assays. Zone diameter of wells (no toxin added) was 13 mm.

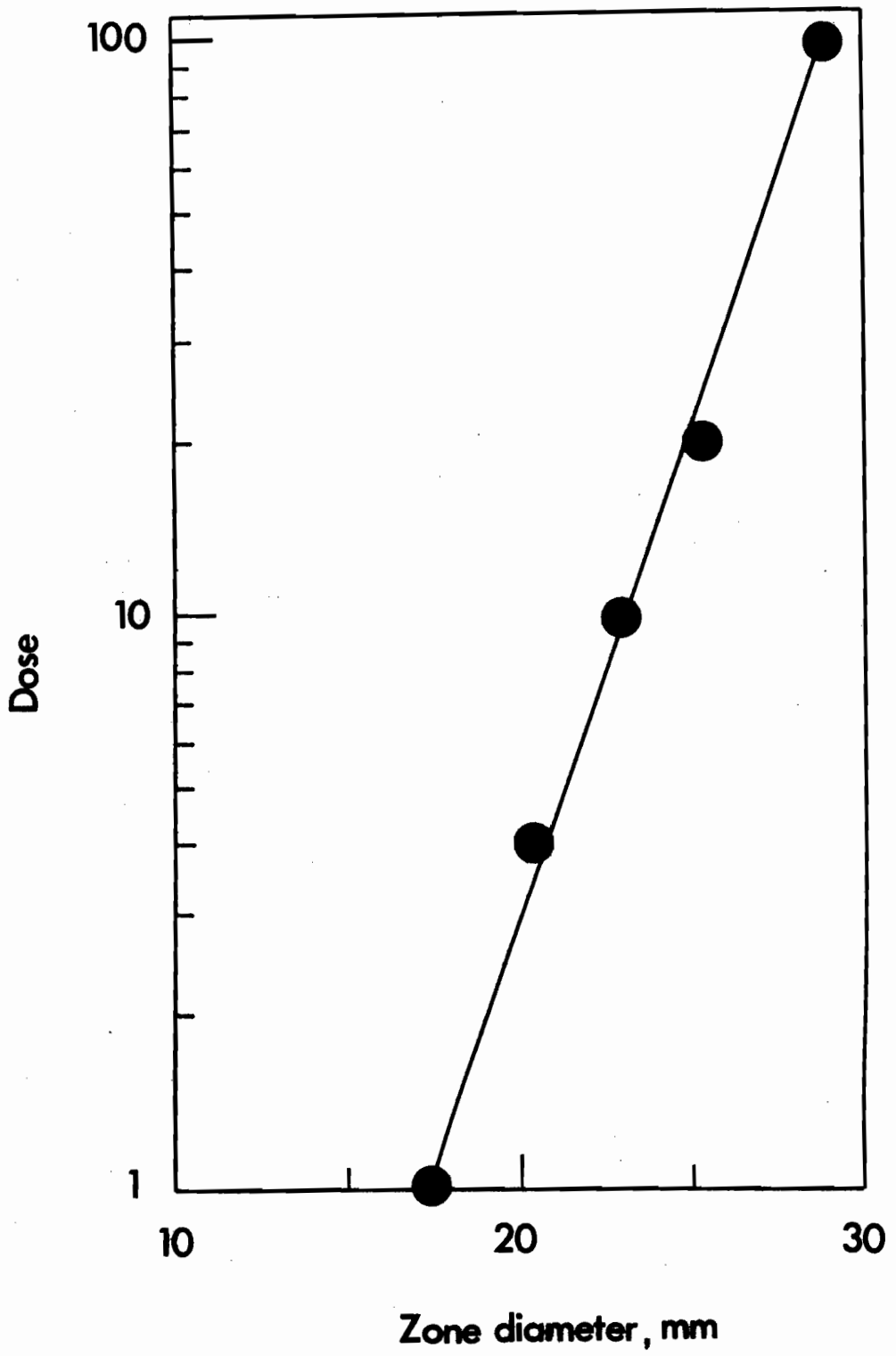
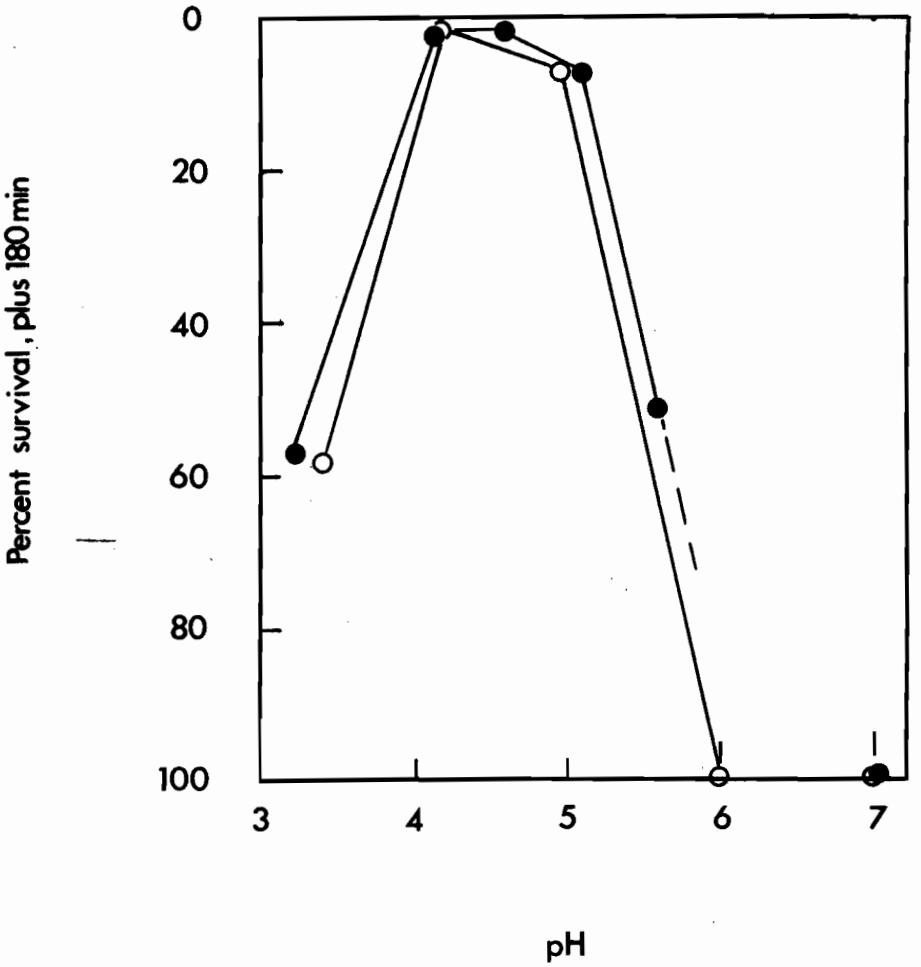

100
10
1
Dose
10
20
30
Zone diameter, mm

Figure 4.7 Effect of pH on PEST action.

Experiment 1 (●): K19.10 cells were grown in pH 4.7 YEPD, washed, resuspended in CB-YEPD media at a range of pH values, incubated with PEST at room temperature and sampled at plus-3 hours for viability on CB-YEPD-agar at the pH at which they had been incubated with toxin.

Experiment 2 (○): K19.10 cells were grown in CB-YEPD at a range of pH values, treated with PEST at room temperature and sampled at plus-3 hours for viability on CB-YEPD-agar at the pH of toxin treatment.


Percent survival, plus 180 min
0
20
40
60
80
100
3
4
5
6
7
pH

in the same way as was the case when the cultures had been grown at the various pH values (Figure 4.7, filled circles).

One interpreation of these results is that the dependence of the toxin-cell interaction on hydrogen ion concentration reflects a reversible inactivation of toxin activity at certain pH values and so is a function of toxin structure. An alternative explanation is that the toxin-cell interaction *per se* is pH-sensitive, between pH 4 and 7.

III. Effect of temperature increase on PEST action

As was true for pH, variation in temperature did not affect PEST activity to the extent that it affected the killer toxins'. At 30°C, close to optimum for maximum growth rate of *S. cerevisiae*, PEST retained its potency. Killer toxin is inactivated rapidly when incubated in normal buffered media at temperatures above 25°C (Woods and Bevan, 1968).

Increasing the temperature at which sensitive cultures were treated with PEST increased the rate of killing, the extent of killing, abbreviated the lag-period, andresulted in the appearance of arginine-derived pool-efflux at a time coincident with the onset of the other events due to the toxin.

Survival curves

Figure 4.1 shows the survival of K19.10 cultures treated with PEST at either 22 to 24°C or at 30°C. The temperature increase both stimulated the rate of loss of culture viability and increased the fraction of the population susceptible to lethal damage.

The lag-period

In Chapter 5, it is suggested that the lag-period between

additions of toxins to sensitive cultures and the onset of metabolic changes in the cells is a measure of the interval between the binding of a potentially-lethal dose of toxin to cells and the completion of a subsequent process that results in the lethal event. At 22 to 24°C the lag-period for PEST action on K19.10 cells was approximately 30 minutes; at 30°C it was less than 10 minutes. Figure 4.8 shows this effect in terms of the toxin-induced change in suspension-turbidity, Figure 4.9 in terms of the ^{42}K-release assay. Perhaps the least equivocal measure of the difference between the lag time before toxin-specific potassium efflux is the time needed for cellular ^{42}K to fall to 50 percent of its initial value: at 22 to 24°C this half-time was about 75 minutes, at 30°C it was about 21 minutes (see Figure 4.9; this figuring of half-times ignores the proportion of ^{42}K-loss due to the "exchange" seen in the untreated portions of cells).

Arginine-pool efflux

At 22 to 24°C, PEST caused a release of [^{14}C] arginine-derived cellular pools, but not a release of macromolecules, from cells of strain K19.10. As was the case with this event in killer toxin-treated K19.10 cells (Figure 3.5, page 36), this effect was "late" relative to effects such as ^{42}K release (data not given). At 30°C, PEST-induced arginine pool efflux was an early event: by plus-60 minutes, virtually all of the arginine-derived pools were found in culture supernatant fluids (Figure 4.10, "wash" fraction, plot *A*; see also the change in the "pools"). Although the onset of this efflux was not timed accurately, it appeared that the efflux was initiated, like that of potassium (Figure

Figure 4.8 Effect of temperature increase on the lag-period in PEST action. I. Culture absorbance assay.

K19.10 cells were grown in YEPD at either 22 to 24°C or 30°C and treated at time zero with either PEST or buffer (control) at the temperature at which they had been grown. Culture absorbance was then measured over time. Plot *A*: 30°C; plot *B*: 22 to 24°C. Symbols: (●), plus PEST; (○), control.

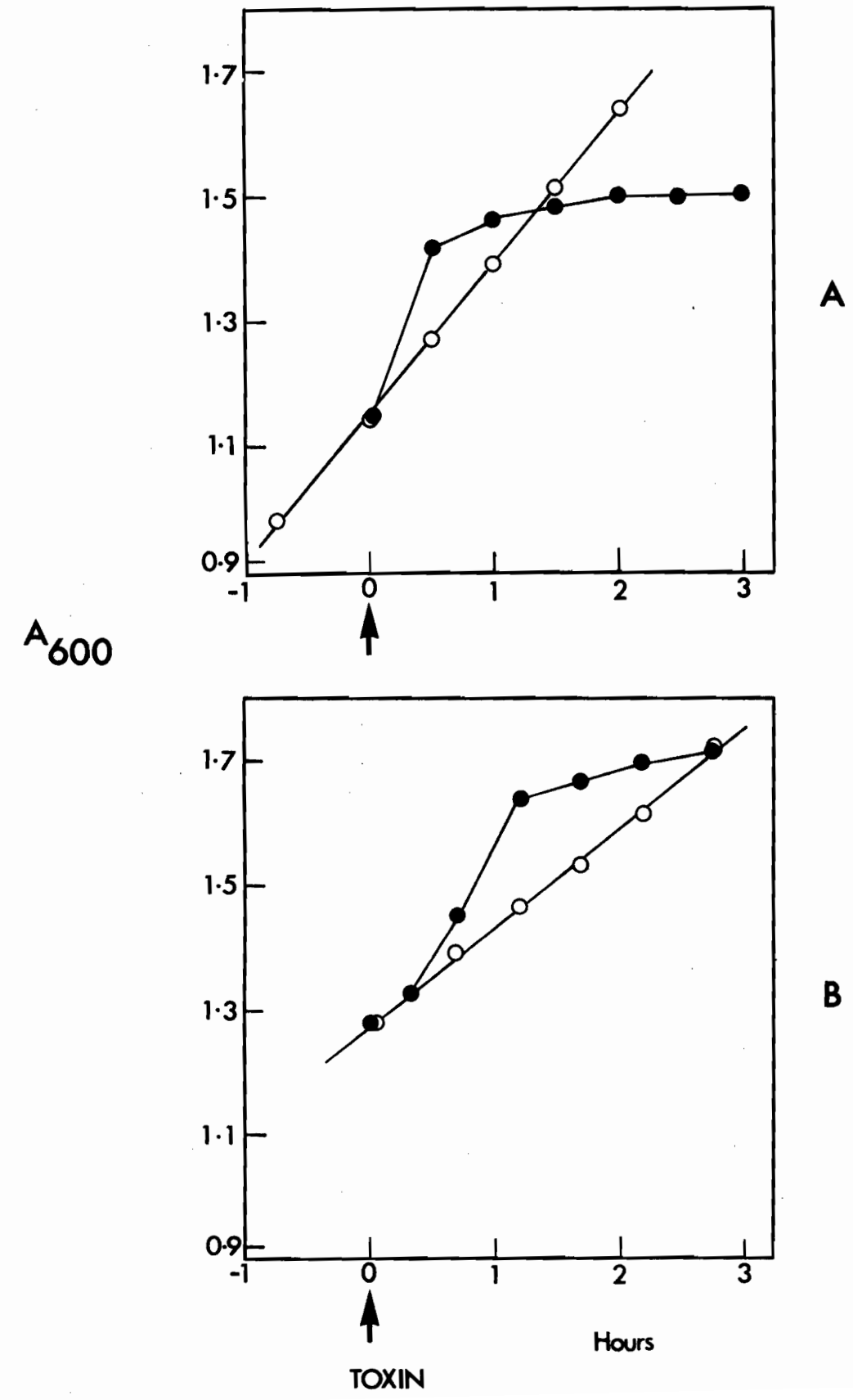
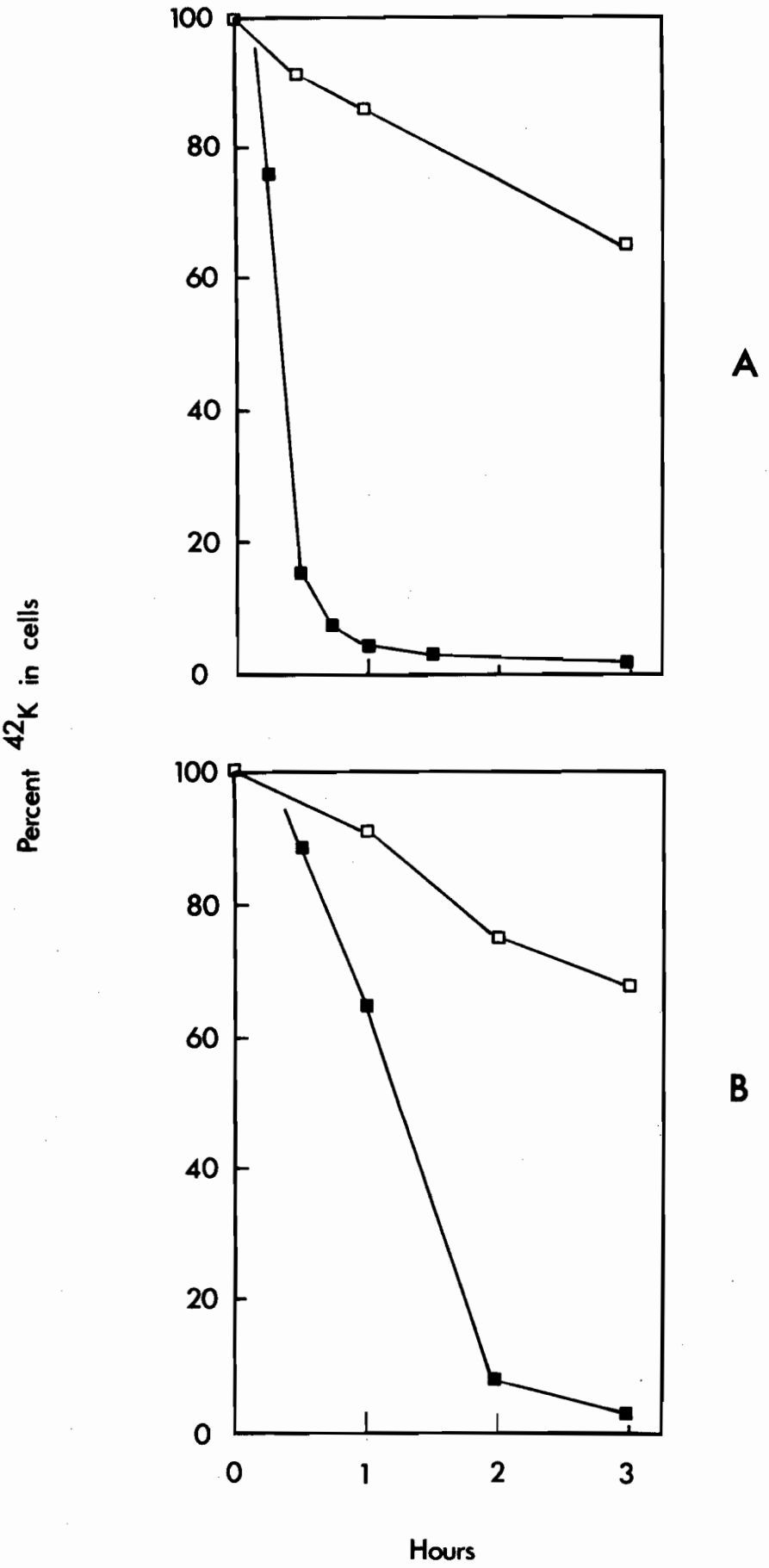

1·7
1·5
1·3
1·1
0·9
-1
0
1
2
3
A
A600
B
Hours
TOXIN

Figure 4.9 Effect of temperature increase on the lag-period in PEST action. II. Potassium-efflux assay.

K19.10 cells were grown and loaded with ^{42}K in NaYEPD at either 22 to 24°C or 30°C, and treated at time zero with either PEST or with buffer (control) at the temperature at which they had been grown. Radioactivity retained by cells was measured, at intervals. Plot *A*: 30°C; plot *B*: 22 to 24°C. Symbols: (■), plus PEST; (□), control.


100
80
60
40
20
0
A
100
80
60
40
20
0
B
0
1
2
3
Hours
Percent ^{42}K in cells

4.9, plot A), within a few minutes of toxin addition (Figure 4.10). Cellular macromolecules labeled with [^{14}C] arginine prior to treatment of cultures with PEST were not released from the cells in response to toxin at 30°C (Figure 4.10, "macromolecules" fraction, plot A). So PEST action, although accelerated by temperature increase, remained selective with respect to the sort of damage it caused to membrane permeability (the name pool efflux-stimulating toxin (PEST) was prompted by the results of these trials done at 30°C). Figure 4.10 also illustrates the resistance of K19.10 cells to PEST at pH 7.0.

In all further experiments done with PEST, note was taken of the actual "room temperature", since the lag-period was clearly very sensitive to temperature; room temperature is specified in the figures and tables, where appropriate.

IV. Dose independence of lag-period

Variation of the dose of killer toxin by a factor of 50 did not alter the lag-period characteristic of the onset of potassium efflux from cells (Chapter 3). Similarly, a 20-fold variation in the dose of PEST did not result in any obvious variation in the delay before postassium release in cells of strain K19.10 (Figure 4.11). In the trial illustrated (room temperature 26 to 27°C), the lag was approximately 10 minutes, irrespective of toxin dose.

Discussion

Table 4.2 is a summary of the effects of killer toxin and PEST on yeast strains at 22 to 24°C and at pH 4.6 to 4.8; it includes the data shown in this Chapter and in Chapter 3, some data not shown here, the

Figure 4.10 Action of PEST on [^{14}C] arginine-derived cellular components, at 30°C.

A 20 ml NaYEPD culture of strain K19.10 (30°C; A_{600} 0.8) was supplemented with 6.2 microcuries of [^{14}C] L-arginine and 5 x 10^{-4} M L-arginine and incubated at 30°C to A_{600} 1.2. The culture was divided into two equal portions and the cells harvested. One portion (cells 1) was washed with pH 4.0 medium, the other (cells 2) with pH 7.0 medium, until the supernatant fluids contained background radioactivity (pH 4.0 medium was pH 4.0 sodium phosphate buffer, 2 percent w/v glucose, 5 x 10^{-4} M arginine; pH 7.0 medium was pH 7.0 sodium phosphate buffer, 2 percent w/v glucose, 5 x 10^{-4} M arginine). The cells were then suspended in either pH 4.0 or pH 7.0 medium (cells 1, cells 2, respectively), treated at time zero with either PEST or water (control), and incubated at 30°C. At intervals each suspension was sampled for radioactivity in the wash, macromolecule and pool fractions (General Materials and Methods). Plot *A*: cells 1 plus PEST; plot *B*: cells 1 plus water; plot *C*: cells 2 plus PEST; plot *D*: cells 2 plus water. Symbols: (■), macromolecules; (x), wash; (O), pools.

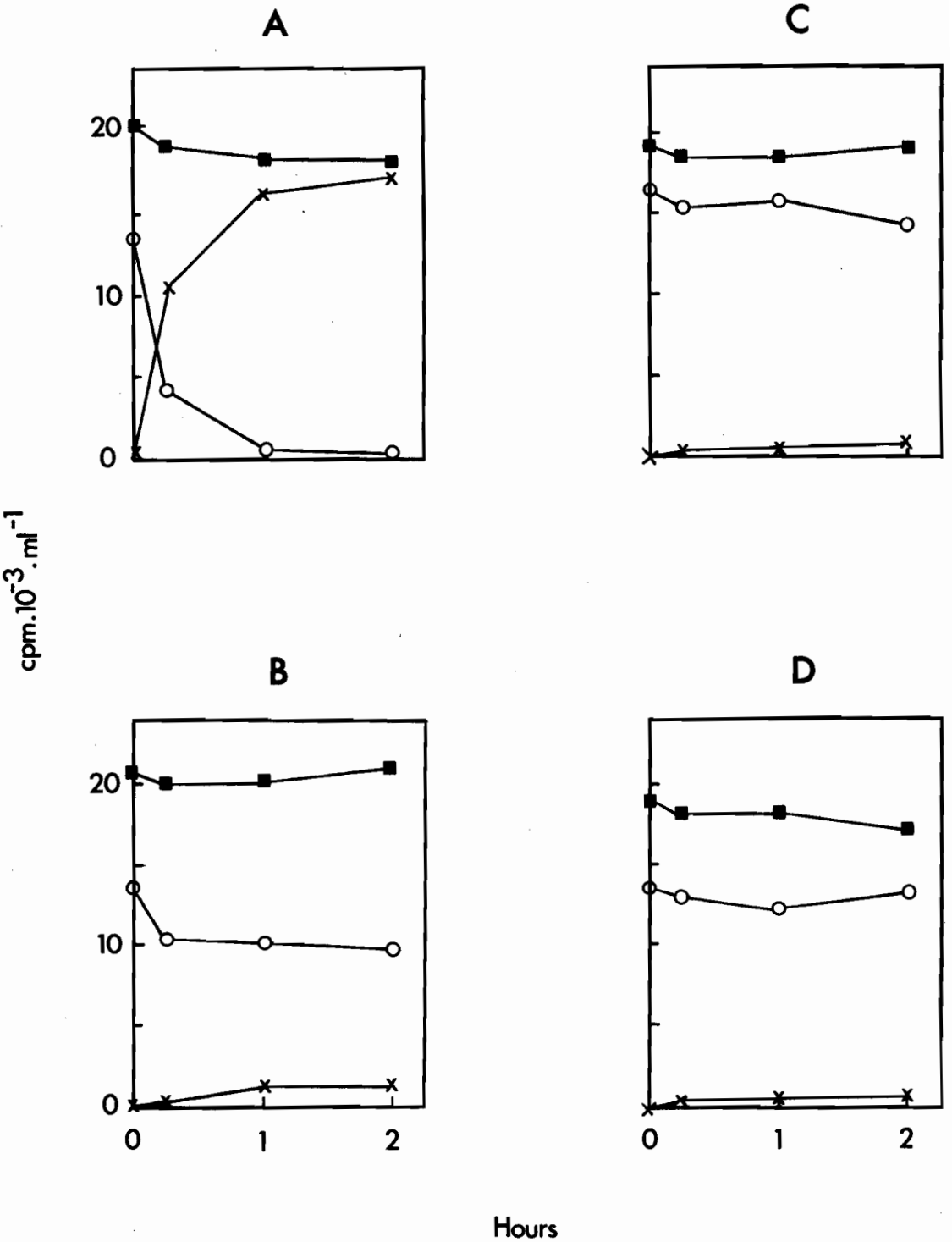
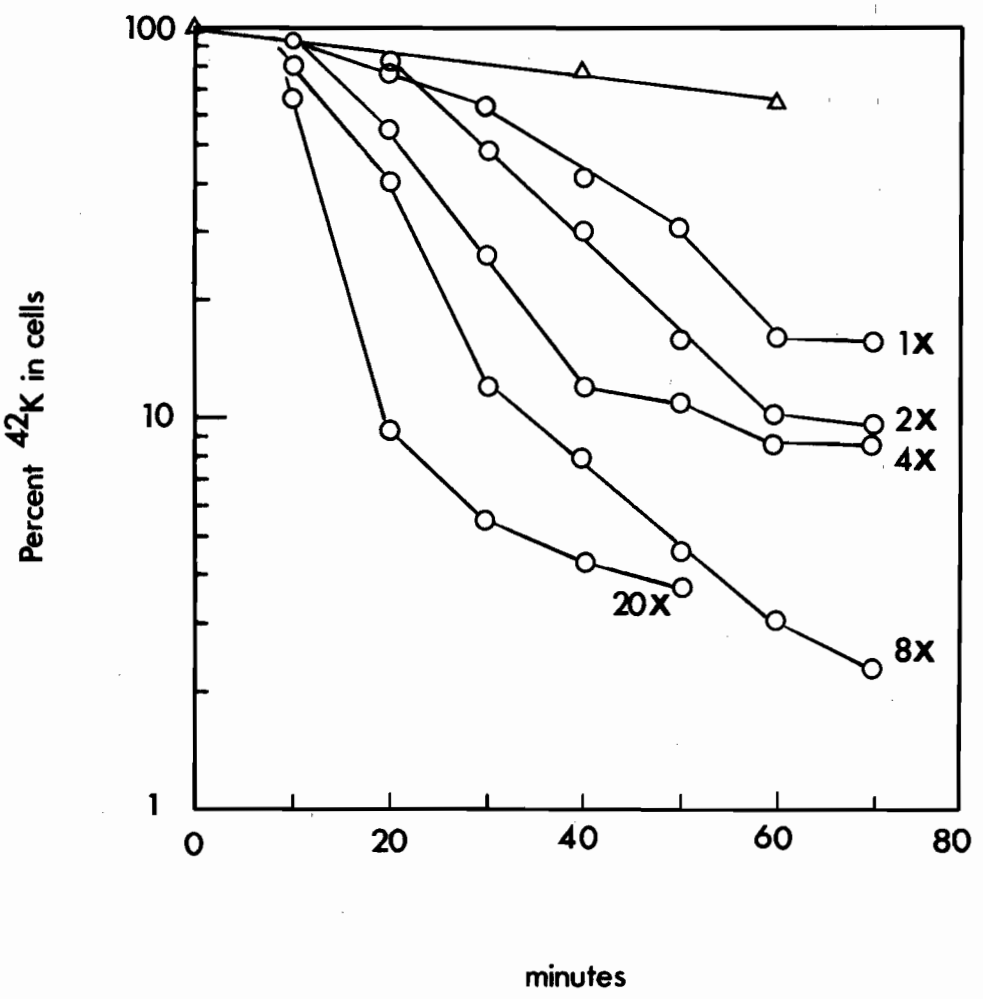

A
C
B
D
20
10
0
0
1
2
cpm.10^{-3}.ml^{-1}
Hours

Figure 4.11. Dose-independence of the lag-period associated with PEST action.

K19.10 cells growing in NaYEPD were loaded with ^{42}K, washed and resuspended with NaYEPD and treated at time zero with either PEST or with buffer (control). At intervals, radioactivity remaining in cells was measured. Symbols: (Δ), control; (○), plus PEST, at dose indicated on Figure. Toxin at 1X was sufficient to kill approximately 98 percent of the cells by plus-70 minutes. Room temperature was 26 to 27°C.


100
10
1
0
20
40
60
80
Percent ^{42}K in cells
minutes
1X
2X
4X
8X
20X

results published by Bussey and Skipper (1976) and some results on strain S14 from the work of Bussey and Sherman (1973).

At pH 4.6 to 4.8 and at 22 to 24°C, the effects of *Torulopsis* toxin on sensitive strains resembled closely those of the killer toxin. Both antibiotics resulted in inhibition of synthesis and uptake of precursors, depletion of cellular pools of ATP and ^{42}K and a culture absorbance increase, and in both cases the lag-period between addition of the toxin and the onset of damage (assessed by ^{42}K release) was independent from the dose of toxin. The only obvious distinction that could be made between the toxins' effects was in the response of yeast adenine nucleotides. In killer toxin treated K19.10 cultures, cellular ATP depletion was accounted for by leakage to the growth medium and by hydrolysis to AMP and ADP; all three adenylates were found in the suspension fluids following toxin treatment (Chapter 3). In PEST treated K19.10 cultures, the ATP reduction in cells was accounted for by an increase in AMP with the nucleotide appearing in the suspension fluids. It is not obvious why the remaining ATP in PEST-treated cultures was not also lost to the fluids -- perhaps the charge difference between the monophosphate and triphosphate forms of the nucleotide is sufficient to determine its passage through the damaged plasma membrane. A further possibility is that the ATP to AMP reaction in PEST-treated cells (if this is in fact the reaction being measured) is due to an enzyme that spans the membrane, hydrolysing ATP at the cytoplasm to AMP in the medium. This proposal is purely speculative.

The stability of PEST allowed an examination of the conditions

affecting culture sensitivity relatively independent of alterations to toxin activity *per se*. From the limited number of trials done it was clear that alterations to both the pH and the temperature at which cultures were treated affected the toxin's effects. Increasing the temperature increased the rate at which cultures lost their viability and apparently the probability that cells that had bound a potentially-lethal dose of toxin would go on to die. Altering the pH resulted in a marked change in culture susceptibility to PEST, a change that appeared to reflect a requirement of the toxin:cell reaction rather than the structure of the toxin (at least between pH 4 and 7). The nature of this pH-sensitivity is difficult to investigate in the absence of a more simple system for examining the specific components necessary to PEST action, but the result shown for cells should provide a guide for future work on PEST action.

Killer toxin-producing strains K12 and K19 were fully sensitive to PEST, as assessed by ^{42}K efflux and culture-absorbance change. If it is assumed that the "biochemical target" of killer toxin and PEST is similar then the immunity of K12 and K19 to killer toxin is presumably not related to an altered target. It is known that strain K12 "binds" as much killer toxin as does sensitive strain S14 (by the activity-removal assay; Bussey, Sherman and Somers, 1973), so it is possible that the immunity is due to modification to the (hypothetical) process that transmits the effects of the initial event, binding, to the target -- the plasma membrane.

In summary, the results suggest that the PEST, like killer toxin,

kills yeast cells by damaging the capacity of the plasma membrane to retain cytoplasmic pools of necessary metabolites and ions. The effects of both toxins can be explained as either being alternative measures of this alteration (^{42}K loss, metabolite loss, alteration to precursor uptake-systems) or consequences of it (inhibition of macromolecule synthesis, loss of viability and, perhaps, ATP hydrolysis -- see General Discussion).

Table 4.2 Summary of effects of killer toxin and PEST on yeast strains[a].

	Killer Toxin					PEST				
Strain	K19.10	S14	TG[b]	K19	K12	K19.10	S14	TG[b]	K19	K12
Effect[c]										
Cell death	✓	✓	✓	X	X	✓	✓	X	✓	✓
Inhibition of macromolecule synthesis	✓	✓[d]	✓	ND	ND	ND	✓	ND	ND	ND
Interference with precursor uptake	✓	✓[d]	✓	ND	ND	ND	✓	ND	ND	ND
Transient absorbance increase	✓	✓[d]	✓	X	X	✓	✓	ND	✓	ND
Depletion of cellular ATP	✓	✓	✓	ND	ND	✓	✓	ND	ND	ND
Efflux of ATP to medium	✓	✓	✓	ND	ND	X	X	ND	ND	ND
Efflux of AMP to medium	✓	✓	ND	ND	ND	✓	✓	ND	ND	ND
Efflux of ADP to medium	✓	✓	ND	ND	ND	ND	X	ND	ND	ND
Efflux of ^{42}K to medium	✓	✓	✓	X	X	✓	✓	X	✓	✓
Efflux of [^{14}C]-derived pools to medium	✓	ND	ND	ND	ND	✓	ND	ND	ND	ND

Table 4.2 - continued

[a]Cells treated with toxin at pH 4.6 to 4.8 and 22 to 24°C.

[b]*T. glabrata*.

[c]All effects, within a given toxin-strain combination, are approximately coincident, except for: (1), culture death, which is seen to proceed continuously from the time of addition of toxin to cultures and (2), [^{14}C] arginine-derived pool efflux, which is initiated at approximately plus-120 min following toxin addition.

[d]Bussey and Sherman (1973).

KEY: ✓ denotes effect;

X denotes absence of effect;

ND means not determined.

Chapter 5

SENSITIVITY OF TOXIN ACTION TO CONDITIONS THAT INTERFERE WITH CELLULAR METABOLISM

Introduction

There have been several results that imply a sensitivity of killer toxin action to the physiological state of the target cells. Stationary-phase cultures are much less sensitive to toxin than actively-growing cultures (Woods and Bevan, 1968; Bussey, 1972), apparently not because of any deficiency in toxin binding (Al-Aidroos, 1975). Bussey (1972) reported that cells growing in a medium containing glycerol as the energy source were ten-fold less sensitive than cells growing in glucose-containing medium; addition of glucose to the glycerol-grown cells resulted in an increase in killing. The relative resistance of glycerol-grown cultures was confirmed by Al-Aidroos (1975) and Rogers (1976) and has been shown to be unrelated to toxin-binding efficiency (Al-Aidroos, 1975). In my own preliminary experiments with the ^{42}K-release assay for toxin action, I had found that the omission of glucose from radioactive cell suspensions suppressed considerably the toxin-specific ^{42}K leakage. Furthermore the reagent 2,4-dinitrophenol appeared to block the killer toxin-induced potassium leakage.

This Chapter contains the results of experiments done to examine the implication that sensitivity of cultures to toxins may be linked to the metabolic status of the cells, particularly the "energised state" -- the cell's capacity to generate and use metabolic energy.

Results

I. Response of toxin action to energy poisons

The mode of energy generation in cultures

Yeasts can be cultured in such a way that they generate ATP from either substrate-level phosphorylations during glycolysis or from oxidative phosphorylations during respiration in the mitochondrion.

To grow cells glycolytically, the cultures were incubated in medium containing glucose as the energy source. At sufficient concentrations, glucose suppresses mitochondrial function (.e.g. Henson *et al.*, 1968), with the result that the cells cannot oxidise the substrate to produce ATP *via* mitochondrial reactions. Strain K19.10 growing in NaYEPD medium was insensitive to potassium cyanide (inhibits cytochrome oxidase; Lardy and Ferguson, 1969) and to antimycin (prevents electron transfer from cytochrome c to b_1; Hagihara, Sato and Yamanaka, 1975) (Table 5.1); by this criterion the glucose-grown cells were not using oxidative phorphorylation to generate energy for growth.

Removal of glucose from growth medium, with the substitution of a non-fermentable substrate such as ethanol or glycerol, results in the synthesis of active mitochondria and therefore the capacity for respiration (Henson *et al.*, 1968). Strain K19.10 grew poorly when inoculated directly from YEPD slant-agar into YEPG or YEPE medium (glycerol or ethanol as energy source, see General Materials and Methods); however inoculation into YEPG containing 0.2 percent w/v glucose produced good growth and subsequent subcultures into glucose-free NaYEPG or NaYEPE were successful. Ethanol-grown K19.10 cells were evidently

Table 5.1 Survival of strain K19.10 after incubation with killer toxin and drugs.

Additions to culture[a]	Concn[b]	Survival[c] (%)	
		Glucose-grown culture	Ethanol-grown culture
None		235 ± 8 (3)	173 ± 7 (5)
Killer	50–60 µg of protein/ml	0.82 ± 0.58 (6)	3.4 ± 3.1 (5)
DNP	0.5 mM	57 ± 20 (3)	100 ± 5 (4)
DNP and killer		0.12 ± 0.06 (3)	1.6 ± 0.9 (4)
CCCP	0.5 mM	0.02 ± 0.02 (4)	37 ± 3 (3)
CCCP and killer		0.34 ± 0.05 (3)	13 ± 7 (3)
Iodoacetate	1 mM	0.040 ± 0.026 (4)	43 ± 17 (4)
Iodoacetate and killer		0.009 ± 0.007 (3)	4.2 ± 2.6 (3)
Cyanide	1 mM	217 ± 19 (4)	98 ± 4 (5)
Cyanide and killer		0.46 ± 0.28 (3)	35 ± 13 (3)
Antimycin	10 µg/ml	243 ± 10 (4)	107 ± 11 (5)
Antimycin and killer		0.27 ± 0.22 (4)	67 ± 8 (3)

Table 5.1 - continued

[a]Additions were made to actively growing cultures (A_{600} 1.2 to 1.3).

[b]Concentrations were as given in the first instance.

[c]Cultures were incubated at 22 to 24°C for 180 min and then plated out on either NaYEPD agar (glucose-grown cultures) or NaYEPE agar (ethanol-grown cultures). Survival is given as the mean $\pm$ standard deviation; the number of determinations is given in parentheses.

using oxidative phosphorylation to generate ATP: either antimycin or cyanide blocked their growth (Table 5.1, ethanol-grown culture). Glycerol-grown K19.10 cultures were, similarly, sensitive to the inhibitors of mitochondrial reactions (data not shown).

Toxin action in glucose-grown and ethanol-grown cultures

Ethanol-grown K19.10 cultures were killer toxin-sensitive, albeit to a lesser degree than glucose-grown cultures (Table 5.1). The difference in toxin-sensitivity is of the same order as that shown by Bussey (1972) for glycerol-grown and glucose-grown S14 cells. It was clear from measurement of toxin-induced ^{42}K-release from cells that the lag-period before metabolic alteration in respiring cultures was considerably longer than in glucose-grown cultures (e.g. Figures 5.1 and 5.2, plots *A*). Survival measurements of respiring and glycolysing K19.10 cultures in the presence of PEST were not made, but the ^{42}K-release from the cells due to the *T. glabrata* toxin suggested that the toxin's action was similar in the two types of culture (Figures 5.3 and 5.4, plots *A*).

The effect of energy-poisons on toxin action

The following drugs were used in experiments designed to measure the response of toxin action to the cell's energised state: antimycin, cyanide, iodoacetic acid (IAA), 2,4-dinitrophenol (DNP) and carbonylcyanide m-chlorophenylhydrazone (CCCP).

Antimycin and cyanide, as discussed, specifically interfere with mitochondrial ATP-production, by blocking the function of the electron-

Figure 5.1 Potassium release in glucose-grown cells treated with killer toxin and drugs.

^{42}K-labeled cultures of strain K19.10 in NaYEPD were incubated at time zero with killer toxin, killer toxin plus drug, the drug alone, or without additions and sampled at intervals for radioactivity remaining in the cells.

Concentrations: antimycin, 10 µg per ml; cyanide, 1 mM; DNP, 0.5 mM; CCCP, 0.5 mM; iodoacetate, 1 mM.

Plot *A*. (△), untreated cells; (□), cells plus toxin; (●), cells plus toxin and cyanide; (⊗), cells plus toxin and antimycin; (○), cells plus cyanide; (×), cells plus antimycin.

Plot *B*. (△), untreated cells; (○), cells plus iodoacetate; (●), cells plus iodoacetate and toxin.

Plot *C*. (△), untreated cells; (○), cells plus DNP; (●), cells plus DNP and toxin.

Plot *D*. (△), untreated cells; (○), cells plus CCCP; (●), cells plus CCCP and toxin.

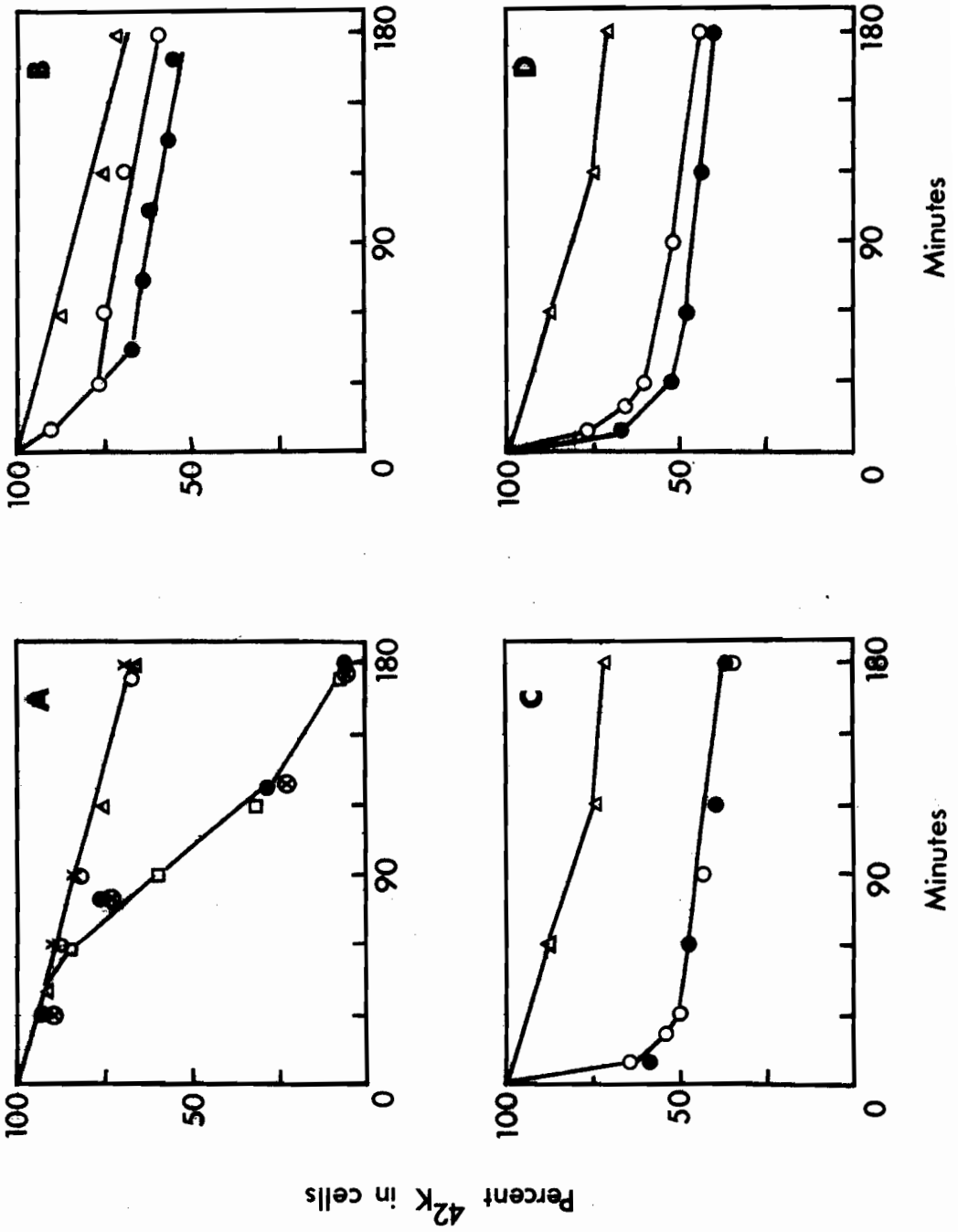

A
B
C
D
100
50
0
90
180
Minutes
Percent ^{42}K in cells

Figure 5.2 Potassium release in ethanol-grown cells treated with killer toxin and drugs.

The experiment was done as described in Figure 5.1, except that the cells were loaded with ^{42}K and incubated with additions in NaYEPE. Drug concentrations as in Figure 5.1.

Plot *A*. (△), untreated cells; (□), cells plus toxin; (○), plus toxin and antimycin; (●), plus toxin and cyanide. The data for cells plus cyanide and cells plus antimycin are given in Figure 5.4.

Plot *B*. (△), untreated cells; (○), cells plus iodoacetate; (●), cells plus iodoacetate and toxin.

Plot *C*. (△), untreated cells; (○), cells plus DNP; (●), cells plus DNP and toxin.

Plot *D*. (△), untreated cells; (○), cells plus CCCP; (●), cells plus CCCP and toxin.

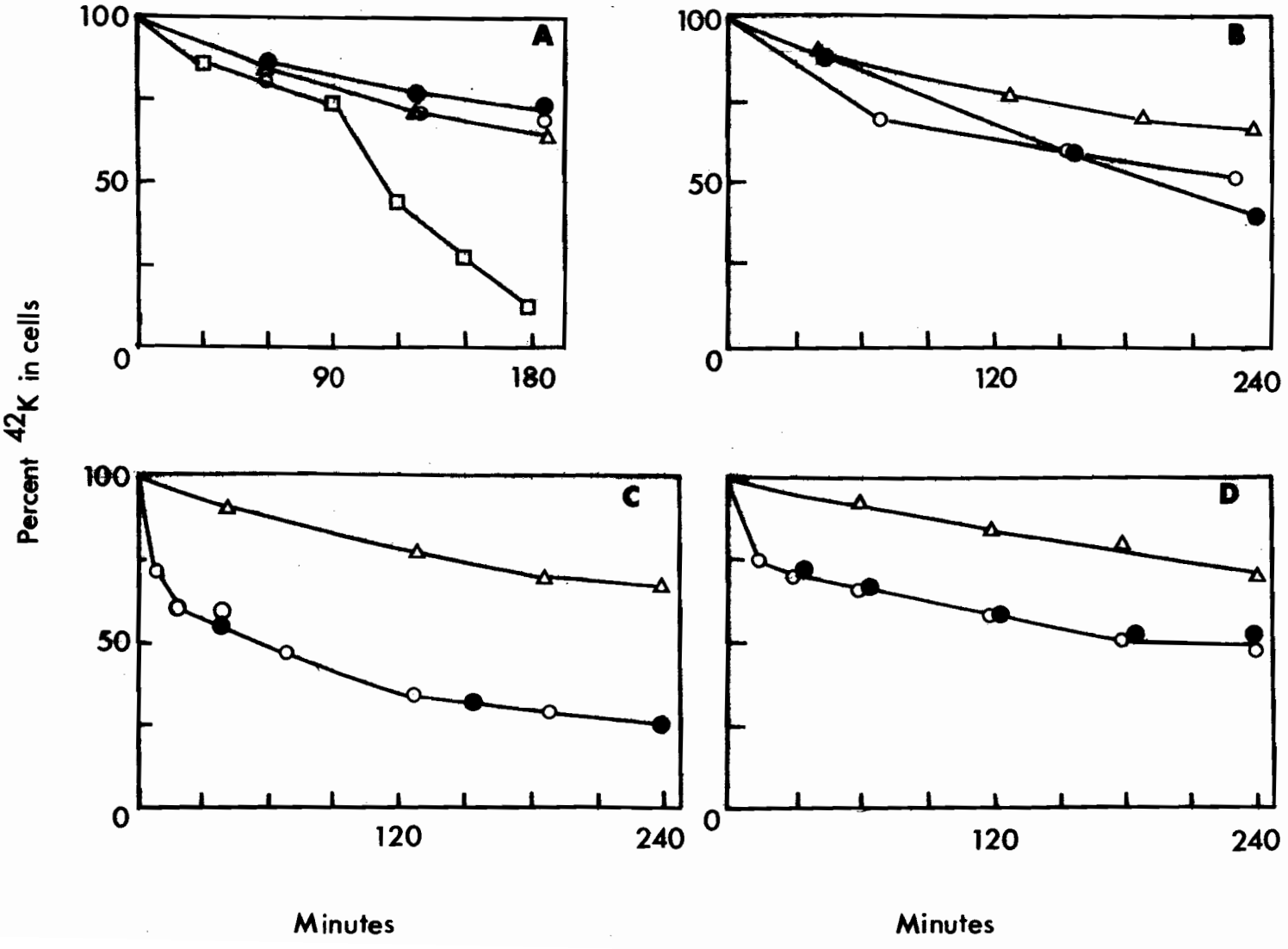

100
50
0
90
180
A
100
50
0
120
240
B
100
50
0
120
240
C
0
120
240
D
Percent ^{42}K in cells
Minutes
Minutes

Figure 5.3 Potassium release in glucose-grown cultures treated with PEST and energy-poisons.

The experiments were done as described in Figure 5.1, except that PEST was the toxin added. Drug concentrations are given in the legend to Figure 5.1. Culture survival in the experiments shown in plot *A* was about 1 percent.

Plot *A*. (△), untreated cells; (□), cells plus toxin.

Plot *B*. (△), untreated cells; (○), cells plus iodoacetate; (●), cells plus iodoacetate and toxin.

Plot *C*. (△), untreated cells; (○), cells plus DNP; (●), cells plus DNP and toxin.

Plot *D*. (△), untreated cells; (○), cells plus CCCP; (●), cells plus CCCP and toxin.

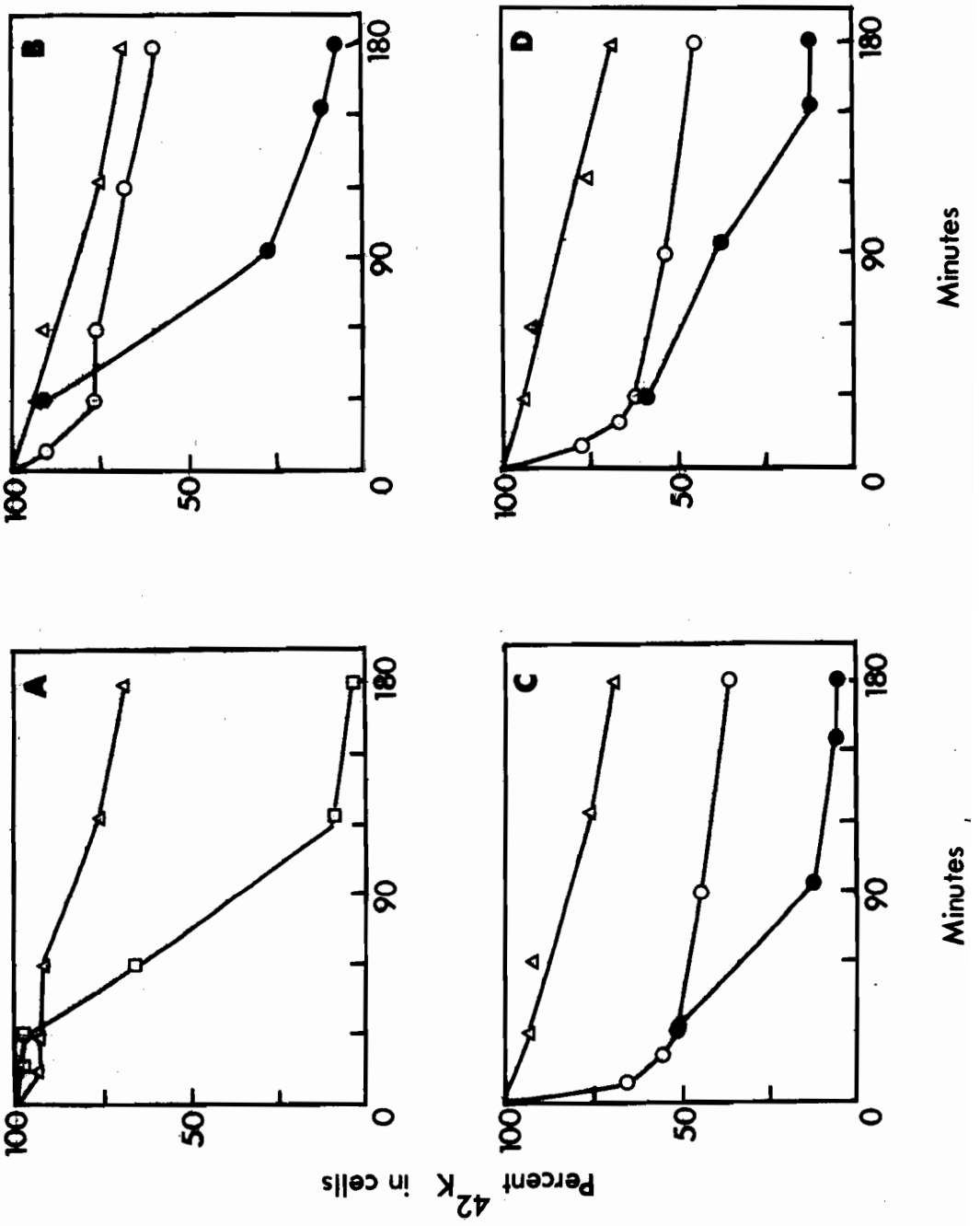

A
B
C
D
100
50
0
90
180
Minutes
Percent ^{42}K in cells

Figure 5.4 Potassium release in ethanol-grown cells exposed to PEST and drugs.

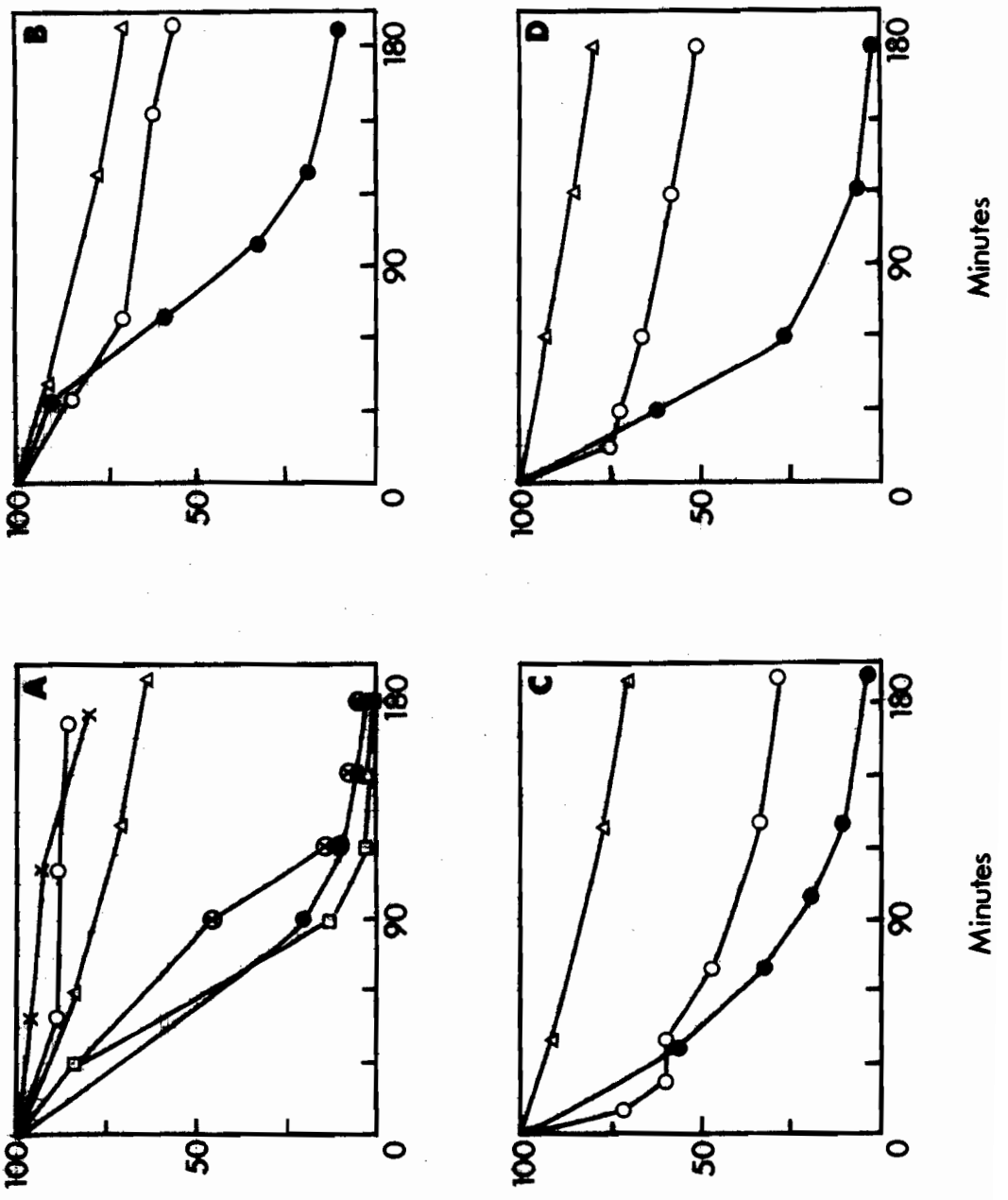
^{42}K-loaded cultures of K19.10 in NaYEPE medium were incubated at time zero with either a drug, PEST, PEST plus a drug, or with no additions, and sampled at intervals for radioactivity retained by the cells. Drug concentrations and PEST concentrations were as used in the experiments described in Figures 5.1 and 5.3.

Plot *A*. (△), untreated cells; (□), cells plus PEST; (⊗), cells plus PEST and cyanide; (●), cells plus PEST and antimycin; (×), cells plus cyanide; (○), cells plus antimycin.

Plot *B*. (△), untreated cells; (○), cells plus iodoacetate; (●), cells plus iodoacetate and PEST.

Plot *C*. (△), untreated cells; (○), cells plus DNP; (●), cells plus DNP and PEST.

Plot *D*. (△), untreated cells; (○), cells plus CCCP; (●), cells plus CCCP and PEST.


A
B
C
D
100
50
0
90
180
Minutes
Percent 42K in cells

transfer chain. Iodoacetic acid, an alkylating reagent, is known to inhibit both glycolysis, by inactivating glyceraldehyde-3 phosphate dehydrogenase (Harris, 1964) and respiration on ethanol, by inactivating alcohol dehydrogenase (Segal and Boyer, 1953). Iodoacetate is also known to cause gross physical damage to yeast membranes (Maxwell and Spoerl, 1972); however the 1 mM concentration used in the experiments described here was insufficient to alter plasma-membrane permeability, based on the retention by cells of ^{42}K (Figures 5.2 to 5.4, plots *B*). DNP and CCCP are proton-conducting reagents, best known for their property of uncoupling oxidative phosphorylation from electron-transfer in the mitochondrion (Harold, 1972; Hirose *et al.*, 1974; Heytler, 1963) but also known to affect the generation and use of metabolic energy in cells that lack the capacity to respire (Harold, 1972).

Cells of strain K19.10 were grown in either NaYEPD or NaYEPE medium, loaded with ^{42}K, and their response to toxins in the presence or absence of the energy poisons measured in terms of the ^{42}K-release assay (Chapter 3). In addition the response of the cells to each poison added alone was measured, to assess the contribution of each drug to ^{42}K-efflux.

Killer toxin-specific potassium efflux was blocked in the glucose-grown cultures when the cells had received either DNP, CCCP or IAA a few minutes before toxin (Figure 5.1). Neither cyanide nor antimycin influenced toxin-induced release of ^{42}K from glucose-grown cells (Figure 5.1). In ethanol-grown cultures, all of the energy-poisons blocked the toxin-specific ^{42}K-release (Figure 5.2). Clearly, the inhibition of

^{42}K efflux by mitochondrial inhibitors was specific to cells growing on a non-fermentable energy source. Glycerol-grown K19.10 cells responded to killer toxin in the presence of the drugs in the same manner as the ethanol-grown cells (results not shown). The inhibition of toxin action in both glucose-grown and ethanol-grown cells by DNP and CCCP (Figures 5.1 and 5.2, plots *C* and *D*) implied that the effects of these reagents were related to their action on sites common to both respiring and non-respiring cells -- perhaps proton-gradients at the yeast plasma membrane (see Discussion).

The drugs used to interfere with cellular energy, particularly the proton-conducting uncouplers, themselves affected cellular retention of ^{42}K (Figures 5.1 and 5.2) and this complicated interpretation of the effects of the drugs on toxin-induced ^{42}K-release. The results of the experiments done with PEST, described in the next paragraph, implied however that in fact none of the effects due to the drugs acting alone could mask the effect of toxins on the cellular pools of potassium.

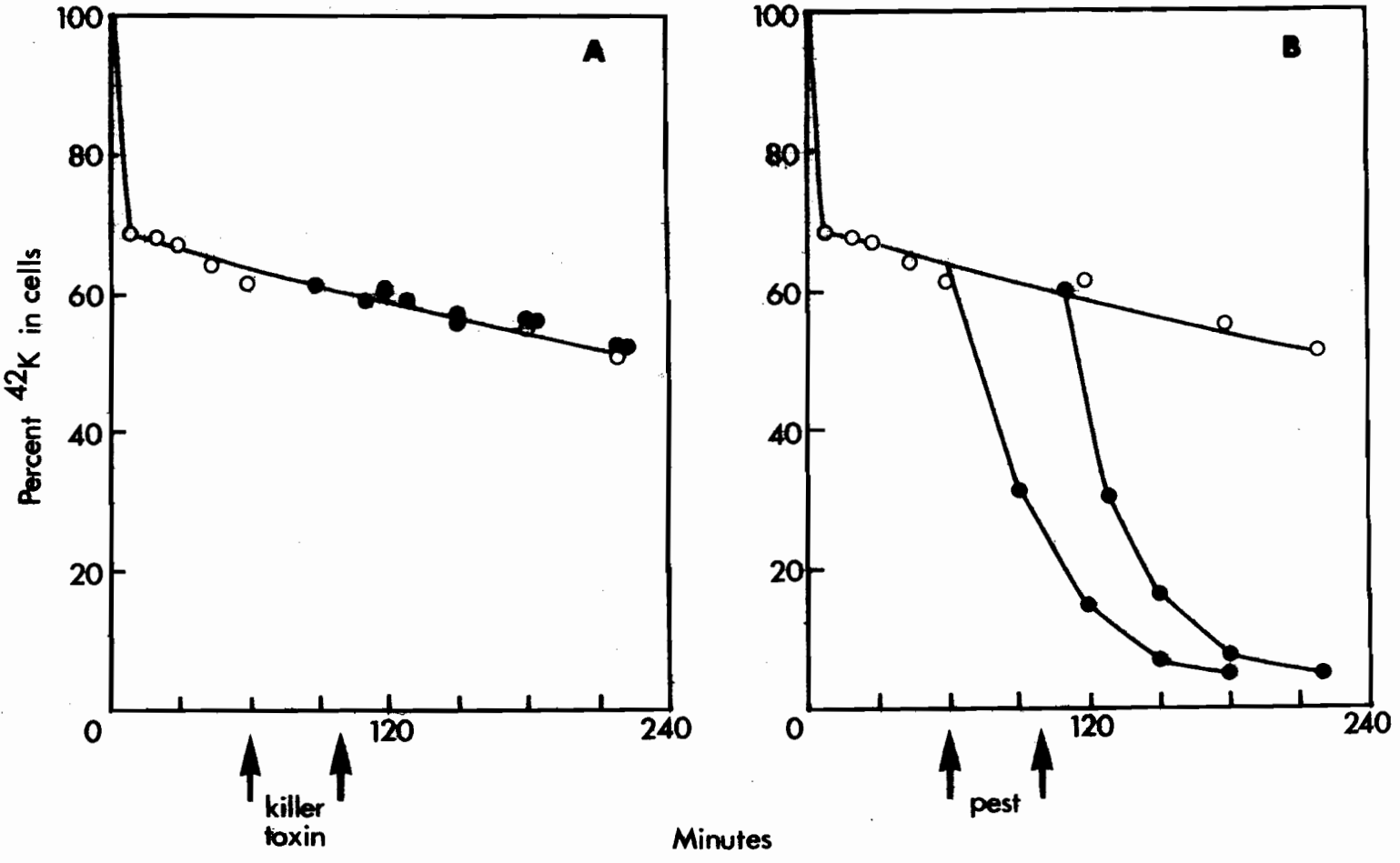
In direct contrast to the results seen in killer toxin-treated cultures, PEST-treated ^{42}K-loaded cells lost their radioactivity in the normal way in the presence of the metabolic poisons: neither DNP, CCCP, IAA, antimycin nor cyanide significantly affected the toxin-induced ion leakage (Figures 5.3 and 5.4). An experiment shown in Figure 5.5 shows with particular clarity the difference between the effect of DNP on killer toxin and PEST action on the yeast ^{42}K pools; in this trial each toxin was added to separate portions of a ^{42}K-loaded, DNP-treated K19.10 glucose-grown culture.

Figure 5.5 Potassium release in a DNP-treated culture treated with either killer toxin or PEST at intervals.

A NaYEPD culture of K19.10 was loaded with ^{42}K, treated with 0.5 mM DNP and incubated at room-temperature. At the times marked by arrows, portions of the culture received either killer toxin or PEST. All suspensions were sampled at intervals for ^{42}K remaining with the cells.

Plot *A*. (O), cells plus DNP; (●), cells plus DNP plus killer toxin at either plus-60 minutes or plus-100 minutes.

Plot *B*. (O), cells plus DNP; (●), cells plus DNP plus PEST at either plus-60 minutes or plus-100 minutes.


A
B
100
80
60
40
20
0
120
240
Percent ^{42}K in cells
killer toxin
pest
Minutes

Resumption of potassium loss from killer toxin-treated cells upon the removal of DNP

The addition of energy poisons to ^{42}K-loaded sensitive cells prevented the ion efflux characteristic of killer toxin action and this was interpreted as meaning that toxin action was blocked by the poisons. This interpretation depends on the validity of the assumption that toxin action always results in potassium efflux. That this assumption is justified was suggested by the results of an experiment in which ^{42}K-loaded cells, treated at time-zero with killer toxin and DNP, were washed at plus-40 minutes to remove the DNP (and unbound toxin) (Figure 5.6). Measurement of radioactivity showed that the washed cells lost their ^{42}K (Figure 5.6, suspension 4). The cells in suspension 4 were in a state equivalent to that described in the following section in which cells treated with DNP and killer toxin were plated for viable-counts: in the one case the washing resulted in ^{42}K efflux from the cells, in the other in loss of viability. There was no evidence, therefore, for toxin action proceeding in the absence of ^{42}K-release from cells.

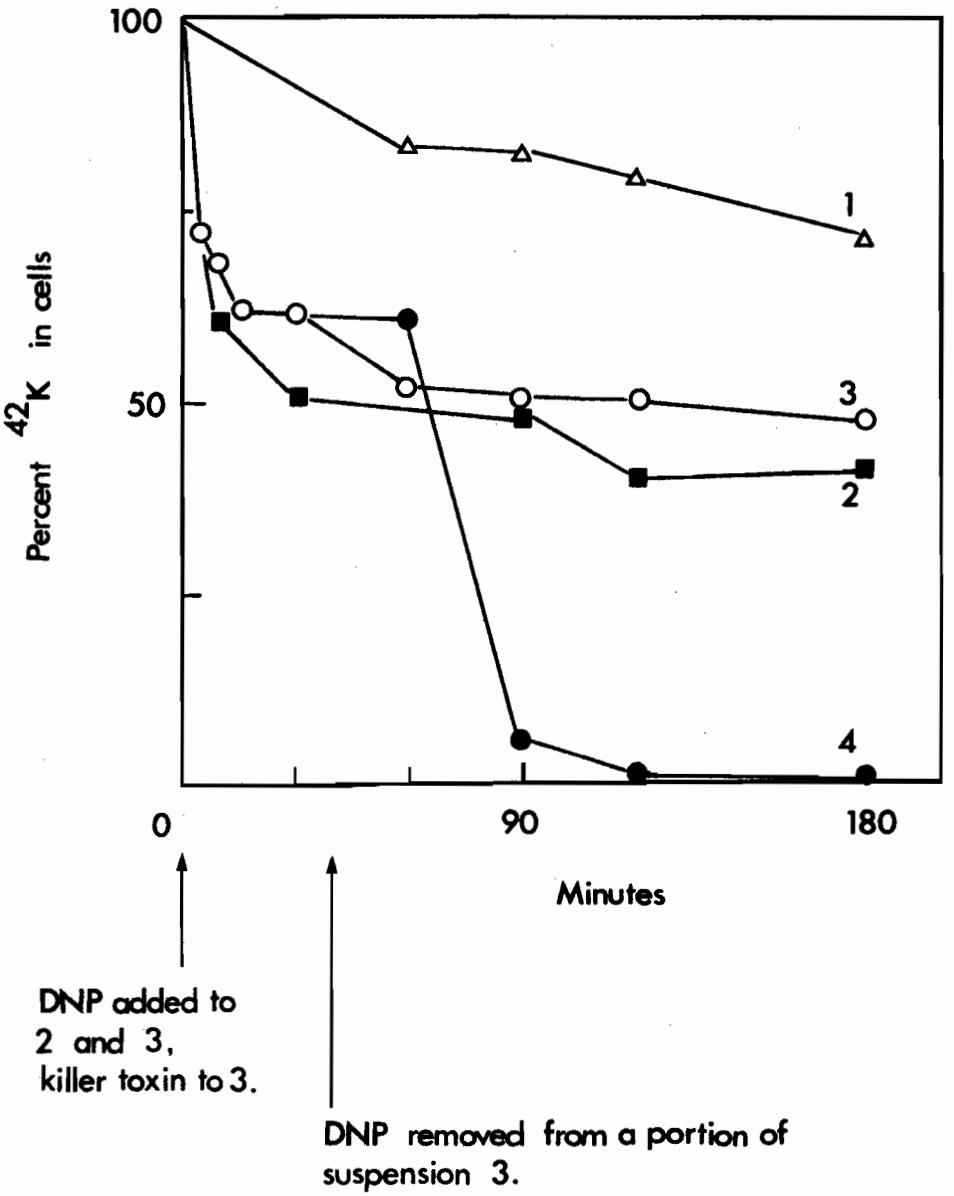
The results shown in Figure 5.6 also imply that DNP does not affect killer toxin-binding; this point will be discussed in the next section.

The stage in toxin action at which energy-poisons act

If a drug interferes with killer toxin action by preventing the binding of toxin to the cells, then its removal, under circumstances such that the cells have no further opportunity to bind toxin, should reveal that the cells have retained their viability (assuming the drug's effects

Figure 5.6 Potassium efflux in killer toxin-treated cells upon removal of DNP.

^{42}K-loaded cells of strain K19.10 were incubated at time-zero in NaYEPD with either no additions (suspension 1), 0.5 mM DNP (suspension 2), or 0.5 mM DNP and killer toxin (suspension 3) and sampled at intervals for radioactivity. At plus-40 min, 4 ml of suspension 3 was centrifuged, the cells washed once with NaYEPD and the cells resuspended with 4 ml NaYEPD (equals suspension 4). All cultures were sampled for ^{42}K in cells. Symbols: (△), suspension 1; (■), suspension 2; (○), suspension 3; (●), suspension 4.


100
50
0
90
180
Percent ^{42}K in cells
Minutes
1
2
3
4
DNP added to 2 and 3, killer toxin to 3.
DNP removed from a portion of suspension 3.

on cellular processes are reversed by its removal). If the drug interferes with some event in toxin action subsequent to binding, the removal of the drug, under the circumstances described, should show that the cells lose their viability.

Of the energy-poisons used here with K19.10 cultures, only DNP, cyanide and antimycin could be effectively removed from cells by washing -- as judged by the viability of the cultures on nutrient agar (Table 5.1: viability of glucose-grown or ethanol-grown cultures treated with DNP alone; viability of ethanol-grown cultures treated with either cyanide or antimycin, alone). CCCP or iodoacetate appeared to damage irreversibly the viability of the glucose-grown cells; in ethanol-grown cultures these reagents killed the cells to a lesser extent (Table 5.1).

In the case of DNP, the interference with killer toxin action (Figures 5.1, plot *C*; 5.2, plot *C*) appeared to be due to inhibition of an event in toxin action subsequent to binding. In both glucose-grown and ethanol-grown cultures, viable-counts of cells treated with both the toxin and the uncoupler showed that at least as much killing had taken place as in cultures treated with toxin only (Table 5.1). Assuming that the dilution for plating cells on agar had reduced unbound toxin to an insignificant concentration, this killing must be attributed to toxin that had bound to the cells in the presence of the DNP. As discussed in the previous section (see Figure 5.6), washing of DNP and toxin-treated cells also resulted in toxin-specific ^{42}K efflux.

The interference by the other drugs on toxin action is not as readily interpreted in terms of a scheme in which an energy poison

inhibits either toxin binding or a subsequent event in toxin action. The killing seen in cyanide or antimycin-treated ethanol-grown cultures when killer toxin was present was reduced considerably over that seen in cultures that had received only the toxin (Table 5.1). Based on this result, the complete suppression of killer toxin-specific ^{42}K-efflux in respiring cultures by antimycin or cyanide (Figure 5.2, plot *A*) could be due in part to an inhibition of toxin binding; direct measurement of toxin binding under these circumstances is necessary to confirm this interpretation. The effects of CCCP or iodoacetate, on toxin-induced potassium efflux in ethanol-grown cells (Figure 5.2, plots *B* and *D*) could, similarly to those of antimycin or cyanide, be due partly to effects on toxin binding and partly to effects on subsequent events in toxin action (Table 5.1).

A model for toxin action

The following simple scheme is proposed as a framework for explaining the known kinetics of toxin action and the role of metabolic energy in killer toxin action. Sensitive cells in the presence of toxin are in either of three states: state 0 in which they have yet to bind sufficient toxin for it to be potentially lethal, state 1 in which they have bound a potentially lethal dose but have yet to be damaged by it, and state 2 in which they have been damaged. In killer toxin action, but not in PEST action, the state 1 to state 2 reaction is a process sensitive to poisoning of metabolic energy.

That there are at least two distinct events in both killer toxin and PEST action is evident from an examination of survival curves and the

measurement of events such as potassium-efflux. Survival curves (Figures 3.1 and 4.1) show that there is no obvious delay between the addition of toxins to cultures and the onset of killing; measurement of "real-time" events, however, demonstrates a delay after toxin addition before any cells become affected, a lag of about 60 min in killer toxin-treated glucose-grown cultures and 30 min in PEST-treated cultures at 22 to 24°C.

The survival-curve data may be considered to be a measure of that fraction of the population in state 0 -- they measure the proportion of the population that has not reached state 1 in toxin action by the time of culture-dilution for plating. Those cells that have reached state 1 proceed to state 2 on the agar-plate and so fail to produce a colony.

The lag-period in toxin action is postulated to be the minimum interval necessary for the state 1 to state 2 reaction to be completed. In these terms, the onset of metabolic alteration in toxin-treated cultures marks the completion of the state 1 to state 2 transition in those cells that bound a potentially-lethal dose of toxin within the first few minutes of exposure to toxin. Interference with the state 1 to state 2 reaction should prevent the fraction of the population that has yet to be damaged from receiving damage: during the lag-period this fraction should be 1.0, i.e. the entire population; subsequent to the lag-period the fraction spared from receiving damage should decrease.

In the case of killer toxin, energy poisons totally suppressed toxin-induced ^{42}K release when added to cultures at the same time as the toxin (Figures 5.1 and 5.2). Figures 5.7 and 5.8 provide evidence that

Figure 5.7 Effect of adding iodoacetate or DNP to glucose-grown cells at intervals after addition of killer toxin.

Plot *A*. A K19.10 culture, loaded with ^{42}K in NaYEPD medium, was treated with killer toxin at time-zero and divided into portions. At each time marked by an arrow, iodoacetate was added to a portion to 1 mM. All suspensions were sampled over time for radioactivity retained by the cells. Symbols: (Δ), untreated cells; (□), plus toxin; (●), plus toxin and iodoacetate. The effect of adding iodoacetate alone to ^{42}K cells has been shown in Figure 5.1, plot *B*.

Plot *B*. K19.10 cultures were treated with killer toxin, as in plot *A*. At the times marked by arrows, portions received DNP to 0.5 mM. Other cultures received DNP alone, at times marked by arrows. All suspensions were sampled over time for ^{42}K retained by cells. Symbols: (Δ), untreated cells; (□), plus toxin; (●), plus toxin, plus DNP; (○), plus DNP (no toxin).

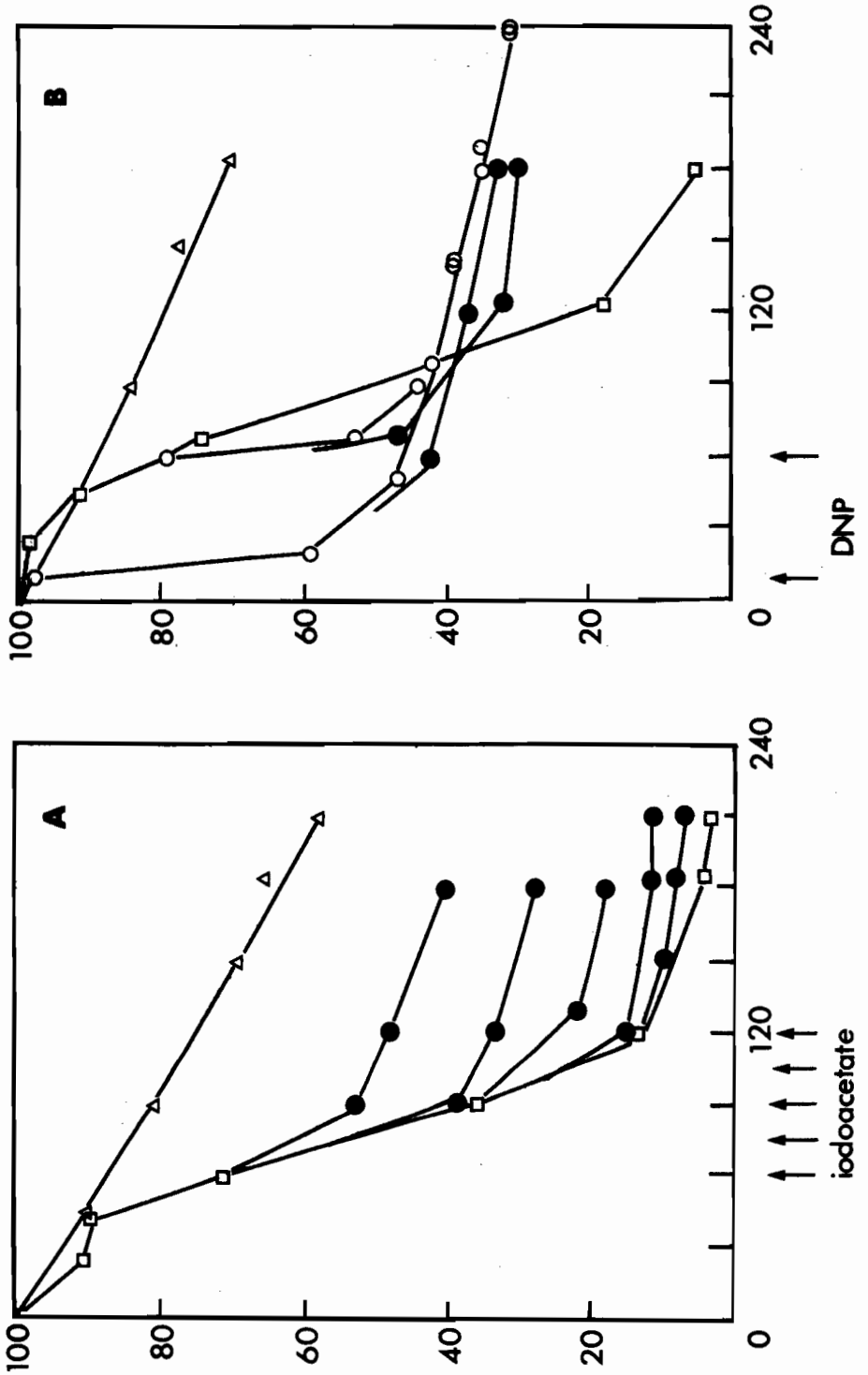
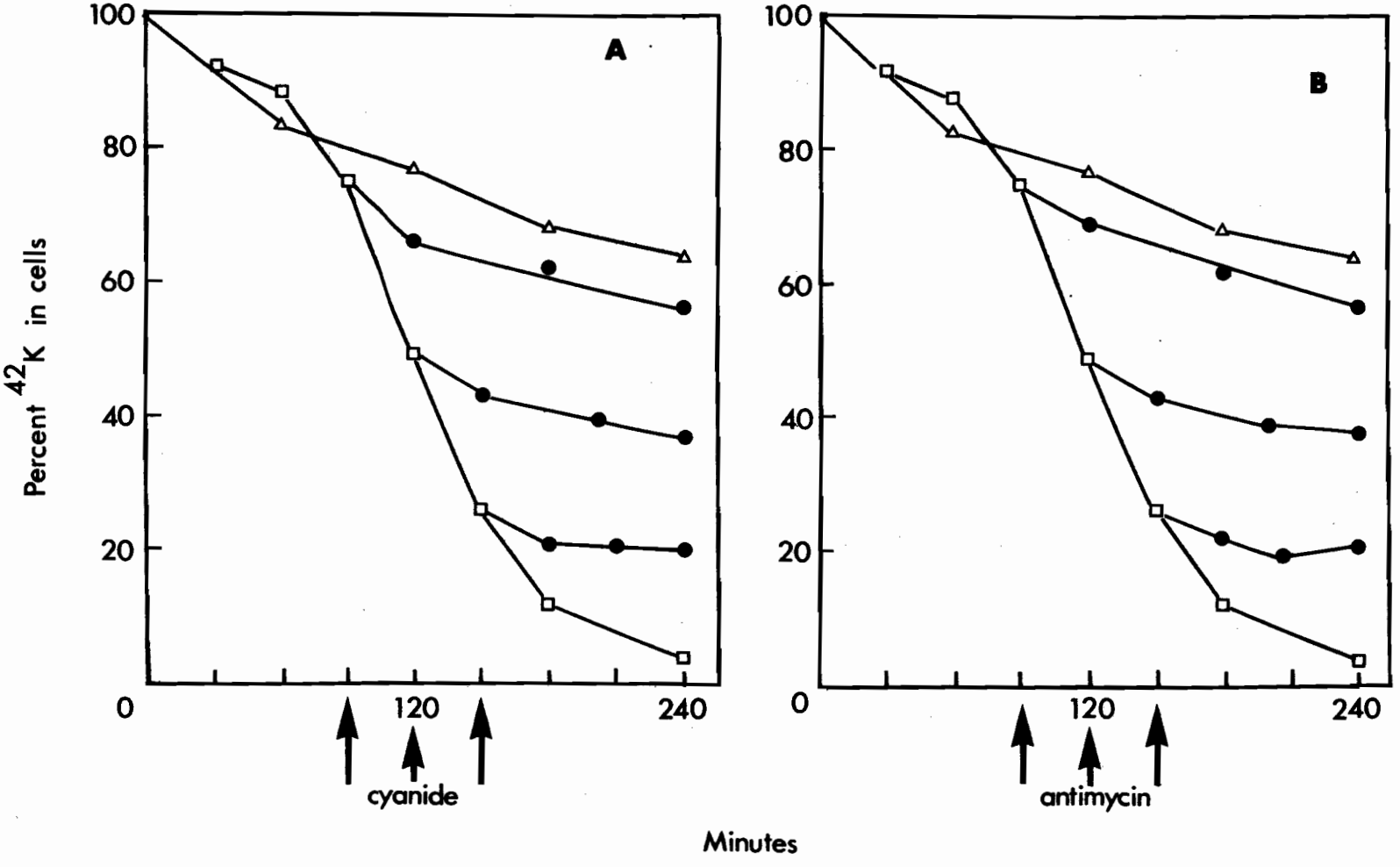

A
B
100
80
60
40
20
0
120
240
iodoacetate
DNP
Minutes
Percent ^{42}K in cells

Figure 5.8 Effect of adding cyanide or antimycin to ethanol-grown cells at intervals after addition of killer toxin.

Plot *A*. K19.10 cells, loaded with ^{42}K in NaYEPE medium, were treated with killer toxin and incubated. At times marked by the arrows, cyanide was added to a portion of the culture to 1 mM. All suspensions were sampled over time for ^{42}K retained by cells. Symbols: (Δ), untreated cells; (□), plus toxin; (●), plus toxin, plus cyanide.

Plot *B*. The experiment was done as described for plot *A*, but antimycin was added in place of cyanide. Final antimycin concentration in cultures was 10 µg per ml. Symbols: (Δ), untreated cells; (□), plus toxin; (●), plus toxin, plus antimycin.


A
B
100
80
60
40
20
0
120
240
cyanide
antimycin
Percent ^{42}K in cells
Minutes

these drugs, when added to cultures at intervals after toxin, arrested the undamaged cells in state 1 or state 0, with the fraction of the population so spared from receiving damage decreasing with time. In these experiments, portions of ^{42}K-loaded cells were treated with killer toxin at time-zero and then with either DNP or IAA (glucose-grown cultures, Figure 5.7) or with cyanide or antimycin (ethanol-grown cultures, Figure 5.8) at various intervals. To distinguish toxin action on ^{42}K release from the effects of the energy-poisons, additional portions of culture received each poison alone. Potassium-efflux due to IAA, cyanide or antimycin was not very different from that due to adding buffer to the cells (untreated cells, see legends to Figures 5.7, plot *A* and 5.8); efflux due to DNP alone was, as expected, significant and the data have been included in Figure 5.7, plot *B* (clear circles). Allowing for the effect of the energy-poisons themselves on cellular ^{42}K pools, the results of these trials are taken to mean that those cells that had yet to undergo damage (lose their ^{42}K) were arrested in this state by the poisons.

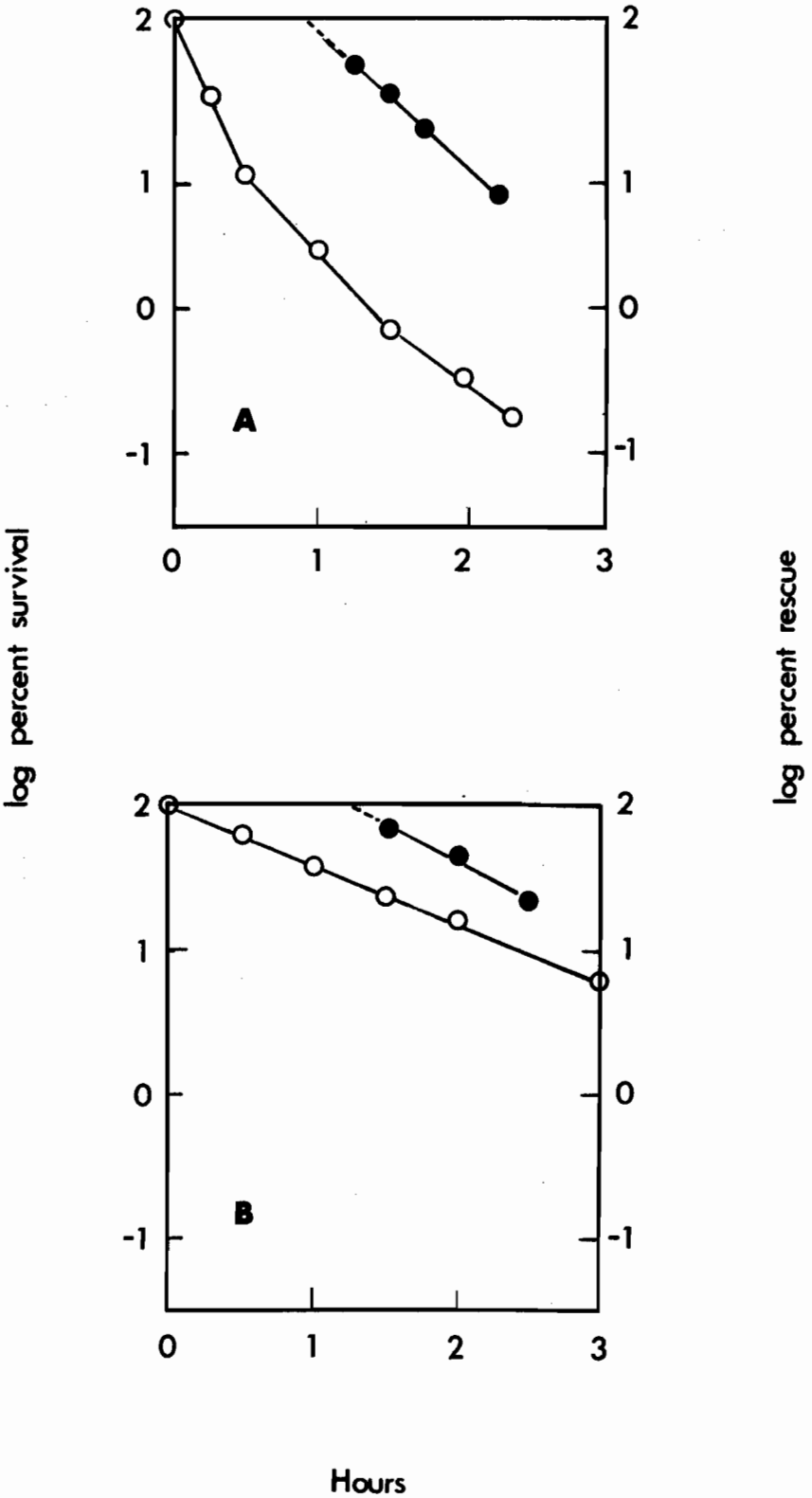
The rate at which cells in a culture bind a potentially-lethal dose of toxin is, at a first approximation, given by the slope of the survival curve; the rate at which the subsequent event in toxin action (the lethal event) occurs may be found from the rate at which the population loses its capacity to be spared from damage by energy-poisons. Figure 5.9 shows the result of this analysis. In this Figure, log percent survival is a simple transformation of the survival curve and is taken to show toxin-binding (the state 0 to state 1 reaction). The data for log percent rescue is from the results of the experiments described in

Figure 5.9 Survival on agar-plates and "rescue" by energy-poisons of killer toxin-treated cultures.

To measure cell survival, K19.10 cultures were treated with toxin and plated at intervals for viable-counts. To measure "rescue", K19.10 cultures were loaded with ^{42}K, treated with toxin and then treated with an energy-poison at intervals and ^{42}K retained by the cells measured; the experiments were those shown in Figures 5.7, plot *A* (glucose-grown cultures) and 5.8, plot *A* (ethanol-grown cultures); the "percent rescue" was derived as described in the text.

Plot *A*. Glucose-grown culture: (O), $\log_{10}$ percent survival on NaYEPD-agar; (●), $\log_{10}$ percent "rescue", by iodoacetate.

Plot *B*. Ethanol-grown culture: (O), $\log_{10}$ percent survival on NaYEPE-agar; (●), $\log_{10}$ percent "rescue" by cyanide.


log percent survival
log percent rescue
A
B
2
1
0
-1
0
1
2
3
Hours

Figures 5.7, plot *A* and 5.8; "rescue" means the block imposed on the transition of cells to state 2, as measured by the suppression of the onset of ^{42}K-release in yeasts that have bound a potentially-lethal dose of toxin. To derive "percent rescue" it has been assumed that the fraction of a toxin-treated population losing cellular ^{42}K at the control-rate (i.e. the rate characteristic of cells treated with the poison alone) is a measure of the fraction undamaged by toxin. For instance, in Figure 5.7, plot *A*, the ^{42}K-efflux time-course of toxin-treated cells treated with IAA at plus-80 min was extrapolated to the y-axis to give 65 percent ^{42}K retained or 65 percent of cells undamaged. This fraction was taken as the recueable-fraction at plus-80 min and plotted accordingly in Figure 5.9, plot *A*, as percent rescue. The data in Figure 5.9, plot *B*, is derived in a similar way, from the results shown in Figure 5.8, plot *A*, and a survival measurement of ethanol-grown cells treated with killer toxin. It may be seen in Figure 5.9 that in either glucose-grown or ethanol-grown K19.10 cultures, the cells were damaged at approximately the same rate as that at which they bound a potentially-lethal dose of toxin and that the time after toxin addition when the cells first become "rescueable" (intersection of rescue data with time-axis; dotted lines) corresponds to the extent of the lag-period (approximately 55 min in glucose-grown cultures and 75 min in ethanol-grown cultures).

II. Sensitivity of toxin action to inhibition of protein synthesis and DNA synthesis

Protein synthesis

One mechanism by which reagents that interfere with the

generation and/or use of metabolic energy may affect killer toxin action is by draining the cell of its capacity to energise the synthesis of macromolecules, macromolecules which may be necessary to the toxin's "biochemical target" (the structure affected in state 2) or part of a hypothetical "transmission" mechanism necessary to the state 1 to state 2 transition. If these components contain proteins, then a direct intervention with protein synthesis should stop toxin action.

Cycloheximide specifically inhibits protein synthesis in the eukaryotic cell by preventing both the initiation and peptide-elongation reactions on the 60 S ribosomal subunit (Pestka, 1971). In cultures of strain K19.10 growing in YEPD medium, the addition of cycloheximide to 40 µg per ml resulted in complete inhibition of [^{14}C] arginine incorporation into cellular macromolecules (ethanol-insoluble radioactivity; data not shown). The effect of cycloheximide on the response of sensitive cells to toxins was examined, in a few experiments.

Preliminary trials in which the ^{42}K-release assay was used to measure toxin action were relatively uninformative, since the addition of cycloheximide to potassium-loaded cells itself resulted in an efflux of the radioactivity that made interpretation of any ion-release due to toxins equivocal (both toxin-producing and toxin-sensitive strains displayed the cycloheximide-induced ^{42}K-release). Nevertheless, the results suggested that killer toxin action was blocked by the inhibitor.

Subsequent experiments were done using arginine-pool efflux and culture-turbidity change as the measures of toxin action.

(a) Arginine-derived pool efflux

Both killer toxin and PEST induced the release from cells of the

low MW cellular pools derived from [^{14}C] arginine at approximately plus-120 minutes, i.e. at about 60 minutes after the onset of events such as potassium efflux (Chapters 3 and 4). At 30°C, the PEST - induced arginine release was an effect approximately coincident with potassium-release (Chapter 4). The presence of cycloheximide in [^{14}C] arginine-loaded K19.10 cells prevented the killer toxin-induced release of radioactive pools (Figure 3.5, page 36); the inhibitor had little effect on the discharge due to PEST action (Figure 5.10; compare with Figure 4.10, plot *A*, page 77). Although not tested here, it is predicted that the addition of cycloheximide to K19.10 cultures at intervals after the addition of killer toxin would, in a manner similar to the energy-poisons, prevent release from cells of the arginine-derived pools in the undamaged fraction of the population; the fraction of cells so spared should decrease as the interval between toxin addition and cycloheximide addition increases.

(b) Culture-turbidity increase

Figure 5.11 illustrates the effect of cycloheximide on the response of culture turbidity to either the killer toxin (plot *A*) or the *T. glabrata* PEST (plot *B*). The effect of killer toxin-induced turbidity increase was abolished by the inhibitor; the PEST induced turbidity-increase was not affected by the presence of cycloheximide.

DNA synthesis

DNA is synthesised during the S phase of the *S. cerevisiae* cell-cycle, the onset of synthesis coinciding with the emergence from

Figure 5.10 PEST-induced [^{14}C] arginine efflux from cells treated with cycloheximide.

A 15 ml NaYEPD culture of K19.10 at 30°C was supplemented with 2.5×10^{-4} M [^{14}C] arginine and incubated for 60 minutes to enable the cells to accumulate the label. The cells were then harvested, washed with NaYEPD plus 40 µg/ml cycloheximide and resuspended at A_{600} 1.2 to 1.3 in NaYEPD plus cycloheximide. PEST was added to a portion of the culture, water (control) to another portion and each culture was incubated at 30°C. At intervals, each culture was sampled for radioactivity in the cells and in the wash (0.5 ml culture was filtered over a glass-fibre disc and washed with 5.0 ml medium: radioactivity in the wash and on the filter was measured. Filter cpm = cell cpm). Plot *A*, cpm in cells; plot *B*, cpm in wash; plot *C*, recovered cpm = $cpm_{cells} + cpm_{wash}$. Symbols: (○), plus PEST; (●), plus water.

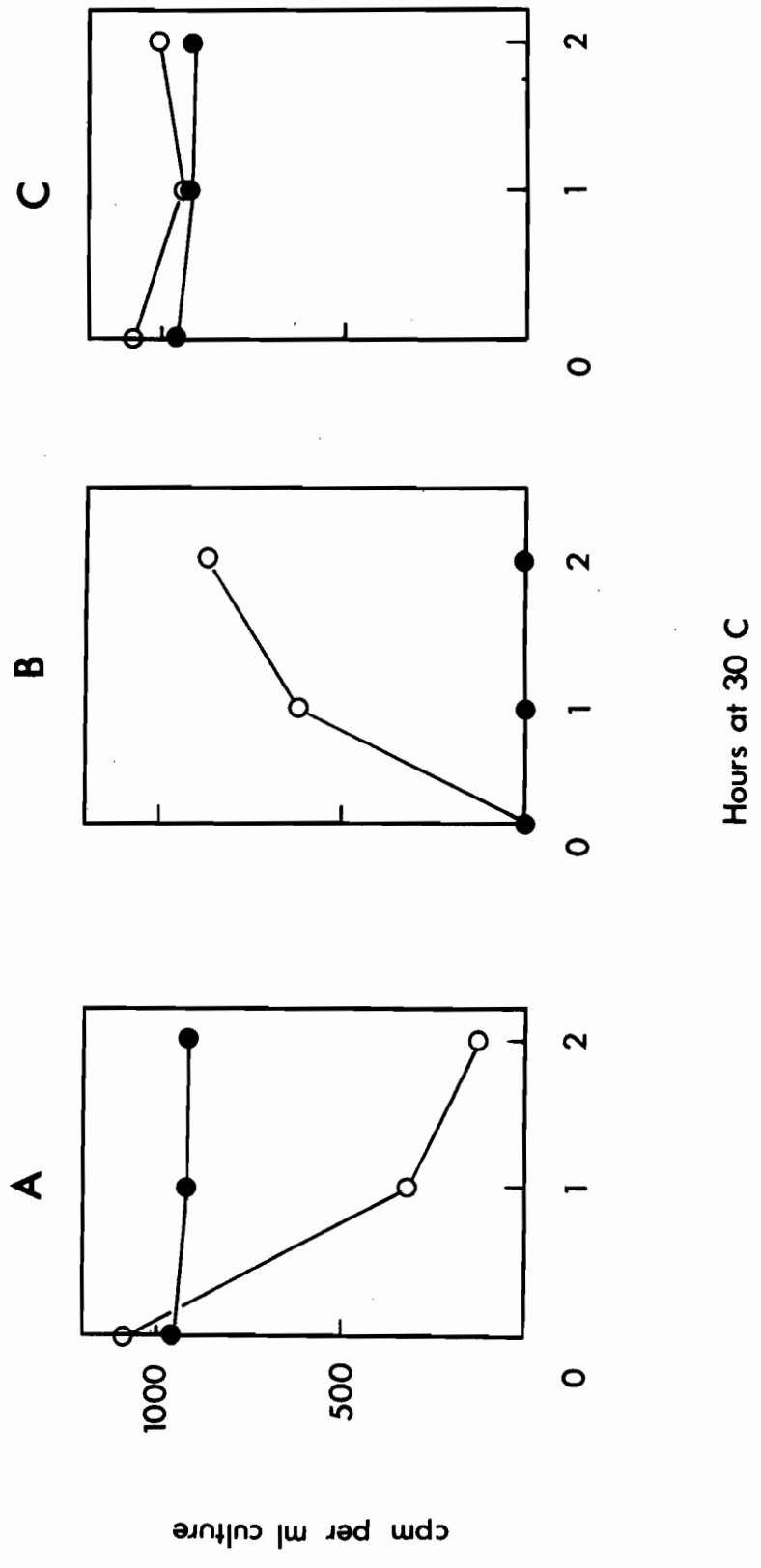
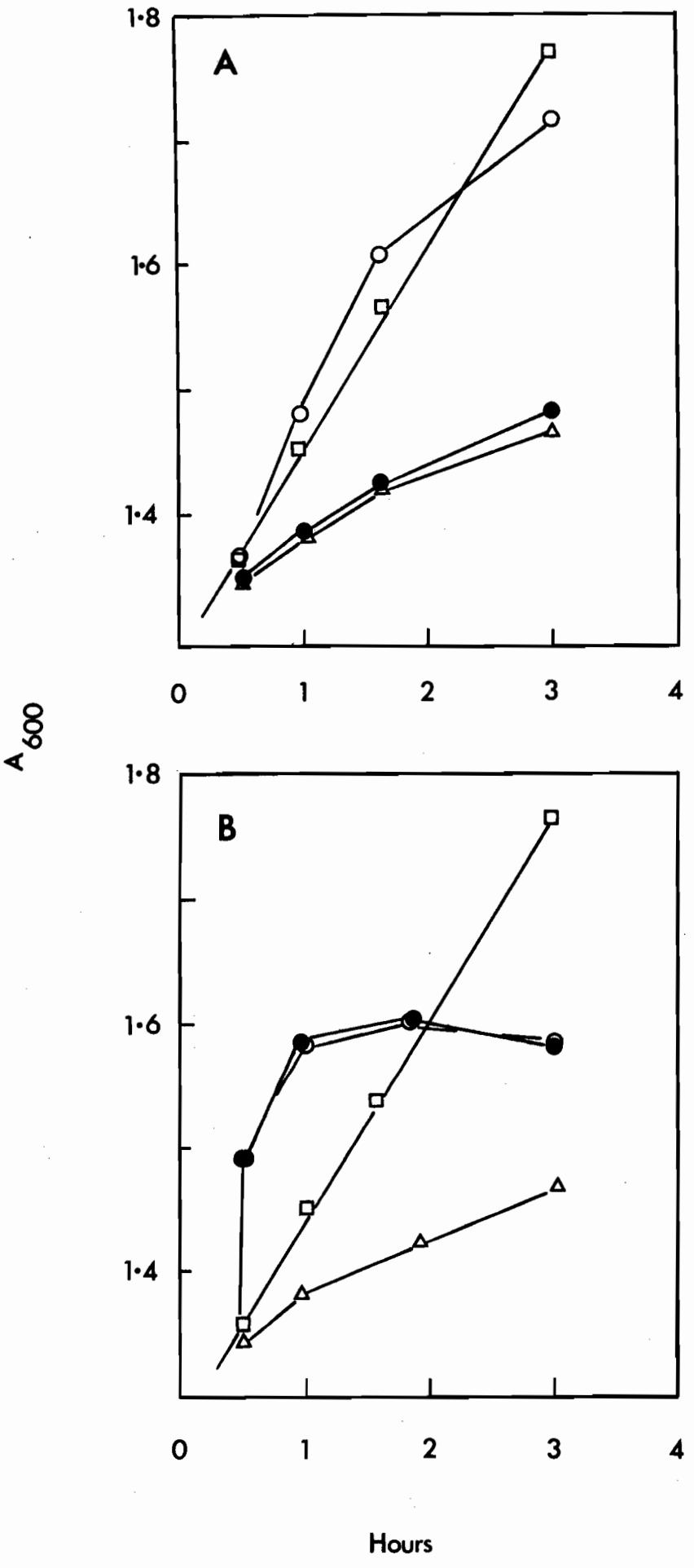

A
B
C
1000
500
0
1
2
cpm per ml culture
Hours at 30 C

Figure 5.11. Effect of cycloheximide on toxin-induced culture-turbidity change.

A NaYEPD culture of strain K19.10 at room temperature was treated at time zero with either no additions, cycloheximide (40 µg/ml), cycloheximide plus killer toxin, cycloheximide plus PEST, or with each toxin alone and incubated at room temperature. The absorbance of each suspension (A_{600} 1.29 at time-zero) was then measured at intervals. Plot *A*, killer toxin; plot *B*, PEST. Symbols: (□), control (no additions); (△), plus cycloheximide; (○), plus toxin; (●), plus cycloheximide and toxin.


A
B
1·8
1·6
1·4
0
1
2
3
4
A_{600}
Hours

the parent cell of the bud (Hartwell, 1974). Hydroxyurea stops culture growth by specifically inhibiting DNA synthesis (Slater, 1973). The hydroxyurea-treated population becomes morphologically synchronised as each cell reaches the block in its cycle, and the drug's effects are reversible by washing since plate-counts remain constant (Slater, 1973).

Addition to K19.10 cultures of hydroxyurea to 75 mM, a dose known to block DNA synthesis completely (Slater, 1973), had no effect on either killer toxin or PEST action, as judged by the potassium-release assay. Figure 5.12 shows the results of an experiment in which ^{42}K-loaded cells had been treated with hydroxyurea for 2 hours before being treated with toxins: the ^{42}K-release was very similar to that seen in normal toxin-treated K19.10 suspensions.

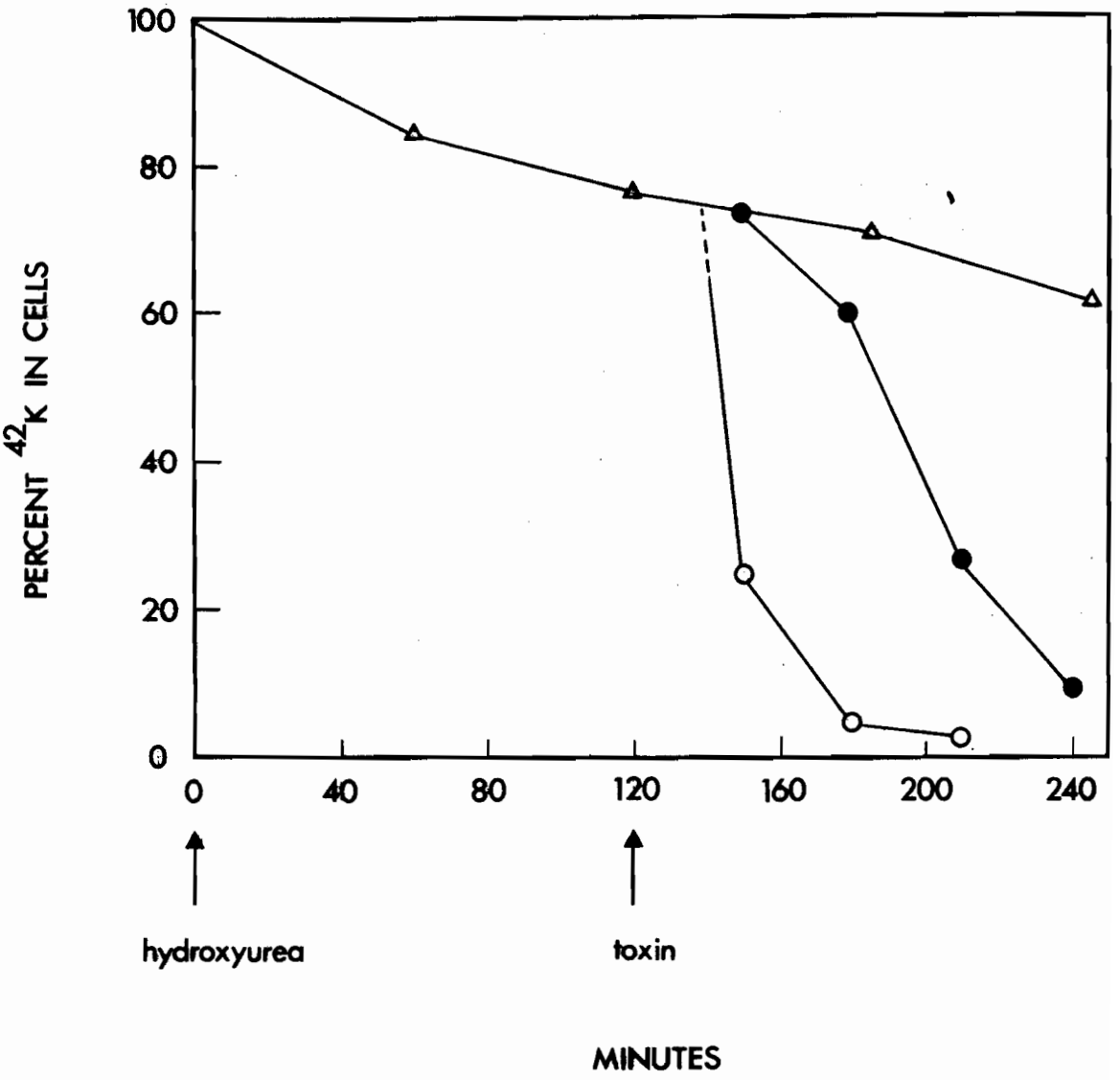
Discussion

These results show that in killer toxin-sensitive cultures growing either glycolytically on glucose or aerobically on ethanol the ^{42}K-efflux from cells characteristic of toxin action is blocked by energy-poisons. PEST action on the potassium pools of the same sensitive strain was not affected by energy-poisons.

On the basis of whether the poisons prevented killer toxin-induced culture death it was possible in principle to distinguish between their effects on the binding to cells of a potentially-lethal dose of toxin and on the events in toxin action subsequent to such binding. The uncoupler DNP clearly affected the onset of ^{42}K-release and not the binding event since the cells were killed on plates, a killing that could

Figure 5.12 Effect of hydroxyurea on toxin action.

A culture of strain K19.10 growing in NaYEPD at room temperature (about 26°C) was loaded with ^{42}K and treated at time zero with 75 mM hydroxyurea. At plus-120 min, portions of the suspension were treated with either killer toxin, PEST or with no additions (control). All suspensions were sampled at intervals for ^{42}K retained by the cells. Symbols: (Δ), control; (●), plus killer toxin; (O), plus PEST.


100
80
60
40
20
0
PERCENT ^{42}K IN CELLS
0
40
80
120
160
200
240
hydroxyurea
toxin
MINUTES

not be attributed to the effects of DNP alone. Iodoacetic acid or CCCP also appeared to block post-binding events in killer toxin action, at least in ethanol-grown cells; in glucose-grown cells these agents were themselves lethal, a result which precludes any such distinction from being made. The reduction in toxin-induced culture-death seen in the ethanol-grown cultures treated with antimycin or cyanide suggests that both of these inhibitors may affect both toxin-binding and post-binding events in these cells.

The addition of energy-poisons to sensitive cultures at intervals after the addition of killer toxin has been found to block ^{42}K-efflux from cells in a way consistent with a scheme in which there is an energy-dependent transition of killer toxin-treated cells from a state in which a potentially-lethal dose of toxin is bound but without effect to a state in which the cells are affected. In these terms, energy-poisons prolong the lag-period indefinitely, the lag-period being defined as the minimum interval necessary for cells that have bound a potentially-lethal dose of toxin to undergo a transition to the damaged state.

It has also been shown that killer toxin action, as measured by either efflux from cells of arginine-derived low MW pools or transient culture-turbidity increase, is prevented by cycloheximide, a specific inhibitor of protein synthesis. Cycloheximide did not, however, prevent PEST from promoting pool-efflux or a turbidity-increase in cells of the same sensitive strain.

These results suggest that killer toxin action, but not PEST

action, depends on continuous protein synthesis by the target-cell. Since protein synthesis is an energy-dependent process it is possible that the inhibition of killer toxin-induced metabolic alteration (potassium efflux) by reagents that interfere with energy metabolism is due to inhibition of protein synthesis. If it can be shown that cycloheximide does not affect the initial event in killer toxin action, the binding to cells of a potentially-damaging amount of toxin, then these results would imply that the components of the sensitive cell necessary to the transition of cells to the state in which they have been damaged by this dose include proteins of high turnover-rate.

Hydroxyurea, an inhibitor of DNA synthesis that results in synchronisation of yeast cells in a batch culture, did not affect killer toxin action on yeast potassium pools. This result implies that simply blocking the growth of sensitive cultures is not sufficient to prevent killer toxin action; it also suggests that the lag period before the onset of membrane-alteration in toxin-treated populations is not a function of the asynchrony of batch-cultured cells.

The effects of iodoacetate, cyanide and antimycin on culture growth and killer toxin action can be reasonably attributed to their common effect of interfering with ATP generation in cells, but those of DNP and CCCP in both glucose-grown and ethanol-grown cultures are less readily explained. In ethanol-grown cells, DNP will deplete ATP by effecting an uncoupling of oxidative phosphorylation from electron-transport in the mitochondrion and by stimulating the activity of mitochondrial ATPase (Slater, 1963) but in cells insensitive to antimycin

or cyanide (glucose-grown cells) the mode of action of DNP is not obvious. Inhibition by DNP of energy-linked functions in organisms generating ATP in a non-respiratory mode is well known (Harold, 1972) and examples are known in yeast cultures (Jarrett and Hendler, 1967; Riemersma, 1968; Borst-Pauwels and Jager, 1969; Ramos *et al.* 1975; Chevallier, Jund and Lacroute, 1975; Sumrada, Gorski and Cooper, 1975). Ramos *et al.* (1975) suggested that DNP stimulates a promitochondrial ATPase to dissipate glycolytic ATP. But it is known that inhibitors of the yeast mitochondrial ATPase (oligomycin or dicyclohexylcarbodiimide) do not block the effects of DNP on energy-linked reactions in fermenting yeast cultures (Galeotti, Kovac and Hess, 1968; Huygen and Borst-Pauwels, 1972). An alternative hypothesis is that reagents such as DNP and CCCP, which dissolve in biological membranes so as to conduct protons, interfere with the maintenance and use of metabolic energy by eliminating proton gradients (Mitchell, 1966; Harold, 1972). In bacteria, mitochondria and chloroplasts, plasma membrane-spanning ATPases generate a proton motive force which is eliminated by "uncouplers" such as DNP (Hamilton, 1975; Hinkle and McCarty, 1978). In yeasts there is no clear evidence for a plasma-membrane ATPase but the effects of DNP and CCCP on glycolysing cells shown here and in other work imply that such an enzyme is present in these cells and functions in energy metabolism.

Chapter 6

METHODS FOR THE PREPARATION OF YEAST SPHEROPLASTS

Introduction

Spheroplasts are cells that have had their cell-wall altered to the extent that when exposed to osmotic stress they swell and burst. For our work, yeast spheroplasts were useful for two main purposes: as a starting material for isolation of plasma membranes and as a substrate for the toxins (see Chapter 7).

The techniques available for preparing spheroplasts have been reviewed (Villanueva and Acha, 1971; Kuo and Yamomoto, 1975). The most widely used technique involves digestion of the yeast cell wall with snail-gut enzymes (such as glusulase). The glusulase procedure (General Materials and Methods), based on the protocol of Eddy and Williamson (1957), has several serious disadvantages: (1) The method is unreliable. In our experience there is no assurance that spheroplasts will be produced from cells on any given day. (2) Yeast strains show a wide variation in their susceptibility to glusulase; many strains from our collection are refractive to the enzyme preparation. (3) The spheroplasts are generally unstable. Glusulase spheroplast preparations often contain a major fraction of broken structures and further damage results upon subsequent handling. (4) The complexity of the snail-gut extract is a potential problem since the spheroplast surface is likely to be altered in numerous ways by the variety of degredative enzymes (Holden and Tracy, 1950, reported 30 distinct activities in a snail-gut extract).

A procedure is described in the following section, the zymolyase procedure, that has proved to be an excellent method for the preparation of spheroplasts from most of our strains. In addition, a minor modification to the glusulase procedure is shown to improve that method's usefulness, at least for strain K19.10.

Results

I. Spheroplasting procedures

The following protocols were found to be useful for preparing spheroplasts from cells of strain K19.10. A subsequent section discusses the usefulness of the methods for other yeast strains and comments on the procedures are given in the Discussion.

(a) The zymolyase procedure

One hundred ml of cells were grown overnight at 30°C in YEPD or NaYEPD to a density of A_{600} 1.2 to 1.4, harvested, washed twice with water and resuspended at 22 ml per g wet weight in zymolyase spheroplasting medium (1.2 M sorbitol, 10 mM tris-HCl, 10 mM $CaCl_2$; pH was 7.5). Freshly dissolved dithiothreitol (DTT) was added to 2 mM, followed by Zymolyase-5000 to 100 µg per ml (the zymolyase, supplied as a powder, was dissolved in 10 mM tris-HCl, pH 7.5, immediately before being added to cell suspensions). The cells were converted to spheroplasts by incubation at 30°C for 120 min, with moderate shaking. Zymolyase-spheroplasts could be harvested quantitatively from suspension by centrifugation at 164 x g. for 5 min; alternatively they could be removed from suspension fluids by filtration over a glass-fibre disc. The spheroplasts were usually stored at 0 to 4°C as suspensions in 1.2 M sorbitol, 10 mM sodium acetate buffer, 5 mM KCl, pH 4.7; under these circumstances they were stable morphologicall

for at least a week.

(b) The modified glusulase procedure

The glusulase procedure (General Materials and Methods) was modified by increasing the concentration of the osmotic stabiliser (sorbitol) from 0.8 to 1.2 M.

Cells, usually 100 ml, were grown overnight at 30°C in YEPD or NaYEPD, harvested, washed twice with water and resuspended to 22 ml per g wet weight in 1.2 M sorbitol. Glusulase was added to 1 percent by volume and the cells converted to spheroplasts by incubation at 30°C, with very gentle shaking. After 60 to 90 min, the spheroplasts were harvested by centrifugation at 650 x g. for 15 min. When stored at 0 to 4°C in 1.2 M sorbitol the spheroplasts were morphologically stable for at least four days

II. Measurement of spheroplast density

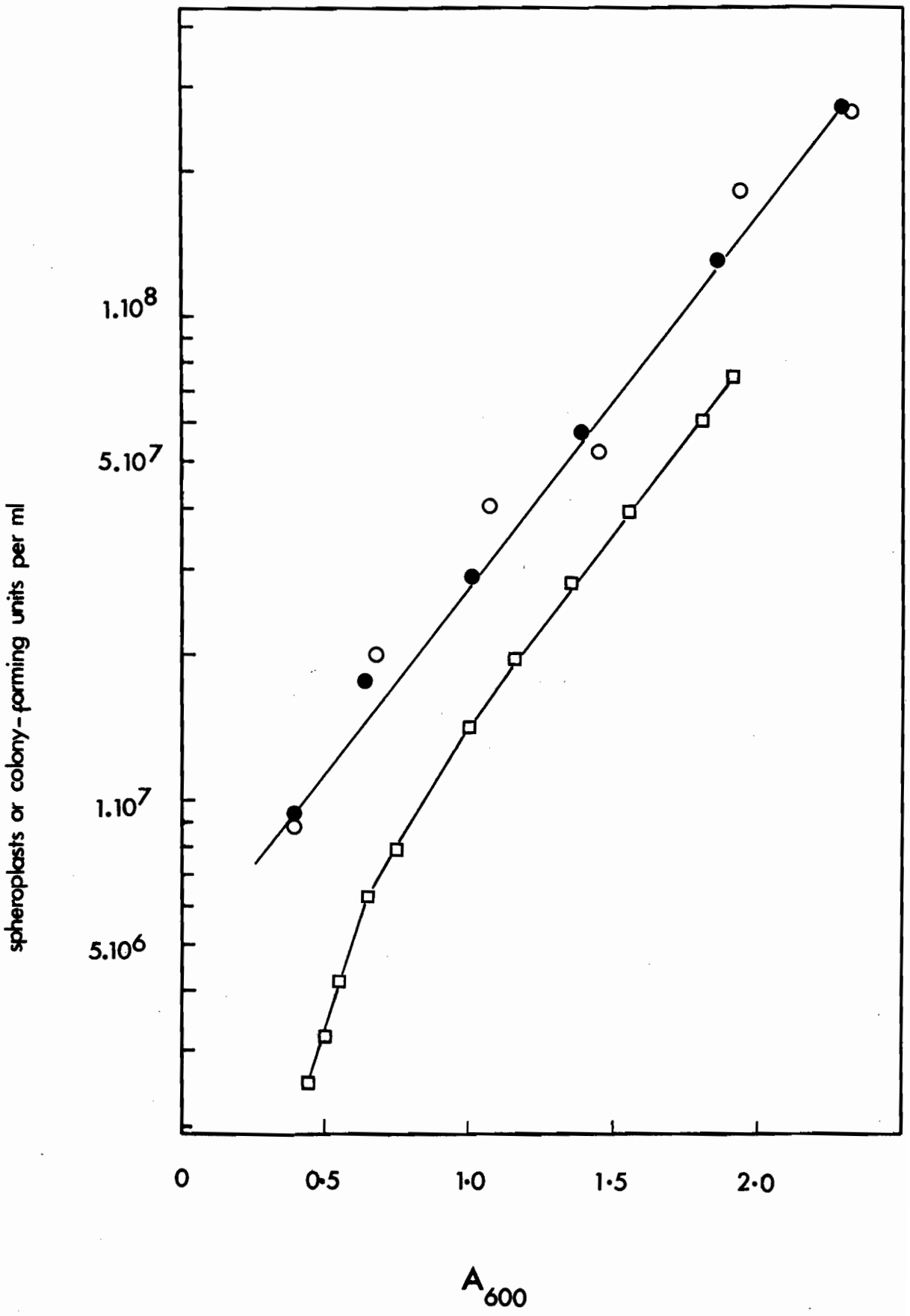
The calibration between spheroplast density and suspension absorbance at 600 nm (1 cm path-length cuvettes) shown in Figure 6.1 was used to determine the concentration of spheroplasts used in the experiments with toxins described in Chapter 7. The counts of zymolyase spheroplasts were imprecise because the spheroplasts remained clumped to a degree after dilution. Also in Figure 6.1 is the relationship between viable-counts of cell suspensions and A_{600} (from Figure 2.1).

III. Integrity of spheroplasts

An assessment was made of both the yield of spheroplasts from cells and the degree to which the spheroplasts were damaged. Ideally, a spheroplasting procedure would result in the quantitative conversion of cells to osmotically-sensitive structures, without altering the structure of the plasma membranes. For the purpose of isolating plasma membranes

Figure 6.1 Relationship between suspension absorbance and cell or spheroplast density.

The data for cells is from Figure 2.1. Spheroplast density was measured by counting dilutions of K19.10 spheroplasts in a hemacytometer. Symbols: (○), zymolyase spheroplasts; (●), modified glusulase spheroplasts; (□), cells.


spheroplasts or colony-forming units per ml
1.10^8
5.10^7
1.10^7
5.10^6
0
0·5
1·0
1·5
2·0
A600

from yeasts, a valuable procedure is to tag the surface of the spheroplast with a radioactive marker, such as ^{125}Iodine, and then isolate the membrane by isolating the radioactivity (Duran, Bowers and Cabib, 1975; Schibeci, Rattray and Kidby, 1975; Santos, Villanueva and Sentandreu, 1978). The validity of such an approach rests on the assumption that the label is restricted to the cell envelope. For this reason it is essential to have an estimate of the upper limit to the damage the spheroplast surface may have received prior to the labeling. Two measures of the process and result of spheroplasting procedures used were made with strain K19.10: visual examination and assay for an internal enzyme.

Visual evidence

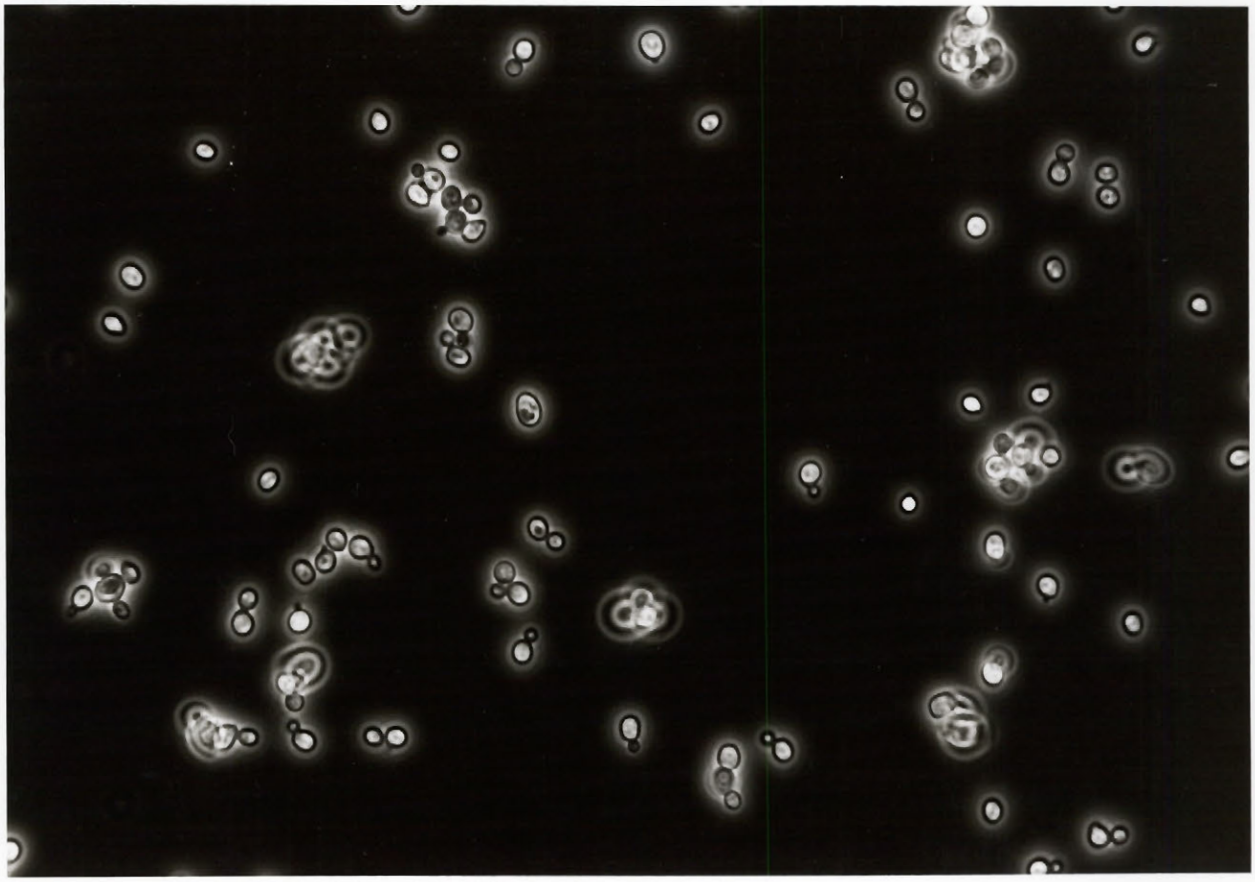
As judged by examination under the microscope, both the zymolyase procedure and the modified glusulase procedure resulted in the quantitative conversion of cells to spheroplasts. At the time of harvesting these spheroplasts, the addition of a drop of suspension to water on a microscope slide resulted in essentially 100 percent lysis (yeast whole-cells are not altered morphologically by resuspension in water).

When examined under phase-contrast optics, the cells from which the spheroplasts were to be prepared were ovoid, budding and surrounded by a bright "halo" (Plate 6.1, photograph *A*). The halo is a measure of cell intactness and is attributed to the difference in the optical properties of the cell contents and the suspension fluid. Typical spheroplast preparations are shown in Plates 6.3 and 6.4: each preparation has been photographed both as is (photograph *A*) and following centrifugation and resuspension of the pellet (photograph *B*). The spheroplasts prepared by either the modified glusulase procedure or zymolyase procedure were

Plate 6.1

Photograph *A*. K19.10 cells (magnification 800 x).
A mid-logarithmic K19.10 culture in NaYEPD medium was centrifuged and the cell pellet washed twice with water and then resuspended in zymolyase spheroplasting medium at 1 g wet weight per 22 ml.

Photograph *B*. Assay of toxins by the well-test (the seeded-plate assay) (magnification 1.15 x).
Killer toxin (right hand side) or PEST (left hand side) was added to a well dug in the surface of a strain S6-seeded plate (General Materials and Methods) and the plate incubated to allow growth of the seed.



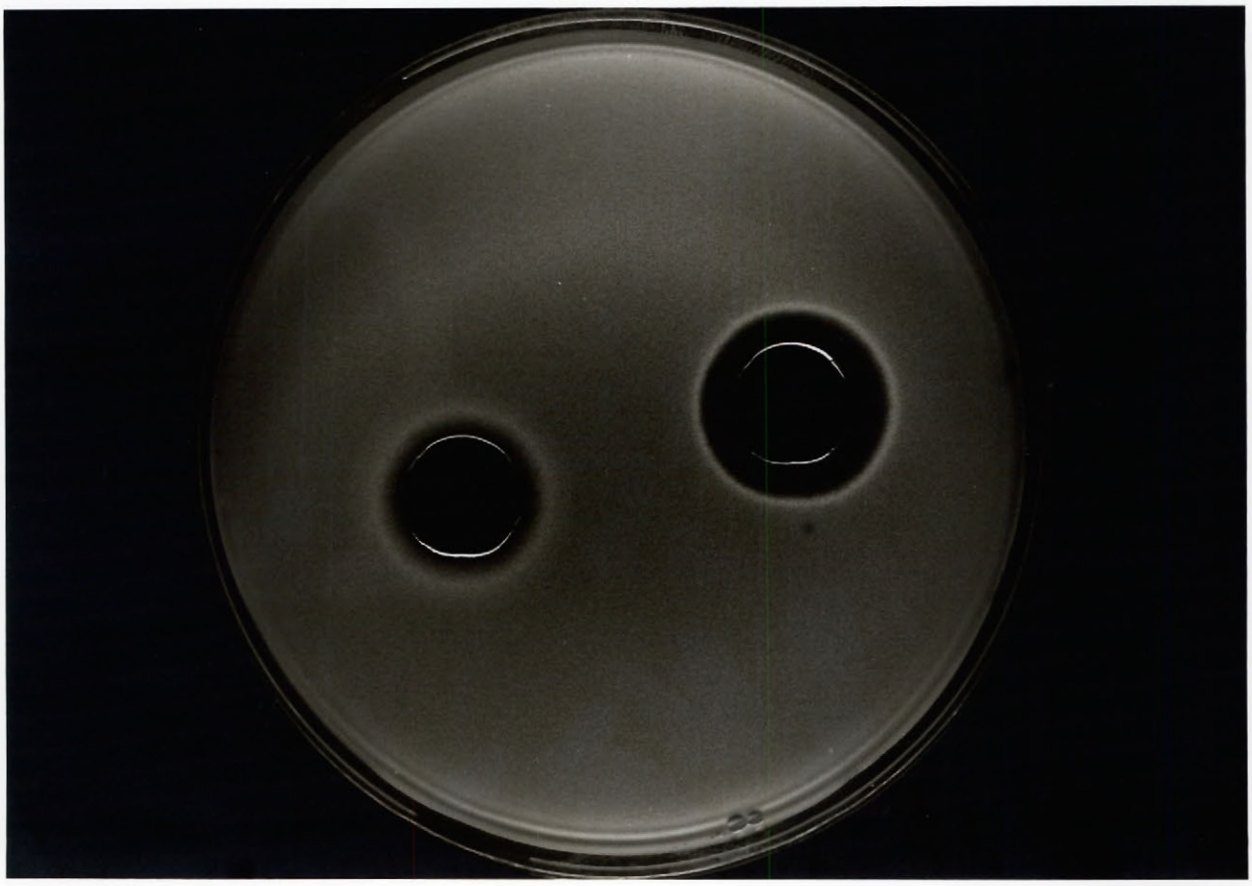
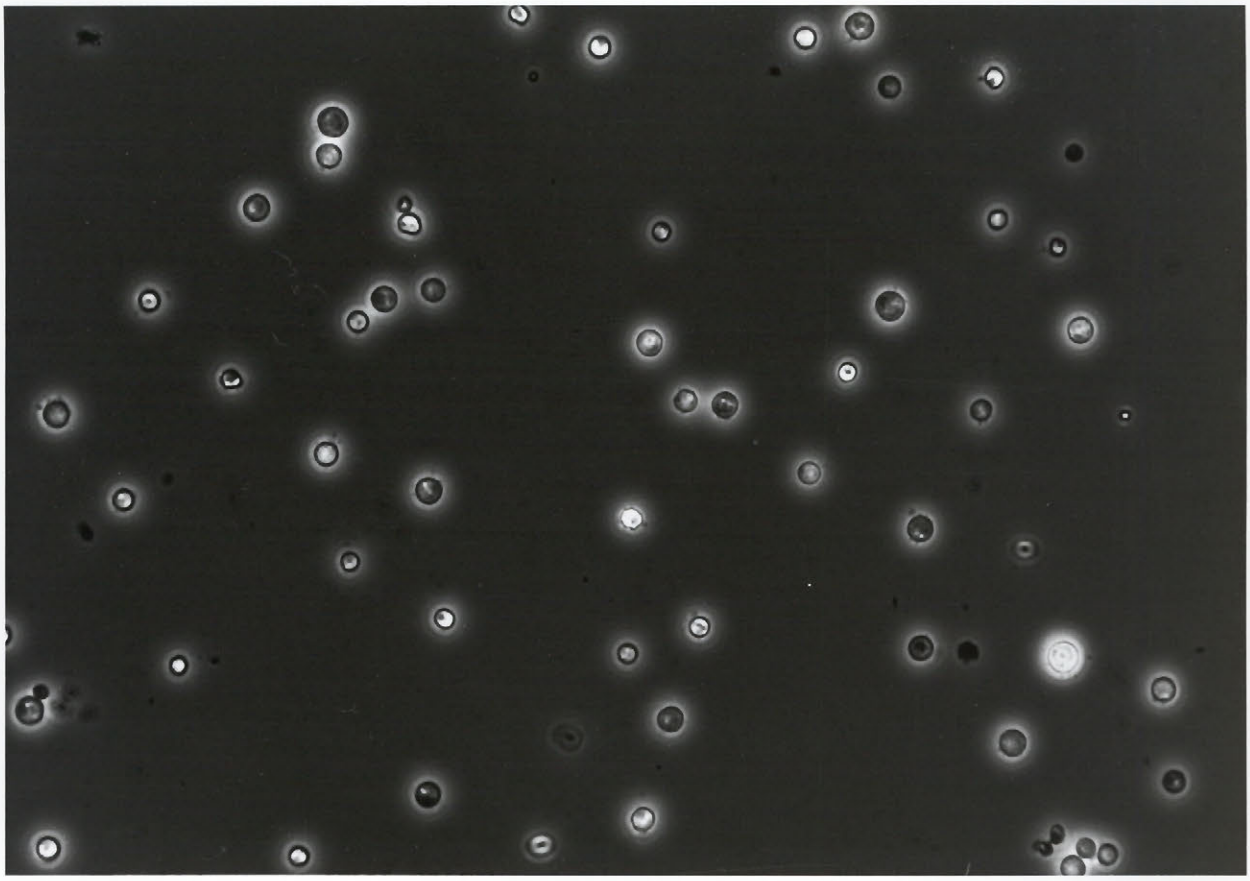


Plate 6.2

K19.10 spheroplasts prepared by the glusulase procedure.

Photograph *A*, spheroplasts before harvesting (magnification 800 A). K19.10 cells were resuspended in 0.8 M sorbitol to 1 g wet cells per 22 ml and incubated with 1 percent v/v glusulase for 120 min at 30°C.

Photograph *B*, spheroplasts after harvesting and resuspension (magnification 800 X).
A portion of the suspension shown in *A* was centrifuged at 650 x g. for 15 min and the pellet resuspended in 0.8 M sorbitol.



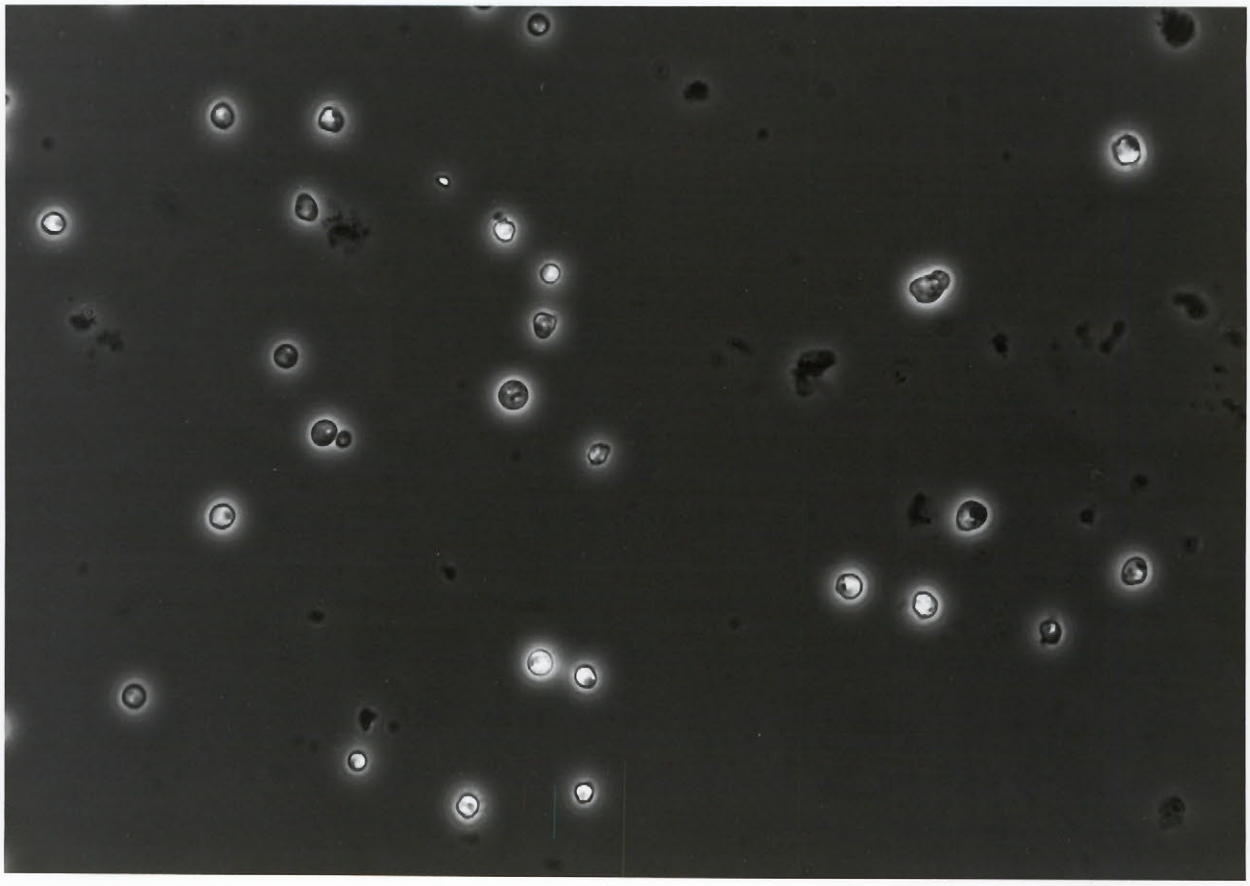
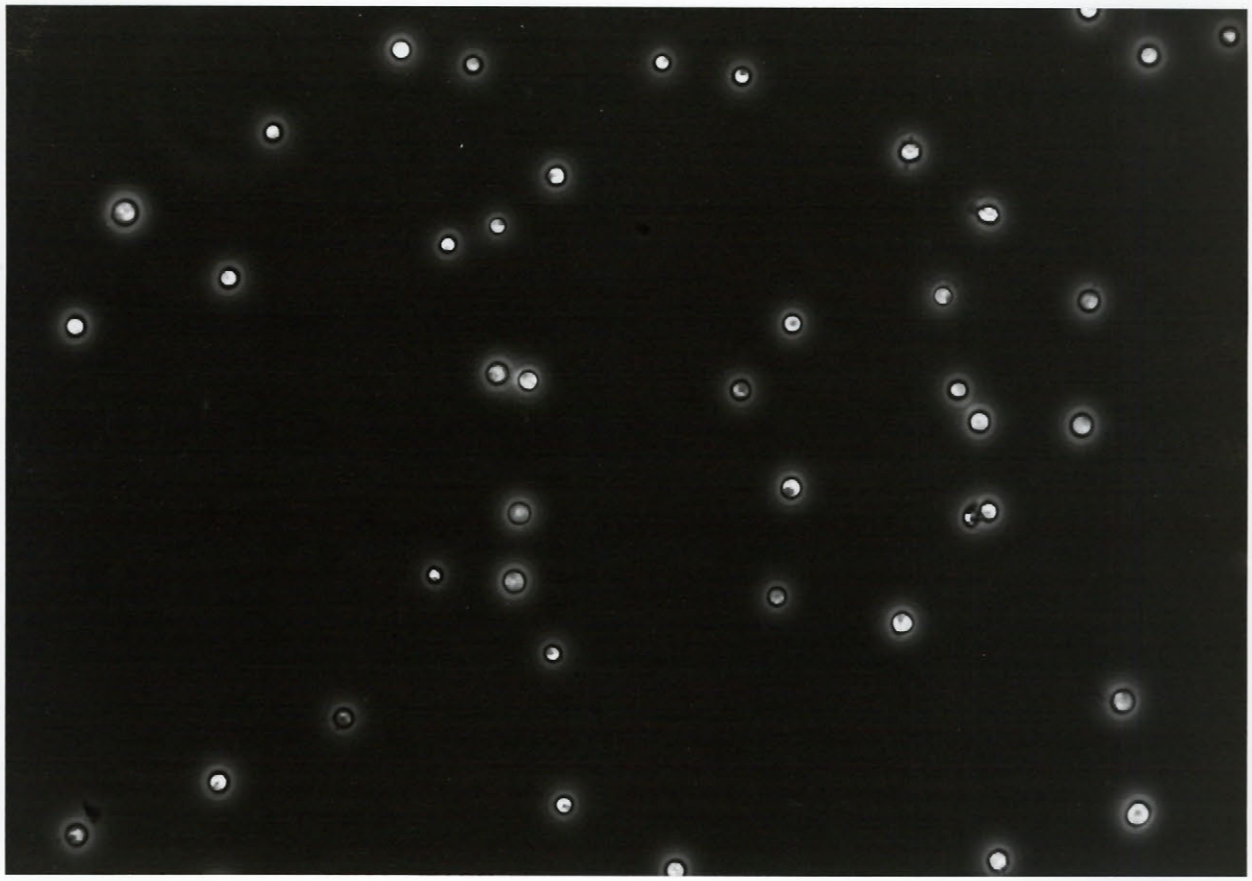


Plate 6.3

K19.10 spheroplasts prepared by the modified glusulase procedure.

Photograph *A*, spheroplasts before harvesting (magnification 800 X). K19.10 cells were resuspended in 1.2 M sorbitol to 1 g wet cells per 22 ml and incubated with 1 percent v/v glusulase for 120 min at 30°C.

Photograph *B*, spheroplasts after harvesting and resuspension (magnification 800 X).
A portion of the suspension shown in *A* was centrifuged at 650 x g. for 15 min and the pellet resuspended in 1.2 M sorbitol.



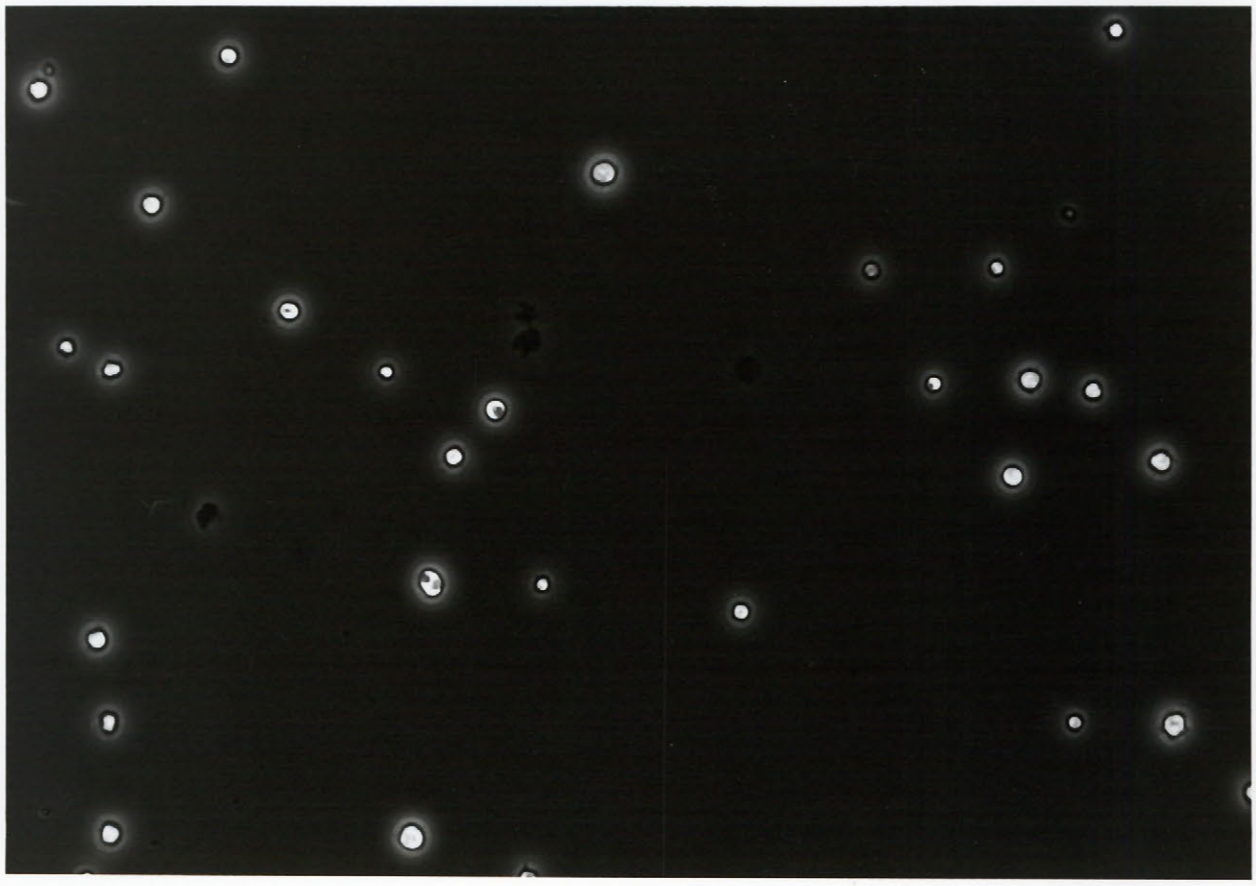
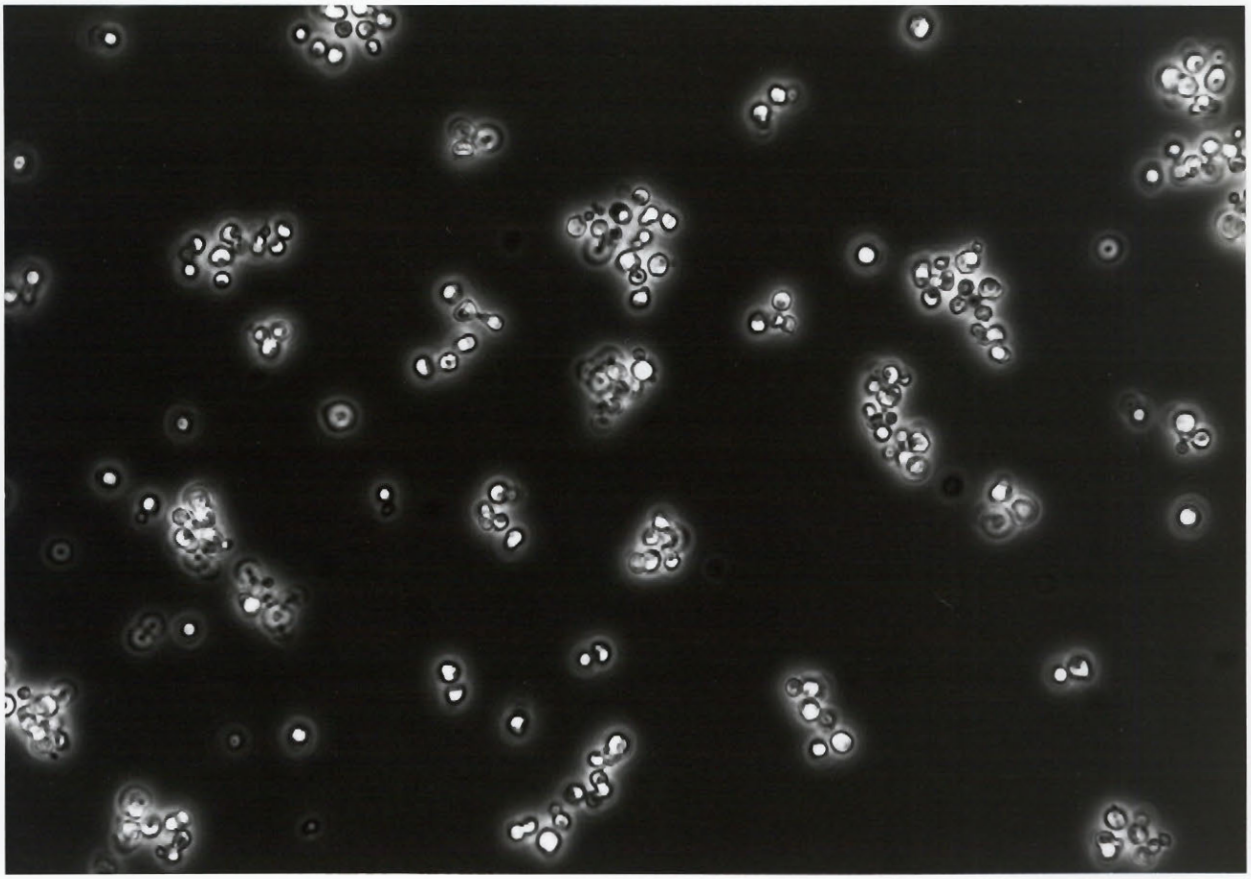


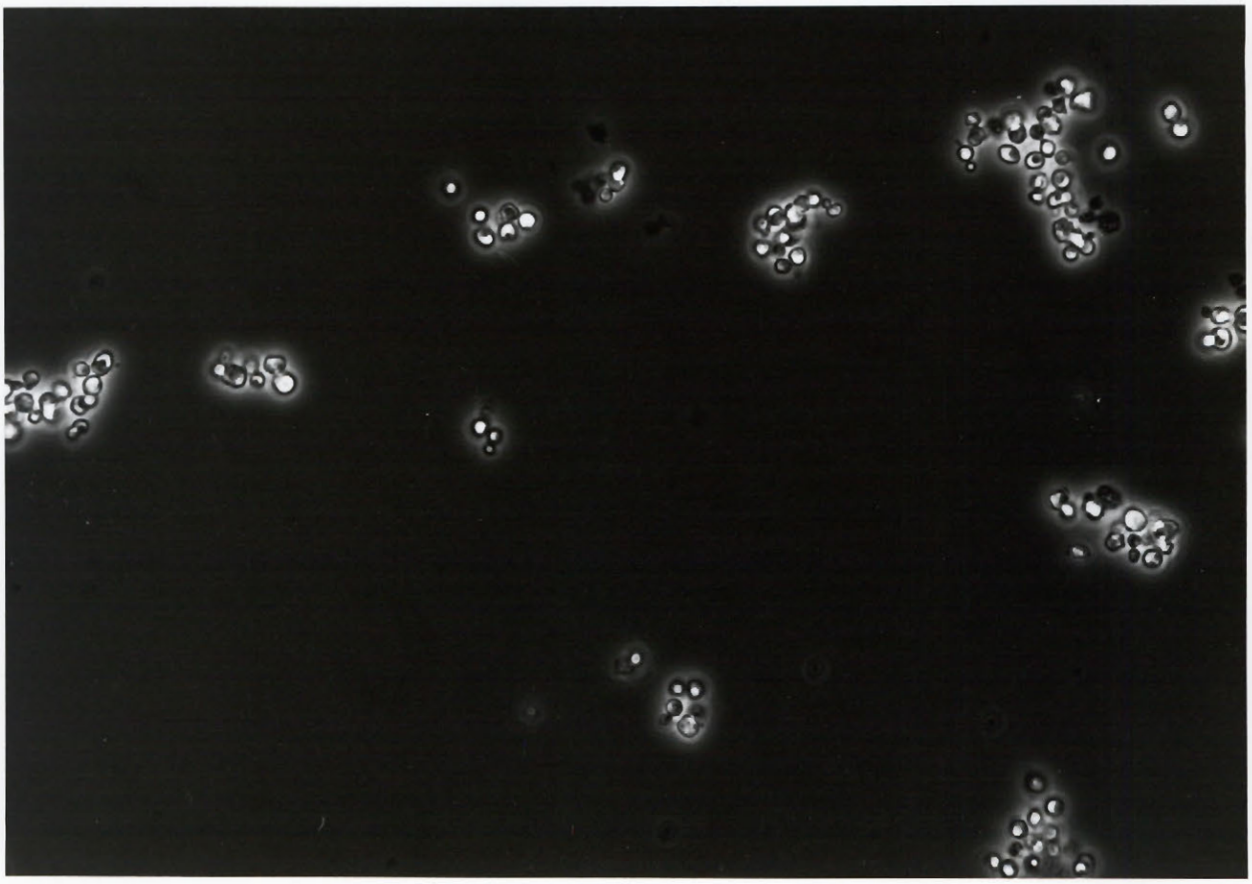
Plate 6.4

K19.10 spheroplasts prepared by the zymolyase procedure.

Photograph *A*, spheroplasts before harvesting (magnification 800 x). K19.10 cells were resuspended in 1.2 M sorbitol, 10 mM $CaCl_2$, 10 mM tris-HCl buffer, pH 7.5, at 1 g wet cells per 22 ml. Dithiothreitol was added, to 2 mM, and Zymolyase-5000, to 100 µg per ml, and the suspension incubated at 30°C for 120 min.

Photograph *B*, spheroplasts after harvesting and resuspension (magnification 800 x).
A portion of the suspension shown in *A* was centrifuged at 164 x g. for 10 min and the pellet resuspended in 1.2 M sorbitol, 10 mM tris-HCl, pH 7.5.





spherical or crenate and intact, i.e. surrounded by a bright halo. They were not damaged appreciably by harvesting. The aggregation of zymolyase spheroplasts (Plate 6.3) was typical for all yeast strains examined. Plate 6.2 shows, for comparison, a typical preparation of glusulase spheroplasts made in 0.8 M sorbitol: harvesting and resuspending these structures resulted in extensive lysis.

Evidence based on an assay for an internal enzyme

Alkaline phosphatase is an intracellular enzyme in *S. cerevisiae*, in contrast to the acid phosphatase that is located at the cell surface, external to the plasma membrane (Mclellan and Lampen, 1963). The presence of alkaline phosphatase in suspensions of cells or spheroplasts is evidence for altered plasma-membrane permeability.

(i) Conditions for assay

Preliminary to assaying spheroplast suspensions, the conditions for assaying alkaline phosphatase in strain K19.10 were examined. Cells were broken with glass beads and a debris-free supernatant fraction taken as the enzyme preparation. The following protocol was used: K19.10 cells were grown at 30°C in NaYEPD to A_{600} 1.4. Two litres of culture (7.5 to 8.0 g wet cells) were harvested, the cells washed twice with water, then resuspended as a slurry in 20 ml water. The cells were added to the glass disruption-vial supplied with the Braun homogeniser (Bronwell Scientific, Rochester, New York), along with 40 g 0.45-0.50 mm diameter glass beads and broken by shaking for 60 seconds, with intermittent cooling with liquid CO_2. The homogenate was decanted and centrifuged at 365 x g. for 15 min; the supernatant was re-centrifuged and the final supernatant diluted ten-fold with water for use in enzyme assays.

Phosphatase was measured by its catalysis of p-nitrophenylphosphate (pNPP) hydrolysis; the product p-nitrophenol (pNP) is measured spectrophotometrically at 420 nm. The following procedure, based on that described by Schurr and Yagil (1971) was used: 50 µl enzyme was added to a solution containing 150 µl 0.04 M pNPP, 100 µl water or other reagents and 500 µl buffer. Reactions were run for up to 10 min at 30°C, then stopped with 500 µl M NaOH. Assay mixtures were then clarified in the centrifuge and the colour in the supernatants measured at 420 nm.

In agreement with the results in the literature, cell extracts showed two peaks of activity: an acid phosphatase (pH 3.5) and an alkaline phosphatase (pH 8 to 10) (Figure 6.2). The acid enzyme-activity is taken as an indication that the surface-located phosphatase was at least partially solubilised by the violence of the disruption procedure (see also the results of Mclellan and Lampen, 1963). Suspensions of K19.10 cells in water did not contain any alkaline phosphatase activity (data not shown). Both acid and alkaline activities in the cell-extracts were stimulated by $MgCl_2$ and inhibited by EDTA (Figure 6.2). Figure 6.3 shows the result of a measurement of the dependence of alkaline phosphatase on the $MgCl_2$ concentration; 10 mM $MgCl_2$ was subsequently adopted for assays. With or without $MgCl_2$, the production of pNP from pNPP was directly proportional to enzyme concentration (Figure 6.3, plot *A*) and to reaction time (results not shown).

The following protocol was used to assay alkaline phosphatase in spheroplast suspensions: 50 µl sample was added to a solution containing 150 µl 0.04 M pNPP, 400 µl 2.4 M sorbitol, 100 µl 0.3 M glycine-NaOH buffer (pH 9.0 when diluted 8-fold), and 80 µl 0.1 M $MgCl_2$; final pH was 9.0. Assays were run for 10 minutes at 30°C, the reactions stopped by

Figure 6.2 Effect of pH on yeast phosphatase activity in the presence and absence of $MgCl_2$ or EDTA.

A cell-extract from a culture of strain K19.10 (see text) was assayed for phosphatase activity either without additions, with 10 mM $MgCl_2$, or with 1 mM EDTA, over a range of pH value. Buffers were all phosphate-free and were prepared on the basis of data supplied in handbooks (Dawson *et al.*, 1969; Sober, 1968). Symbols: (○), no additions; (●), plus $MgCl_2$; (■), plus EDTA.

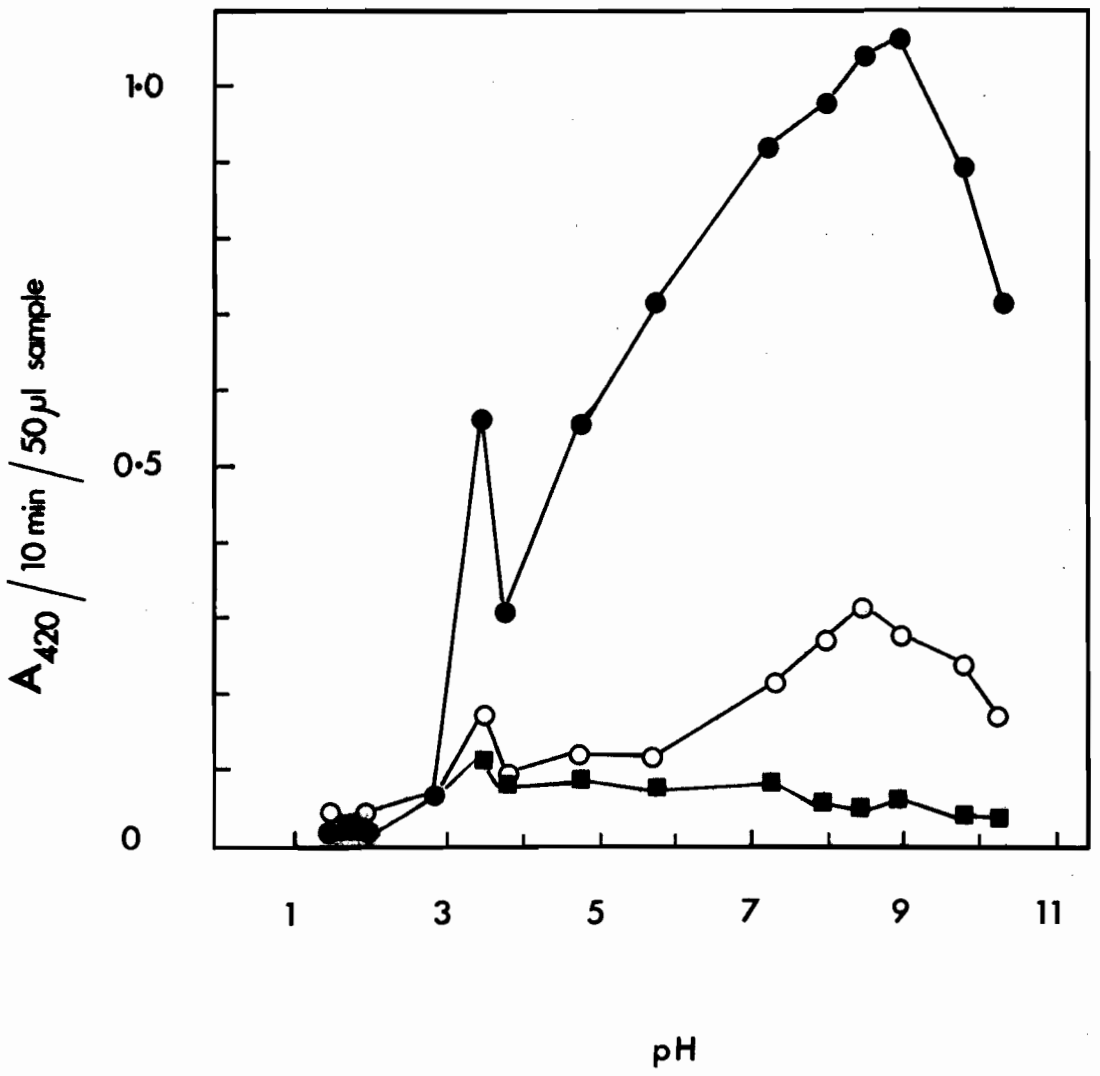
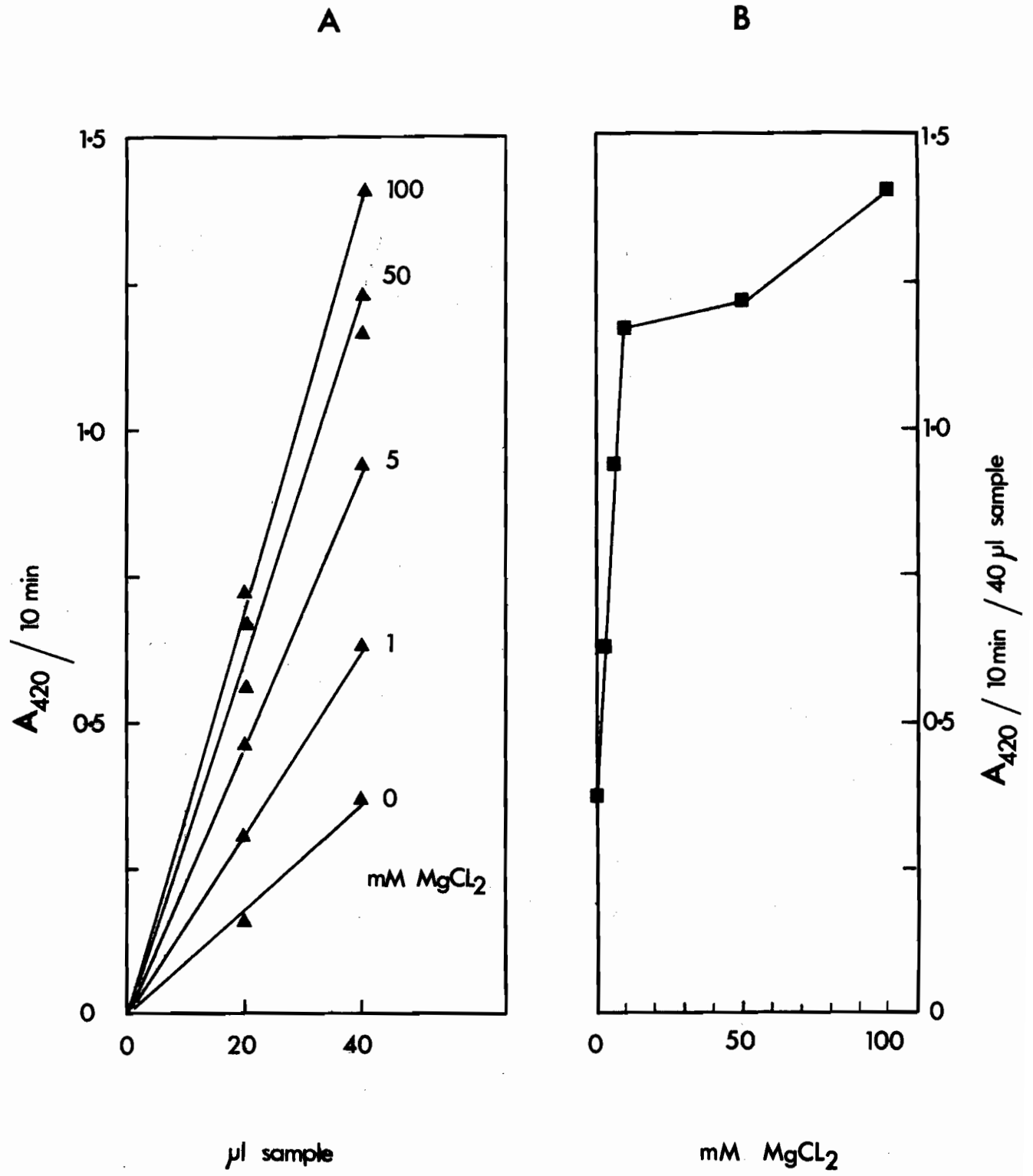

1·0
0·5
0
A_{420} / 10 min / 50 µl sample
1
3
5
7
9
11
pH

Figure 6.3 Effect of $MgCl_2$ concentration on yeast alkaline phosphatase activity.

A K19.10 cell-extract, prepared as described in the text, was assayed at pH 9.0 for phosphatase activity in the presence of $MgCl_2$ from 1 to 100 mM, final concentration.

Plot *A*: enzyme activity as a function of $MgCl_2$ concentration and enzyme concentration; reaction time was 10 min.

Plot *B*: derived from the data in plot *A*.


A
B
1·5
1·0
0·5
0
100
50
5
1
0
mM MgCl2
A420 / 10 min
0
20
40
µl sample
0
50
100
mM MgCl2
A420 / 10 min / 40 µl sample

addition of 0.5 ml M NaOH to each tube, and the tubes centrifuged. The absorbance of supernatant fluids was measured at 420 nm. All samples were assayed in duplicate, the two A_{420} values averaged, corrected for a blank value (assay mixture with water in place of sample; typical value A_{420} 0.03 against water-reference), and the result expressed as A_{420} per 10 minutes per 50 µl sample.

ii. Assays

Several classes of damage to the cell can be envisaged during spheroplast formation. (1) The plasma membrane may be disrupted, due to the degredative effects of the added enzymes and to membrane-swelling once the wall is weakened. Such damage should release alkaline phosphatase to the suspension fluids and the enzyme will be detectable both in supernatant fluids and in suspension *per se*. (2) Less severe membrane damage may occur, leaving the envelope intact but "leaky". Leakiness to small molecules, implying restricted damage, could result in alkaline phosphatase being detectable in spheroplast suspensions but not in supernatant fluids from the suspensions: the membrane would be permeable to pNPP, pNP and P_i (inorganic phosphate), but not to the enzyme. Leakiness to larger molecules suggesting more extensive damage, could result in loss of internal proteins to the suspension medium and both spheroplast suspensions and supernatant fluids would contain alkaline phosphatase. Any distinction between damage of this type and damage due to lysis (class (1)) would be equivocal on the basis of enzyme assay; the distinction would need to be made on the basis of visual examination of spheroplast suspensions.

In order to place an upper limit on the degree of lysis in spheroplast populations and to assess the type of damage incurred where

lysis was not indicated, K19.10 cells were converted to spheroplasts by each of the three procedures (glusulase, modified glusulase, zymolyase) and the process monitored by measuring the alkaline phosphatase present in each of three fractions: (I) the cell/spheroplast suspension; (II) the filtrate from a sample of the suspension filtered over glass-fibre; and (III) the supernatant from a centrifuged sample of suspension. "Control" suspensions, i.e. cells incubated without spheroplasting-enzymes, were also assayed in this way. To arrive at a measure of the total available alkaline phosphatase, spheroplasts were assayed after having been deliberately lysed with water.

Table 6.1 summarises the results of these measurements, the enzyme activities being expressed as the percentage of the total available (see footnote to Table for examples of actual 100 percent-activities). From this data, the percent of total activity present in the filtrates or supernatants (the lower value is used, since it presumably reflects the least destructive method for separating spheroplasts from suspension fluids) is taken to show the upper limit to spheroplast-lysis, while the difference between these values and those found in suspensions is taken to indicate the extent to which the spheroplast populations contain structures with plasma-membranes leaky to pNP and pNPP (see previous discussion).

Spheroplasts made with glusulase

The preparation of K19.10 spheroplasts with glusulase in 0.8 M sorbitol was, clearly, a very destructive process. By plus-60 minutes, 40 percent of cellular alkaline phosphatase was detectable in spheroplast

Table 6.1 Summary of assays for alkaline phosphatase in K19.10 suspensions.

"Medium"[a]	Cell wall degrading preparation	Time (min)	Alkaline phosphatase activity: percent of total in lysed spheroplasts[b]			Protocol number[f]
			suspension[c]	filtrate[d]	supernatant[e]	
Water	none	0	0.6	0.2	0.4	
		60	0.9	0.5	0.6	1
		120	1.7	0.5	0.6	
0.8 M sorbitol	none	0	1.2	0.3	0.4	
		60	1.2	0.2	0.8	2
		120	1.2	0.2	0.2	
0.8 M sorbitol	glusulase	0	0.1	0.0	0.0	
		60	31.0	15.0	3.0	3
		120	37.0	13.0	15.0	
1.2 M sorbitol	none	0	1.0	0.7	0.5	
		60	1.0	0.4	0.8	4
		120	1.3	0.2	0.4	
1.2 M sorbitol	glusulase	0	0.0	0.0	0.0	
		60	9.0	-	1.6	5
		120	9.0	3.6	2.7	
1.2 M sorbitol,	none	0	1.0	0.8	0.8	
10 mM tris-HCl,		60	1.1	0.3	0.7	6
2 mM DTT		120	1.3	0.4	0.4	
1.2 M sorbitol	zymolyase	0	4.8	2.7	4.2	
10 mM tris-HCl		60	32.5	9.2	15.9	7
2 mM DTT		120	41.8	12.0	15.9	

Table 6.1 - continued

1.2 M sorbitol,	none	0	0.8	0.8	2.4	
10 mM tris-HCl,		60	1.5	0.9	-	8
10 mM $CaCl_2$,		120	5.3	0.8	1.2	
2 mM DTT						
1.2 M sorbitol,	zymolyase	0	5.0	1.9	3.5	
10 mM tris-HCl,		60	10.3	2.6	4.5	9
10 mM $CaCl_2$,		120	13.3	3.2	5.2	
2 mM DTT						
1.2 M sorbitol,	zymolyase	0	4.9	1.7	3.3	
10 mM tris-HCl,		60	18.8	5.9	11.3	10
10 mM $MgCl_2$,		120	27.2	9.0	13.1	
2 mM DTT						

[a]Media buffered with tris-HCl were pH 7.5.

[b]Typical <u>total</u> alkaline phosphatase activity was 1.5 to 1.7 A_{420} units per 50 µl sample per 10 min (see text), where the sample was spheroplasts lysed in water at a concentration equal to that of the un-lysed suspensions.

[c]Cell/spheroplast suspension assayed as is.

[d]Cell/spheroplast suspension filtered over glass-fibre and the filtrate assayed.

[e]Cell/spheroplast suspension centrifuged and the supernatant fluids assayed. Centrifugation was done at the minimum force.time needed to pellet the structures.

[f]Protocol number 3 = glusulase procedure; number 5 = modified glusulase procedure; number 9 = zymolyase procedure.

suspensions; of this activity, about 50 percent could be attributed to lysis (Table 6.1, protocol number 3).

Increasing the sorbitol concentration to 1.2 M reduced substantially the extent of damage in spheroplast preparations. At the most, 4 percent of total alkaline phosphatase was recovered in suspension fluids, and about 9 percent of spheroplasts appeared to have suffered changes in their permeability to small molecules (Table 6.1, protocol number 5).

Spheroplasts made with zymolyase

The original protocol used involved incubation of cells with 1.2 M sorbitol, 10 mM tris-HCl, 2 mM DTT, pH 7.5 (protocol 7, Table 6.1). Visually, this method resulted in a high yield of spheroplasts, but also in considerable lysis. Based on alkaline phosphatase assays, 12 to 16 percent of spheroplasts were broken and about 40 percent were permeable to pNP and pNPP (Table 6.1).

Addition of $CaCl_2$ to the spheroplasting medium resulted in a dramatic reduction, visually, in the proportion of spheroplasts that was damaged. From the phosphatase assays it is estimated that no more than 5 percent of spheroplasts were broken; about 14 percent appeared to be permeable to pNP and pNPP (Table 6.1, protocol number 9). Magnesium chloride was not as effective as $CaCl_2$ in stabilising zymolyase spheroplasts (Table 6.1, protocol number 10).

IV. Versatility of the procedures

The zymolyase procedure, as described for strain K19.10, was successful with all of the yeast strains listed in Table 2.1 (page 8),

with a few exceptions (see below). "Success" means that, based on a visual examination, the cells were converted quantitatively to osmotically-sensitive structures, with less than 1 percent lysis at the time of harvesting the spheroplasts.

Exceptions were as follows: (I) Strains S30, R23 and S14.93 were partially-resistant to zymolyase-5000 -- the spheroplast preparations contained whole cells. (II) *T. glabrata* ATCC 15126 spheroplast preparations contained a significant fraction of broken structures, presumably as a result of relatively high sensitivity of the wall of this strain to the enzymes. (III) Preparing spheroplasts from ethanol-grown K19.10 cells (see Chapter 7) required approximately 400 µg zymolyase-5000 per ml of suspension, a four-fold higher level than that in the standard protocol. The increased enzyme level also resulted in considerable lysis of spheroplasts.

The modified glusulase-procedure appeared no more versatile than the original procedure using glusulase. This was expected, since the refractivity of certain yeasts to snail-gut enzymes must reflect their cell-wall structure, a structure not expected to be altered by simply increasing the sorbitol concentration.

Discussion

An effective and versatile method for converting cells to spheroplasts with zymolyase has been developed. The method has proven to be useful both for preparing toxin-sensitive spheroplasts (Chapter 7) and for preparing spheroplasts for the isolation and characterisation of the yeast cell-envelope (Bussey, Saville, Chevallier and Rank; manuscript in

preparation). The following discussion is concerned with the mechanism by which Zymolyase-5000 is effective, some observations on the effects of $CaCl_2$ and other substances on the spheroplasting procedures and the nature of the envelope surrounding the zymolyase spheroplast.

Preparation of spheroplasts requires a weakening of the cell wall so that the cells can no longer resist the osmotic stress of being exposed to solutions of low solute concentration. The *S. cerevisiae* wall has been shown to consist of glucans (polymers of glucose) and mannans (polymers of mannose), with lesser amounts of chitin, lipid and polypeptide; the mannans are often covalently linked to polypeptides (Phaff, 1971). The arrangement of these components is not known. Current models envisage the mannan-proteins to constitute the outermost layers of the wall and the glucans the layers closest to the plasma membrane (Lampen, 1968; Kidby and Davies, 1968, 1970). Manners, Masson and Patterson (1973) and Manners *et al.* (1973) have shown that the wall glucans are heterogeneous, there being two classes of polymer: approximately 85 percent consists of molecules in which the backbone is β-(1→3)-linked, with side-chains attached to the backbone; the remainder is β-(1→6)-linked in the backbone, with side-chains attached *via* β-(1→3)-glucosidic bonds. Glucanases are effective in producing spheroplasts from whole cells and it is clear that the glucans are the primary determinant of the rigidity of the wall and so the shape of the cell. All of the many enzyme preparations found to be effective for spheroplast production contain β-(1→3)-glucanases and/or β-(1→6)-glucanases (Kuo and Yamamoto, 1975; Rambourts and Phaff, 1976a) and in those cases where the glucanases have been isolated from the extracts

they can be shown to effectively lyse viable cells or at least degrade isolated cell walls (e.g. Rambourts and Phaff, 1976a, 1976b).

The effectiveness of Zymolyase-5000 is attributed to its glucanase activities. According to its supplier, the preparation contains a β-(1→3)-glucanse, zymolyase (also a β-(1→3)-glucanse, with preference for long glucan molecules; Kitamura and Yamamoto, 1972) and minor activities of protease, mannanase and acid phosphatase (Kirin Brewery Co., Ltd., Takasaki, Gumma Pref., Japan). The mannanase and protease activities may have a role in the action of Zymolyase-5000, but according to Kitamura and Yamamoto (1972) purified zymolyase is very effective in degrading yeast cell-walls.

The inclusion in zymolyase spheroplasting medium of dithiothreitol, as recommended by the supplier of Zymolyase-5000, was essential to the production of spheroplasts from cells of strain K19.10. Facilitation of cell-wall degredation by thiols is well known (Burger, Bacon and Bacon, 1961; Duell, Inoue and Utter, 1964; Schwenke, Magana-Schwenke and Laporte, 1977) and may be due to the splitting by the reagents of disulphide bridges between cystine residues in the wall proteins (mannan-proteins). Presumably agents such as dithiothreitol facilitate glucanase action on the wall by opening up the mannan-proteins to allow the enzymes access to glucans.

Inclusion of calcium chloride in the zymolyase spheroplasting medium overcame to a large degree two drawbacks to the original protocol (protocol 7, Table 6.1): a gross agglutination of spheroplasts and a considerable degree of spheroplast-lysis. Calcium chloride was not

necessary in solutions with which the spheroplasts were washed, stored or treated with toxins (Chapter 7) -- spheroplasts remained intact and only mildly aggregated in $CaCl_2$-free media. The effects of other salts on the spheroplasting procedure were not examined systematically but equimolar $MgCl_2$ or $MgSO_4$ substituted for $CaCl_2$, partially in preserving spheroplast intactness and completely in precluding gross agglutination. In a study of *S. carlsbergensis* glusulase-spheroplasts, Indge (1968) found that magnesium chloride at 1 mM preserved the structure of lysed-spheroplasts and retarded the destructive effects of chelating agents on the plasma-membrane of intact spheroplasts, results that suggest that a divalent metal cation is necessary to the physical integrity of the spheroplast envelope. However, the modified-glusulase spheroplasts prepared here from strain K19.10 were as stable as were the zymolyase spheroplasts, and the glusulase procedure did not involve addition of divalent metal cations (1-10 mM $CaCl_2$ completely suppressed spheroplast formation in glusulase-treated K19.10 cell-suspensions, data not shown; see also Hutchison and Hartwell, 1967, for a similar effect of $MgCl_2$). It appears that Mg^{2+} or Ca^{2+} is necessary to the integrity of the spheroplast membrane only while zymolyase and DTT are present. The cations may alter the nature of zymolyase-susceptible wall components or the enzyme activities themselves; then again they may protect the plasma membrane from some of the various activities present in Zymolyase-5000, perhaps by binding to negatively-charged groups on membrane-proteins.

No effort was made to determine the nature of zymolyase-induced spheroplast agglutination but the effect of $CaCl_2$ or $MgCl_2$ in preventing

most of the clumping is presumably a result of their modifying the Zymolyase-5000-susceptible wall components or neutralising negative charge. Glusulase spheroplasts showed no aggregation; the surface structure of zymolyase and glusulase spheroplasts is clearly quite different.

Zymolyase spheroplasts were bounded by a thick "wall" which may have contributed to their unusual resistance to damage during preparation and handling. While not obvious from examination of intact spheroplasts (Plate 6.4) the "wall"s were quite apparent in spheroplast suspensions which had been osmotically-lysed with water or low concentration buffers. If intact spheroplasts were transferred from medium containing 1.2 M sorbitol to an excess volume of 0.2 to 0.3 M sorbitol or sucrose, the naked spheroplasts could be seen to pop-out from the "wall"s; after a few minutes the released spheroplasts, swelling in the low concentration solution, burst. Plate 6.5 (photograph *A*) shows zymolyase spheroplasts in the process of being released from the "wall". Centrifuging an osmotically-lysed spheroplast suspension through a dense cushion of a material such as Renografin-76 pelleted the "wall"s, leaving the spheroplast-membranes (and other material) at the interface. Plate 6.5 (photograph *B*) shows a crude "wall" pellet from spheroplasts of strain K19.10.

I did not manage to isolate the spheroplasts from the "wall"s in a way which left the naked spheroplasts intact. The reduction in the osmotic potential of the suspension fluid necessary to induce the release of the spheroplast from the "wall" appeared to damage the spheroplast-

Plate 6.5

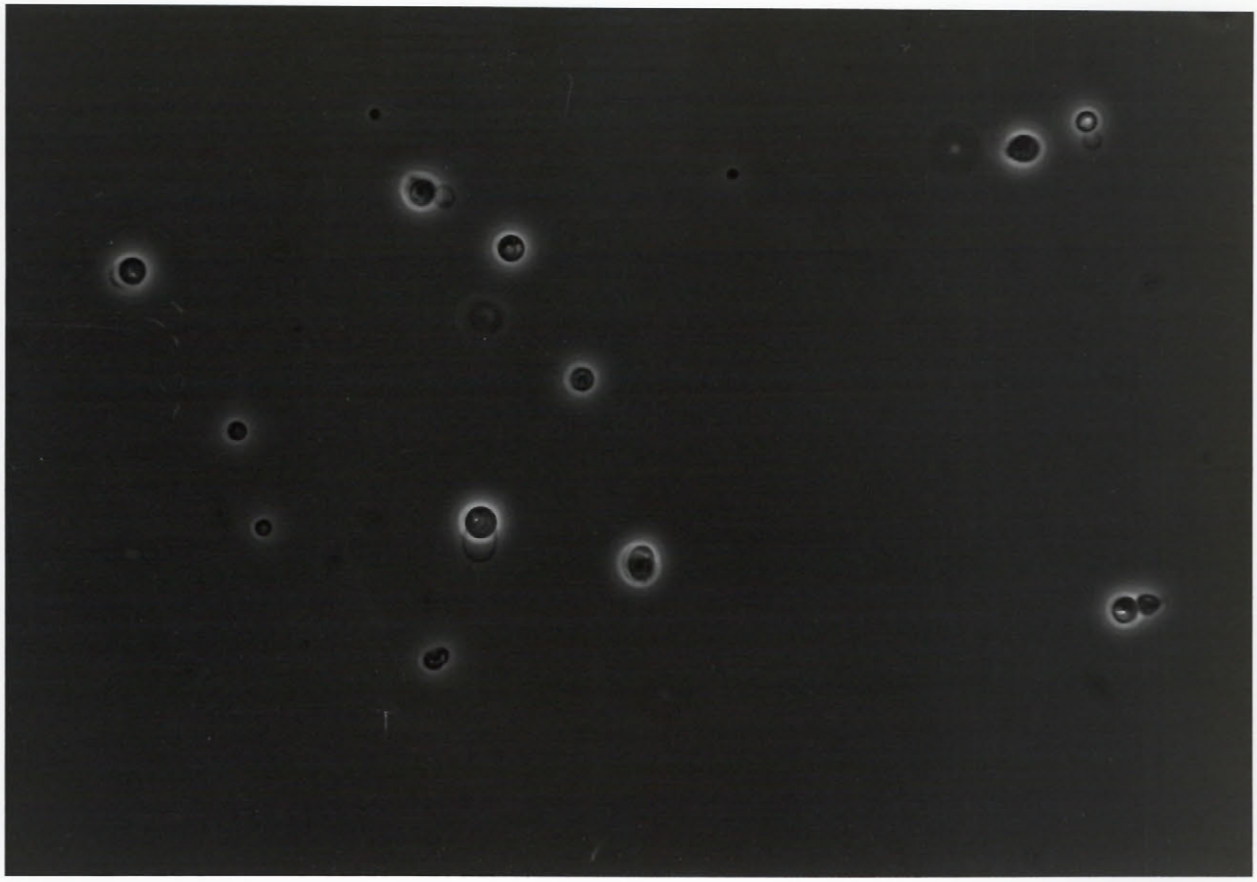
The "wall" left on spheroplasts after treatment of K19.10 cells with zymolyase.

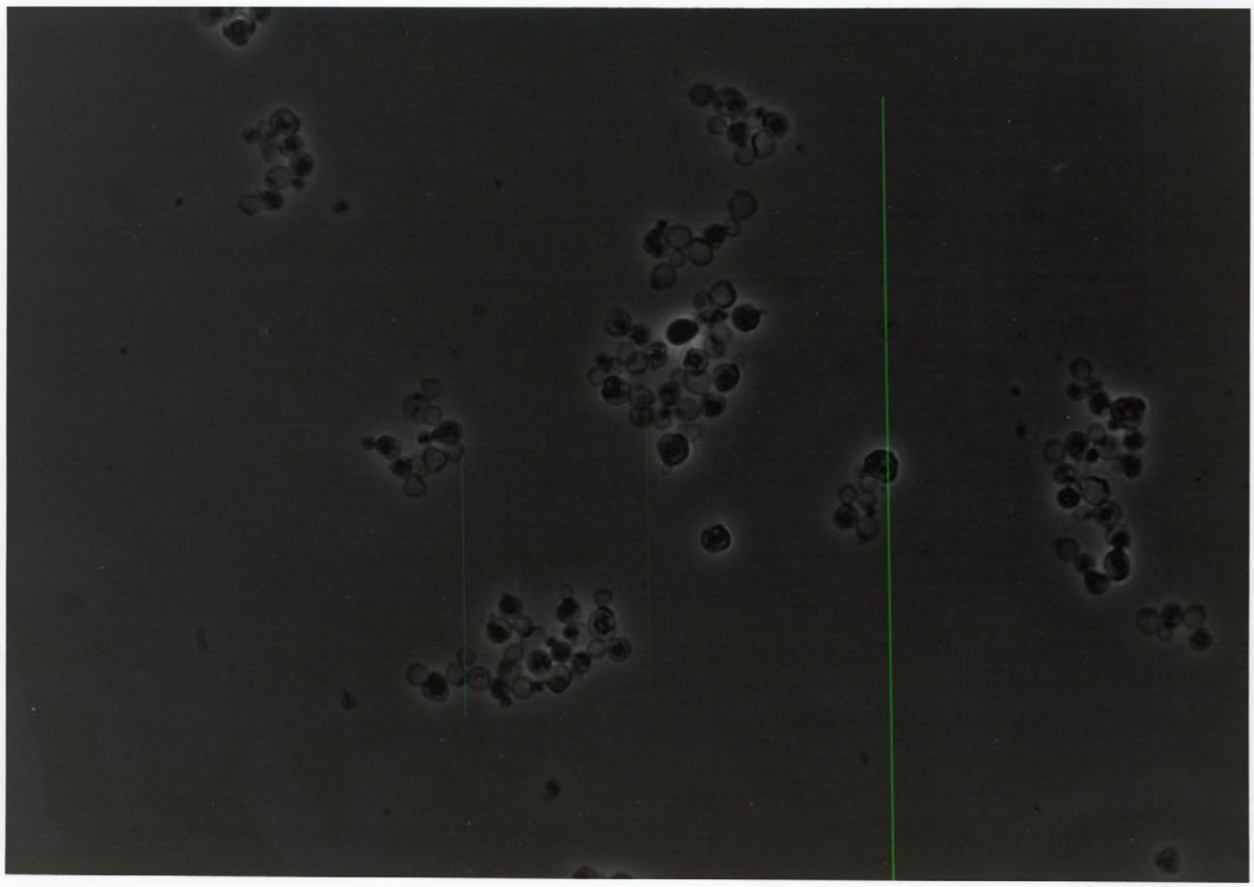
Photograph *A*, separation of "wall" from spheroplast (magnification 800 x).

K19.10 zymolyase-spheroplasts in 1.2 M sorbitol, 10 mM $CaCl_2$, 10 mM tris-HCl, pH 7.5, were added to an excess volume of 0.2 M sucrose. The spheroplasts can be seen to be in various stages of being released from the overlying "wall".

Photograph *B*, crude "wall" fraction (magnification 800 x).

K19.10 zymolyase-spheroplasts were lysed with water, layered over a cushion of 40 percent v/v Renografin-76 and centrifuged at 10,000 x g. for 5 min in a swinging-bucket rotor. The pellet was resuspended in 10 mM tris-HCl buffer, pH 7.5 and was the "wall"preparation.





membrane: restoring the sorbitol concentration to 1.2 M did not completely prevent lysis in the population of naked spheroplasts.

In summary, it is clear that incubating cells with Zymolyase-5000 results in limited alteration to wall-structure. Nevertheless the alteration is sufficient to render the cells susceptible to osmotic stress. It is also evident that the majority of our laboratory strains of *S. cerevisiae* contain Zymolyase-5000-susceptible components in their walls, a comment that cannot be made with respect to susceptibility to snail-gut enzymes. Nothing is known of the structure of the zymolyase-"wall", although Rank (personal communication) has found that ghosts isolated from lysed zymolyase-spheroplasts contain no mannose (ghosts are cell-shaped envelopes of high density and are thought to contain both the "wall" and the spheroplast plasma-membrane). The spheroplasts used as substrates for yeast toxins (Chapter 7) were enveloped by the "wall" and it would be useful to know the composition and structure of the layer, in view of the results obtained in the experiments described in Chapter 7.

Chapter 7

EFFECTS OF TOXINS ON SPHEROPLASTS PREPARED FROM TOXIN-SENSITIVE, TOXIN-PRODUCING AND TOXIN-RESISTANT CELLS

Introduction

The alteration to the yeast cell-wall necessary to spheroplast formation might be expected to modify the response of cells to toxins, given that the initial event in toxin action is binding to cell-wall receptors.

Bussey, Sherman and Somers (1973) examined killer toxin-binding (as defined by the activity-removal assay, General Introduction) and killer toxin action on cells and glusulase-spheroplasts from toxin-producing, sensitive and resistant strains. They found that although glusulase removed at least 99 percent of the toxin binding capacity of S14 cells, the S14 spheroplasts were toxin-sensitive. Spheroplasts from resistant mutants R14 and R18, strains deficient as whole-cells at binding toxin, were also killer toxin sensitive, suggesting that the sites available to toxin on glusulase-spheroplasts are distinct from those available on whole-cells. Spheroplasts from the toxin-producing strain K12 remained toxin-insensitive. The implication of the results with R14 and R18, that resistance of whole-cells to killer toxin can be due to a masking of sensitive spheroplast-sites by modification to the cell-wall, could not be properly tested by Al-Aidroos (1975) with her collection of toxin-resistant mutants since many of the mutants proved to be refractive to snail-gut enzymes.

It was decided to take advantage of the new method for spheroplast preparation, and the sensitive ^{42}potassium-release assay for toxin action, to examine the role of the native cell-wall in the response of sensitive strains to toxins and to examine a selection of resistant mutants for the possibility that their resistance was due to masking of sensitive surface-sites.

Results

I. Spheroplasts from toxin-sensitive cells

The protocol used to demonstrate toxin-sensitivity in glusulase spheroplasts involved the fresh preparation of spheroplasts, incubation of spheroplasts in a nutrient medium from 2 to 3 hours and then a 3 hour incubation with toxin (Bussey, Sherman and Somers, 1973). The following procedure for testing toxin-sensitivity of zymolyase spheroplasts, developed for strain K19.10, was less tedious. The spheroplasts could be prepared several days in advance of their use and extended incubation before challenge with toxins was not necessary.

Spheroplasts were tested for sensitivity to toxins by measuring their ability to retain pools of ^{42}K.

Potassium loading

Zymolyase spheroplasts, prepared as described in Chapter 6, were harvested and resuspended in 1.2 M sorbitol, 10 mM sodium acetate buffer, 5 mM KCl, pH 4.7, ("medium"), to a density of A_{600} 1.9 to 2.1 (1.4 to 2.0 x 10^8 spheroplasts per ml; see Figure 6.1). Two ml of spheroplasts were allowed to equilibrate to room-temperature, then loaded with ^{42}K by adding glucose to 2 percent w/v (or ethanol to 2 percent v/v in the case

of spheroplasts prepared from respiring cells) and ^{42}KCl. After 30 to 45 minutes the suspension was diluted with about 10 ml medium and the spheroplasts harvested by centrifugation. The supernatant was discarded and the pellet resuspended with 10.0 ml medium containing either glucose or ethanol as the energy source. Labeled spheroplasts were kept on ice until used in experiments.

Potassium-loading in spheroplasts, as in cells (Chapter 3), was measured by filtering a sample over a glass-fibre filter, washing the filter with unlabeled medium and counting the radioactivity trapped by the filter. Spheroplasts were retained quantitatively by glass-fibre filters.

Potassium retention

Labeled spheroplasts at a density of 2.8 to 4.0 x 10^7 per ml were incubated at room-temperature with appropriate additions and sampled at intervals for ^{42}K. Radioactivity remaining in spheroplasts was defined as the cpm retained on glass-fibre filters (see potassium-loading). The results were expressed as the percent ^{42}K remaining in spheroplasts, where 100 percent was the activity at time zero.

Toxin dose

Toxins were added to spheroplast suspensions at a dose which in suspensions of K19.10 whole-cells was sufficient to kill approximately 95 percent of the population by plus-180 min. No attempt was made to measure the survival of spheroplasts on agar plates.

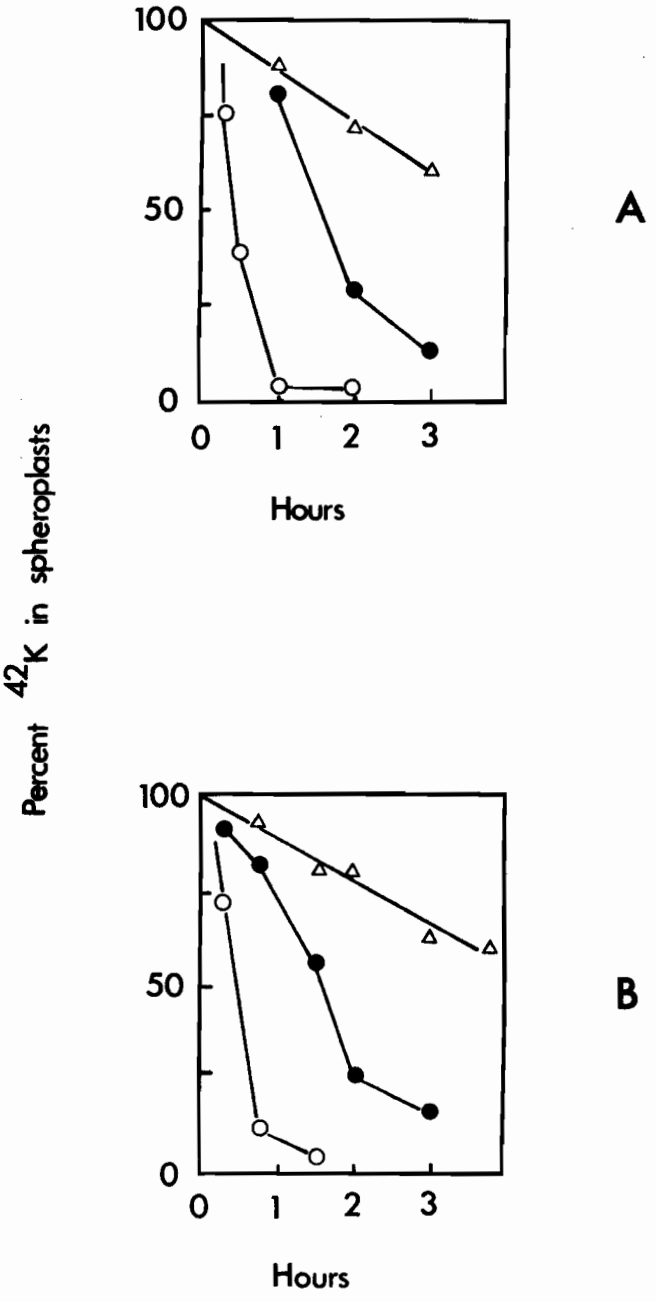
Response of spheroplasts to toxins

Figure 7.1 shows the result of incubating zymolyase spheroplasts

Figure 7.1 Potassium efflux as a measure of toxin action on spheroplasts.

Spheroplasts prepared with zymolyase from NaYEPD-grown cultures of strains K19.10 and S14 were loaded with ^{42}K and treated at time zero with either killer toxin, PEST or with no additions (control). Radioactivity remaining in spheroplasts was measured with time. Symbols: (●), plus killer toxin; (○), plus PEST; (△), control. Room temperature was 27°C.

Plot *A*: K19.10 spheroplasts; plot *B*: S14 spheroplasts.


100
50
0
0 1 2 3
Hours
A
Percent ^{42}K in spheroplasts
100
50
0
0 1 2 3
Hours
B

from strains K19.10 and S14 with either killer toxin or PEST. As was the case with whole-cells of these strains, the spheroplasts lost ^{42}K in the presence of the toxins. Control (untreated) spheroplasts, like untreated cells, lost radioactivity at a slow rate indicative of a $^{42}K/^{39}K$ exchange reaction across the plasma membrane.

Effect of energy-source and energy-poisons on the response of spheroplasts to toxins

In the scheme for toxin action described in Chapter 5, the lag-period was postulated to be the time required for the state 1 to state 2 transition to occur in cells that had bound a potentially-lethal dose of toxin within the first few minutes after toxin addition. Energy poisons were considered to interfere with killer toxin action by prolonging this transition indefinitely. Killer toxin-treated zymolyase spheroplasts, like glusulase spheroplasts (Bussey, 1974), displayed a lag-period similar to that characteristic of sensitive whole-cells (Figure 7.1). There was no reason then to suppose that energy-poisons should not arrest killer toxin action on zymolyase spheroplasts.

Figure 7.2, plots *A* and *B*, shows that omission of the energy-source (glucose) from K19.10 spheroplast suspensions blocked the ^{42}K-release characteristic of killer toxin action. This dependence of killer toxin action on glucose was also indicated in earlier experiments, with ^{42}K-loaded whole-cells, but in a much less clear-cut way than with the spheroplasts (data not shown). Given that the cell/spheroplast must be "energised" to be damaged by killer toxin, then this difference

in the degree to which spheroplasts and cells responded to toxin in the absence of added glucose may reflect a difference in energy reserves. Zymolyase spheroplasts had been effectively starved prior to toxin-treatment (120 min at 30°C during preparation, then several days at 0 to 4°C during storage, in media devoid of an energy source). Energy-reserve materials such as glycogen and trehalose (Suomalainen and Oura, 1971) might be expected to be exhausted in the spheroplasts, relative to the whole-cells.

In the presence of glucose, DNP or iodoacetate blocked killer toxin-induced ^{42}K-release from spheroplasts; potassium cyanide did not (Figure 7.2, plots *C*, *D* and *E*; spheroplasts were from glucose-grown cells).

Figure 7.3 illustrates the result of adding killer toxin to ^{42}K-loaded zymolyase spheroplasts isolated from ethanol-grown K19.10 cells. As was the case for respiring whole-cells, the spheroplasts did not lose potassium in the presence of toxin if either cyanide or antimycin (mitochondrial inhibitors), iodoacetate (inhibitor of alcohol dehydrogenase) or DNP (proton-conductor) was present. However the omission from the spheroplast suspensions of ethanol did not prevent killer toxin-induced potassium release (plot *A*, compare with plot *B*). It is difficult to reconcile this result with that found in spheroplasts from glucose-grown cells (Figure 7.2) without advocating that the spheroplasts from respiring cells retain energy-reserves during what appears to be a starvation condition.

Potassium efflux from K19.10 spheroplasts treated with PEST was

Figure 7.2 Potassium efflux in spheroplasts isolated from glucose-grown K19.10 cells: effect of energy source and energy poisons on response of spheroplasts to killer toxin.

^{42}K-loaded spheroplasts suspended in 1.2 M sorbitol, 10 mM sodium acetate, 5 mM KCl, pH 4.7, were incubated at time zero under each of the following regimes and sampled at intervals for radioactivity.

Plot *A*, with or without killer toxin;

plot *B*, plus glucose, with or without killer toxin;

plot *C*, plus glucose and DNP, with or without killer toxin;

plot *D*, plus glucose and iodoacetate, with or without killer toxin;

plot *E*, plus glucose and cyanide, with or without killer toxin.

Concentrations: glucose, 2 percent w/v; DNP, 0.5 mM; iodoacetate, 1 mM; cyanide, 1 mM; toxin, 10 µl per ml (see text). Symbols: (●), with toxin; (○△), without toxin.

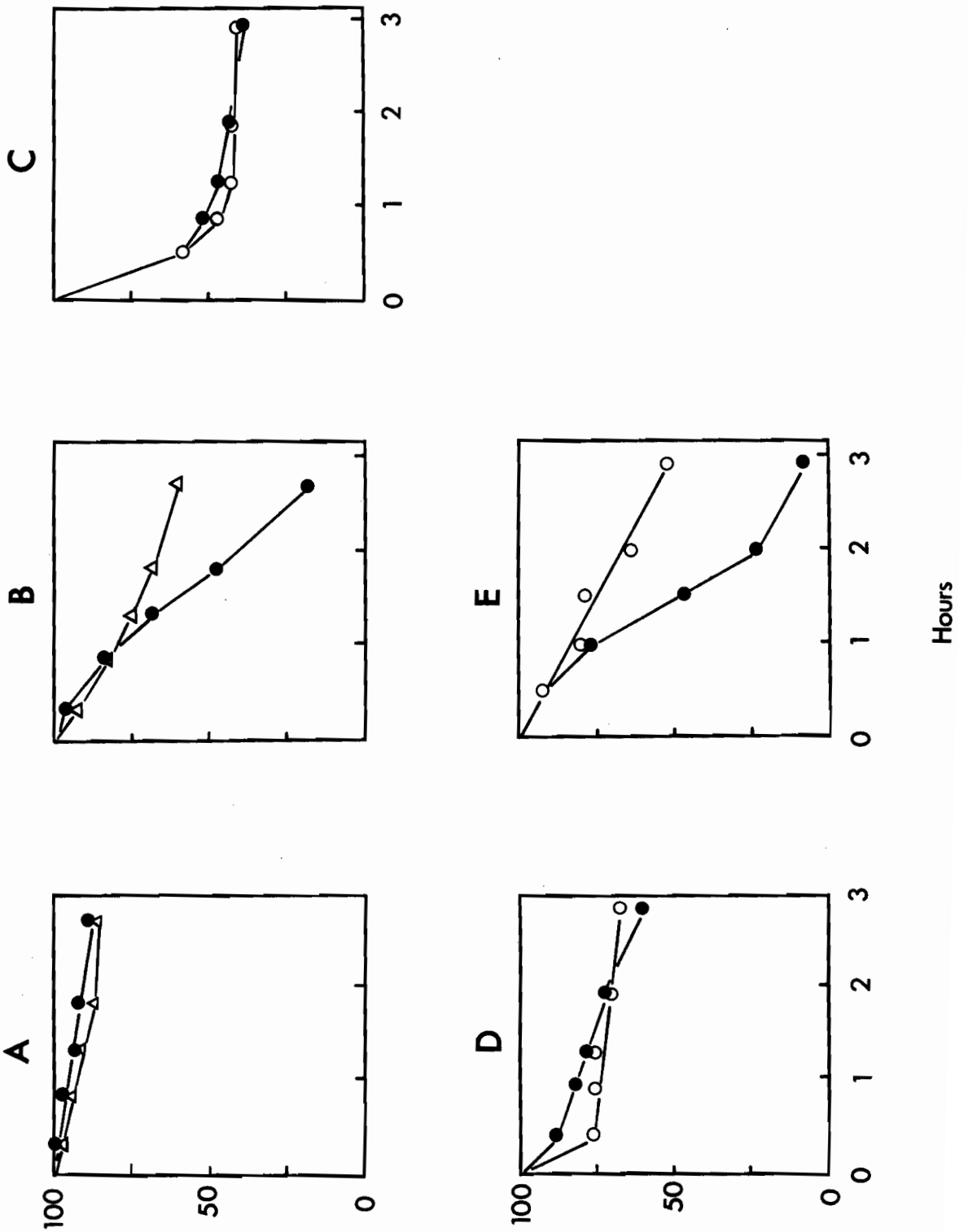

A
B
C
D
E
100
50
0
0
1
2
3
Hours
Percent ^{42}K in spheroplasts

Figure 7.3 Potassium efflux in spheroplasts isolated from ethanol-grown cells: effect of energy source and energy poisons on response of spheroplasts to killer toxin.

^{42}K-loaded K19.10 zymolyase spheroplasts suspended in 1.2 M sorbitol, 10 mM sodium acetate, 5 mM KCl, pH 4.7, were incubated at time zero under each of the following regimes and sampled at intervals for radioactivity.

Plot *A*, with or without killer toxin;

plot *B*, plus ethanol, with or without toxin;

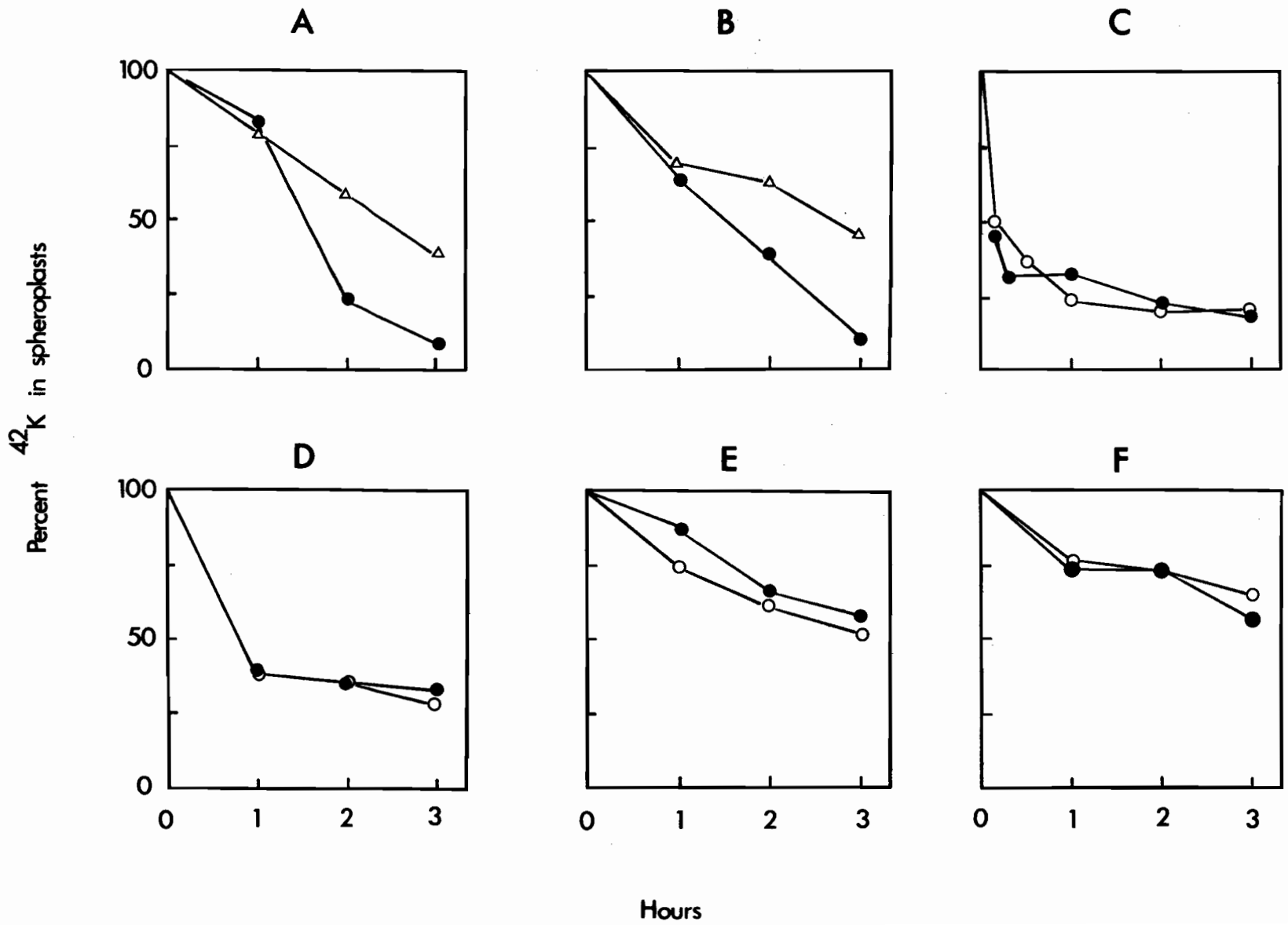
plot *C*, plus ethanol and DNP, with or without toxin;

plot *D*, plus ethanol and iodoacetate, with or without toxin;

plot *E*, plus ethanol and cyanide, with or without toxin;

plot *F*, plus ethanol and antimycin, with or without toxin.

Concentrations: ethanol, 2 percent v/v; DNP, 0.5 mM; iodoacetate, 1 mM; cyanide, 1 mM; antimycin, 10 µg per ml; toxin, 10 µl per ml. Symbols: (●), with toxin; (△○), without toxin.


A
B
C
D
E
F
100
50
0
0
1
2
3
Percent ^{42}K in spheroplasts
Hours

not blocked by either DNP, CCCP, iodoacetate, cyanide or antimycin. Figure 7.4 shows this result for spheroplasts from glucose-grown cells, Figure 7.5 for spheroplasts from ethanol-grown cells.

While the effect of energy poisons on killer toxin action in spheroplasts appeared to be identical to that on whole cells (Chapter 5), a distinction between inhibition by the poisons of toxin-binding and subsequent events in toxin action was not possible with spheroplasts since no attempt was made to measure the viability of toxin-treated spheroplasts.

II. Spheroplasts from toxin-producing cells

Glusulase spheroplasts from strain K12 retained the resistance of K12 cells to killer toxin (Bussey, Sherman and Somers, 1973). Similarly, zymolyase spheroplasts from cells of strain K19 or K12 and from *T. glabrata*, were insensitive to the appropriate toxin (Figure 7.6). The spheroplasts remained sensitive to the toxin to which the cells were sensitive (Figure 7.6).

III. Spheroplasts from toxin-resistant cells

Ten of the mutants isolated from sensitive strain S14 on the basis of resistance to killer toxin by Al-Aidroos (1975) and three of the mutants isolated from sensitive strain SS14 by Bussey (personal communication) as killer toxin resistant were converted to zymolyase spheroplasts, loaded with ^{42}K and challenged with toxins. The methods for these experiments were as described for strain K19.10 (glucose-grown cells).

Figure 7.4 Potassium efflux in spheroplasts isolated from glucose-grown cells of strain K19.10: effect of energy poisons on response of spheroplasts to PEST.

^{42}K-loaded zymolyase-spheroplasts in 1.2 M sorbitol, 10 mM sodium acetate, 5 mM KCl, pH 4.7, plus 2 percent w/v glucose, were incubated at time zero under each of the following regimes and sampled at intervals for radioactivity.

Plot *A*, plus PEST; no additions (control);

plot *B*, plus DNP; plus DNP and PEST;

plot *C*, plus CCCP; plus CCCP and PEST;

plot *D*, plus iodoacetate; plus iodoacetate and PEST;

plot *E*, plus antimycin; plus antimycin and PEST;

plot *F*, plus cyanide; plus cyanide and PEST.

Concentrations: drugs, as given in legend to Figure 7.3; CCCP was 0.5 mM; PEST, 10 µl per ml. Symbols: plus PEST (●), without PEST (○△), (Plots *A*, *B*, *D*, *E* and *F*); plus PEST (○), without PEST (●), (Plot *C*).

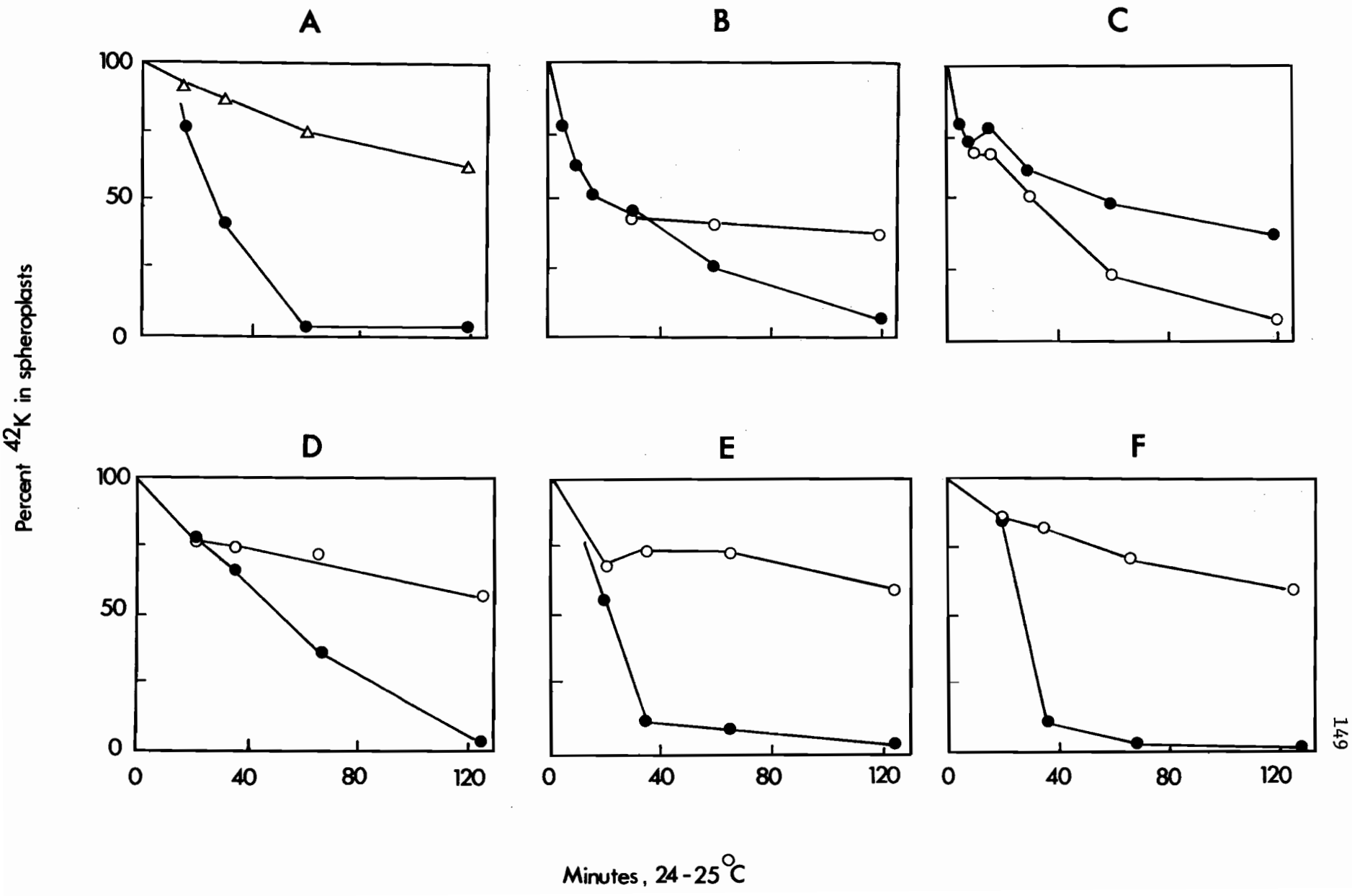

A
B
C
D
E
F
100
50
0
0
40
80
120
Percent ^{42}K in spheroplasts
Minutes, 24-25°C

Figure 7.5 Potassium efflux in spheroplasts isolated from ethanol-grown cells of strain K19.10: effect of energy poisons on response of spheroplasts to PEST.

The experiment was done as described in the legend to Figure 7.2 except that spheroplast suspensions contained 2 percent v/v ethanol in place of glucose.

Plot *A*, plus PEST; no additions (control).

Plot *B*, plus DNP; plus DNP and PEST.

Plot *C*, plus CCCP; plus CCCP and PEST.

Plot *D*, plus iodoacetate; plus iodoacetate and PEST.

Plot *E*, plus antimycin; plus antimycin and PEST.

Plot *F*, plus cyanide; plus cyanide and PEST.

Symbols: (●), plus PEST; (○), without PEST.

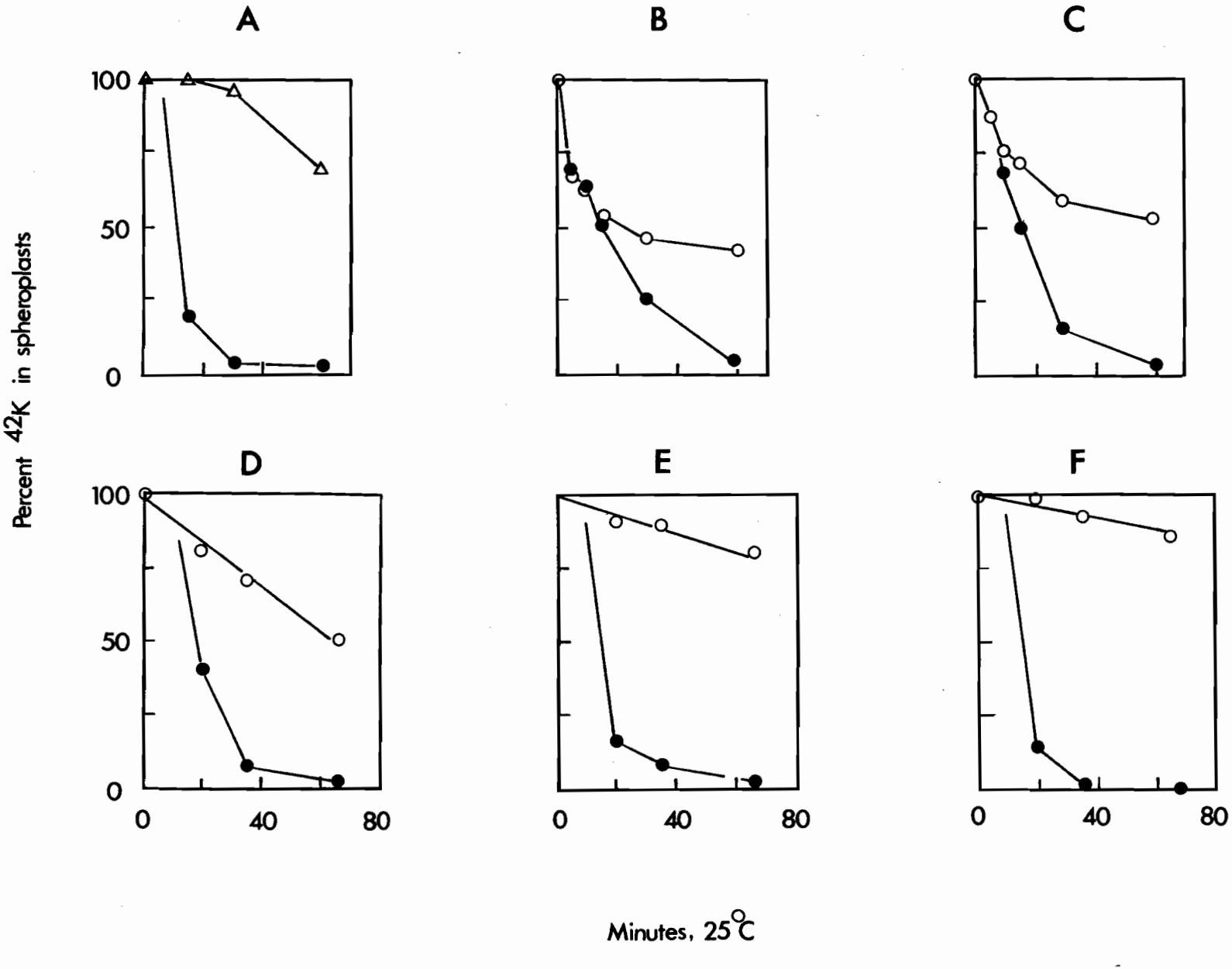

A
B
C
D
E
F
100
50
0
0
40
80
Percent ^{42}K in spheroplasts
Minutes, 25°C

Figure 7.6 Response of potassium pool of spheroplasts from toxin-producing strains to killer toxin and PEST.

^{42}K-loaded zymolyase-spheroplasts, prepared from *S. cerevisiae* K12 and K19, and *T. glabrata* ATCC 15126, were suspended in 1.2 M sorbitol, 10 mM sodium acetate, 5 mM KCl, pH 4.7, plus 2 percent w/v glucose, and at time zero treated with either killer toxin, PEST or no additions. At intervals the suspensions were sampled for ^{42}K remaining in spheroplasts. Symbols: (●), plus killer toxin; (○), plus PEST; (△), no additions. Plot *A*, K19; plot *B*, K12; plot *C*, *T. glabrata*. Toxin concentrations were sufficient to kill at least 95 percent of cells of toxin-sensitive strain K19.10 within 3 hours.

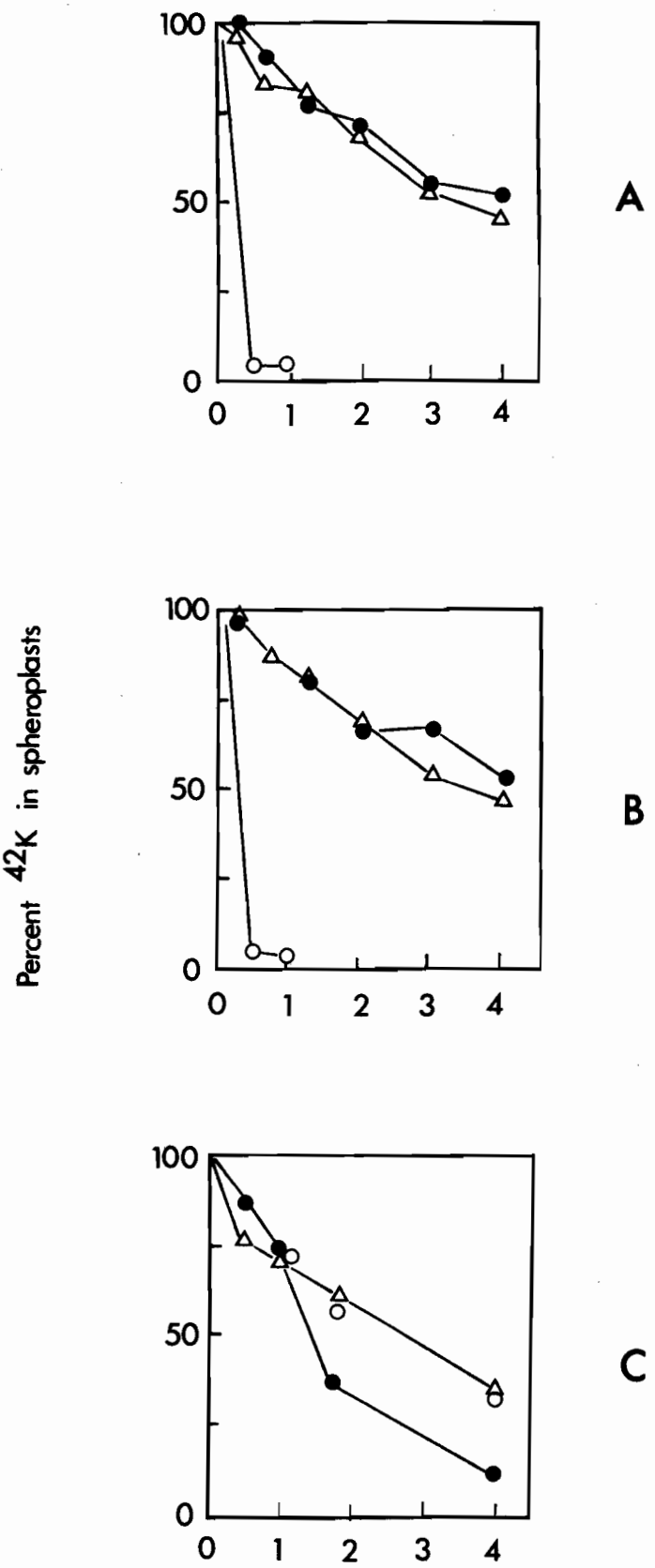

100
50
0
0 1 2 3 4
A
B
C
Percent ^{42}K in spheroplasts
Hours

Table 7.1 summarises the results. The table includes also the results of challenging ^{42}K-loaded whole-cells of the resistant mutants with toxins and the results of assaying whole cells and spheroplasts of the toxin-producing and toxin-sensitive strains. The response of cells or spheroplasts to toxins is given as either resistance, partial-resistance or sensitivity, as judged by the kinetics of ^{42}K-release. Figure 7.7 illustrates each response; all of the ^{42}K-release measurements used to construct Table 7.1 are given either in the text or Appendix A. In Figure 7.7, plot *A*, R94 cells were resistant to either toxin, plot *B*, R94 spheroplasts were sensitive to PEST, plot *D*, S14.14 spheroplasts were sensitive to killer toxin and in plot *F*, S14.96 spheroplasts were partially resistant to killer toxin.

Discussion

Spheroplasts from sensitive cells

With the aim of reducing the complexity of the toxin-cell interaction, we had intended to examine the effects of killer toxin and PEST on plasma-membrane vesicles isolated from zymolyase spheroplasts. Isolation of a vesicle fraction with some function that may have proved to be useful as an assay for toxin action was not achieved. As a prelude to work with isolated plasma membranes the response of spheroplasts to toxins was examined.

The ability of either killer toxin or PEST to efflux ^{42}K from sensitive whole cells was clearly not affected by the cell-wall modification implicit to the cell to spheroplast conversion. In addition, the energy poison sensitivity of killer toxin action on potassium pools and the poison

Table 7.1 Summary of sensitivity of cells and spheroplasts to toxins (^{42}K-release assay).

Strain	Complementation group[a]	Resistance gene[a]	Sensitivity to toxin[b,c]			
			Killer toxin		PEST	
			Cells	Spheroplasts	Cells	Spheroplasts
K12	-	-	R	R	S	S
K19	-	-	R	R	S	S
T. glabrata	-	-	S	S	R	R
S14	-	-	S	S	S	S
K19.10	-	-	S	S	S	S
S30	-	-	S	S	S	S
S14.94	1	-	R	R	R	S
S14.96	1	kre 1	R	R^p	R	S
S30.5	1	-	R^p	R	S	S
S30.16	1	-	R^p	R	S	S
S14.14	11	kre 2	R	S	R	S
S30.49	11	-	R	R	S	S
S30.53	11	-	R	R	S	S
S14.MB6	111	kre 3	R	R	R^p	S
S14.75	-	-	R	S	R	S
S14.93	-	-	R	R	R	S
R18	-	-	R^p	S	R^p	S
R23	-	-	R	S	R	S
R94	-	-	R	R	R	S

Table 7.1 - continued

[a] Al-Aidroos and Bussey, 1978.

[b] As measured by ^{42}K-release assay.

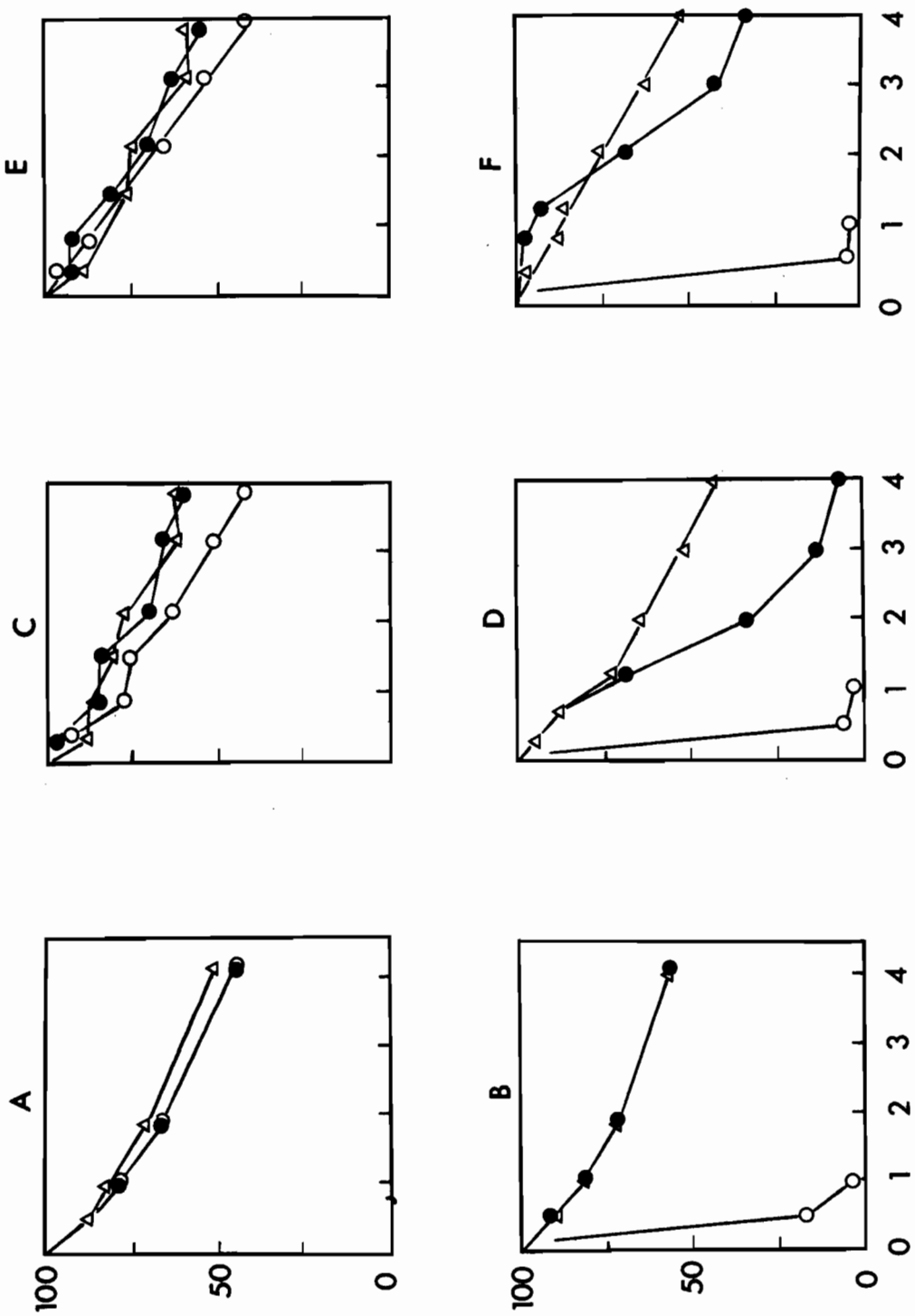
[c] R: resistant; R^p: partially-resistant; S: sensitive.

Figure 7.7 Response of cells and spheroplasts from toxin-resistant mutants to toxins.

^{42}K-loaded cells of strains R94, S14.14 and S14.96 resuspended in NaYEPD medium and ^{42}K-loaded zymolyase-spheroplasts of the same strains suspended in 1.2 M sorbitol, 10 mM sodium acetate, 5 mM KCl, pH 4.7, plus 2 percent w/v glucose, were incubated at time zero with either killer toxin, PEST or with no additions (control). At intervals suspensions were sampled for ^{42}K retained by cells or spheroplasts.

Plot *A*, R94 cells; plot *B*, R94 spheroplasts; plot *C*, S14.14 cells; plot *D*, S14.14 spheroplasts; plot *E*, S14.96 cells; plot *F*, S14.96 spheroplasts.

Symbols: (●), plus killer toxin; (○), plus PEST; (△), control.


A
B
C
D
E
F
100
50
0
0 1 2 3 4
Percent ^{42}K remaining
Hours

insensitivity of PEST action on these pools, was retained in spheroplasts. These results, like those of Bussey, Sherman and Somers (1973) with glusulase spheroplasts of strain S14, imply that the native structure of the cell wall in sensitive whole-cells plays no essential role in toxin action on these strains. It should be possible to isolate toxin-sensitive plasma-membrane vesicles from spheroplasts of sensitive strains.

Spheroplasts from toxin-producing cells

The nature of autoimmunity of toxin-producing cells is not understood, although in the case of *S. cerevisiae* killer strains immunity is known to be determined by the M dsRNA genome (General Introduction). On the basis of the resistance to killer toxin of zymolyase spheroplasts (Table 7.1) and glusulase spheroplasts (Bussey, Sherman and Somers, 1973), the autoimmunity of killer strains is not related to the structure of the cell wall. A similar argument can be made for the immunity of *T. glabrata* to PEST.

Spheroplasts from toxin-resistant cells

The results support the conclusion of Bussey, Sherman and Somers (1973), that resistance of whole cells to toxin can be due to a masking of sensitive spheroplast sites. They also support the comment of Al-Aidroos and Bussey (1978) that the genetic background of the cell that carries a resistance mutation can modify the phenotype of the mutant.

I. Killer toxin resistance

As was the case with glusulase spheroplasts of these strains (Al-Aidroos and Bussey, 1978), zymolyase spheroplasts of the group 1 S14-derived mutants were resistant (Table 7.1). Strain S14.96 spheroplasts were,

however, partially-sensitive to killer toxin, and it is possible that the group 1 mutants have toxin-sensitive spheroplast sites but that neither glusulase nor Zymolyase-5000 alters the cell wall sufficiently to expose these sites to toxin. The S30-derived group 1 mutants are distinct phenotypically from the S14-derived strains; in terms of the potassium-release assay the spheroplasts were more resistant to toxin than the whole-cells (Table 7.1).

The killer toxin-sensitivity of the spheroplasts from the group II S14-derived mutant S14.14 suggests that this strain carries a mutation that, as in mutants R18 and R14 in the report of Bussey, Sherman and (1973), modifies the S14 cell surface so as to mask the access of toxin to spheroplast sites. Mutations in the same gene, as defined by a complementation test, did not result in the S30-derived mutants having toxin-sensitive spheroplasts (Table 7.1), a result that is discussed in a subsequent paragraph.

The single known example of a group III mutation, strain S14.MB6, is the only resistant mutant known to have wild-type capacity to bind killer toxin (by the activity-removal assay, General Introduction) (Al-Aidroos and Bussey, 1978). The resistance of S14.MB6 zymolyase spheroplasts is consistent with a hypothesis that this strain is tolerant, i.e., resistant because of an alteration to a component that is necessary to an event in toxin action that is subsequent to toxin-binding to the wall. It is possible that S14.MB6 is a "target"-mutant.

The other mutants examined here are genetically ill-defined, however strains S14.75 and R23, like the group II mutant S14.14, appear

to retain toxin-sensitive spheroplast sites since their zymolyase spheroplasts effluxed ^{42}K in the presence of toxin (Table 7.1). Strain R18 was not fully toxin resistant as a whole-cell and appears to have an altered phenotype from that described by Bussey, Sherman and Somers (1973).

II. PEST resistance

Killer toxin resistance that is due to modification to a post toxin-binding event would not be expected to result in a co-resistance to PEST, given that the post-binding events are distinct in killer toxin and PEST action (Chapter 5). Co-resistance might be expected from modification to wall structure if the initial surface-binding events are common to both toxins or if the surface receptors share components.

All of the S14-derived killer toxin-resistant mutants were at least partially-resistant to PEST as whole-cells and the PEST-resistant strains were all PEST-sensitive as spheroplasts (Table 7.1). These results suggest that the group I, II and III mutations each alter the S14 cell wall so as to mask access of PEST to sensitive surface-sites. While group I and II mutants are known to have altered walls, as defined by subnormal killer toxin-binding capacity (Al-Aidroos and Bussey, 1978), the group III mutant, S14.MB6, is not altered in this respect. It is not obvious how a mutation in a single gene could alter both a component necessary to post-binding events in killer toxin action (see above) and a cell-surface component so as to block the access of PEST to spheroplast sites.

III. The problem of genetic background

The results shown in Table 7.1 for toxin-resistant mutants are difficult to interpret in terms of a one complementation group -- one function hypothesis. While the properties of mutants in each of groups I and II are consistent within one genetic background, S30 or S14, the properties are not shared across the two series of strains. As discussed by Al-Aidroos and Bussey (1978), S30 and S14 are distinct, both in terms of cell wall structure and response to killer toxin. Strain S30 is refractive to both snail-gut enzymes (Al-Aidroos, 1975) and Zymolyase-5000 (Chapter 6) and a cross between S30 and S14 showed that more than one gene was involved in the difference between S30 and S14 in glusulase-sensitivity (Al-Aidroos and Bussey, 1978). A further cross between S30 and S14 yielded spores whose sensitivity to killer toxin ranged from 10 to 100 percent of either of the parents' (Al-Aidroos and Bussey, 1978). It seems likely then that the expression of the group I and II mutations is different in S30 and S14 because of the difference in genetic background.

Chapter 8

GENERAL DISCUSSION

The killer toxin's effects on K19.10 cultures and *T. glabrata* ATCC 15126 cultures are consistent with the conclusion of Bussey and Sherman (1973), that the *S. cerevisiae* toxin damages the plasma membrane in sensitive cells.

Events such as toxin-induced potassium efflux may be sufficient to kill cells, but it is not known whether this or any other of the known events is a measure of the primary toxin-induced alteration in cells. The lag-period in either killer toxin or PEST action implies that a relatively slow post-binding event preceeds the membrane alterations. If all of the known effects of the toxins are in fact consequent to a more primary damaging event then it should be possible to block any or all of them without altering the primary damage. If any of the known metabolic alterations is also unnecessary to the lethal effect of the toxins, then such blocks should not affect the death of toxin-treated cultures (see footnote on page 161 for an example of an apparently unnecessary event, in colicin action).

As mentioned in Chapter 3, hydrolysis of cellular ATP in toxin-treated cells is not readily explained in terms of alterations to plasma membrane permeability. The membrane-acting colicins also induce ATP hydrolysis in sensitive cells although, in contrast to killer toxin-treated cells, the nucleotide is not released to the growth medium (Fields and Luria, 1969; Hirata *et al.*, 1969; Feingold, 1970). Plate

et al. (1974) have suggested that the ATP hydrolysis due to colicin action is the result of a futile effort by the cell to re-energise the plasma membrane*, the colicin's primary effect being to disrupt the energised-state of the membrane. If yeast cells, like bacteria (Hamilton, 1975), use a membrane-spanning ATPase to energise membrane-linked processes via ion-gradients then disruption of the gradients, and as a result of the energised-state, by toxins may result in ATP hydrolysis via ATPase stimulation. The effects of DNP and CCCP on glucose-grown yeast cells (Chapter 5) suggest that these cells do use proton-gradients to energise membrane function.

The molecular mechanism by which killer toxin or PEST, either directly or indirectly, modifies the permeability of the yeast plasma membrane is not known. If the toxins cause structural alterations to the membrane then such alterations are subtle: two-dimensional (sodium dodecyl sulphate) polyacrylamide-gel electrophoresis of the ghost-proteins isolated from cells treated with either toxin reveal no

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*Colicin-induced ATP hydrolysis is eliminated by either using ATPase-negative mutants or cells treated with the ATPase-inhibitor, N,N^1 dicycylohexylcarbodiimide as the sensitive cells (Feingold, 1970; Philips and Cramer, 1973; Plate *et al.*, 1974). This prevention of ATPase stimulation does not prevent the onset of other colicin-induced events such as potassium efflux (Feingold, 1970) or death (Plate *et al.*, 1974). ATP hydrolysis is thus an effect of the colicin-induced damage and not a cause of the damage.

alteration to the surface proteins (Bussey and Saville, unpublished data). If the toxins disrupt membrane-transport in a specific way, by modifying the coupling of metabolic energy to transport, then it should be possible to isolate mutants whose resistance to the toxins is due to a modified coupling mechanism.

While the metabolic alterations seen in PEST or killer toxin-treated cells are similar, implying a similarity between the biochemical target of each toxin, the results of treating toxin-sensitive cells with energy-poisons or cycloheximide suggest that the events leading to the onset of these alterations are distinct. The K19.10 cell appears to be a passive target for PEST action, but in killer toxin action only the binding to cells of a potentially-lethal dose of toxin is passive. The inhibition of killer toxin-induced metabolic alterations by cycloheximide suggests that the energy poison-sensitivity of toxin action may be due to a requirement for cell-surface proteins of high turnover-rate.

Colicin action, like that of killer toxin, is blocked by energy-poisons, although not by inhibition of cellular protein synthesis (see Holland, 1975, for review). In colicin-treated populations the cells undergo a transition from a state in which they can be spared from death by an incubation with trypsin to a state in which they cannot (Reynolds and Reeves, 1963, 1969; Plate and Luria, 1972). The rate of the transition corresponds to the rate at which the population is damaged by colicin (Plate and Luria, 1972). This transition is blocked by either energy-poisons or conditions such that the cells are starved for an energy-source i.e., energy starvation results in permanent trypsin-rescueability

presumably because, under this constraint, the potentially-damaging colicin molecules remain trypsin-accessible (Jetten and Jetten, 1975; Okamoto, 1975). Killer toxin-treated cells are known to be spared from being killed by a potentially-lethal dose of bound toxin by treatment with glusulase (Bussey, 1972). If the energy poison-sensitivity of killer toxin action is of a similar nature to that of colicin action, then toxin-treated cells should be maintained in a condition such they can all be spared from death by glusulase for as long as a reagent such as DNP is present.

The results of Bussey, Sherman and Somers (1973) on the role of the cell wall in killer toxin action have been confirmed here and extended to the action of PEST. The analysis of the response of zymolyase spheroplasts to toxins shows that although the toxins act on spheroplasts in the same way as on whole cells, the spheroplast sites, at least in some resistant mutants, are distinct from whole-cell sites. A reasonable explanation for the resistance of these mutants is that their cell wall is altered so that toxins can no longer penetrate the outer envelope to reach the spheroplast sites. The nature of the group I and II resistance mutations is not clear, because of the problem of genetic background of the parent sensitive-strains. Further mutants should be isolated in an effort to analyse the cellular components necessary to toxin action. In particular, target mutants (tolerant mutants) should be looked for.

Two other yeast toxins have been examined recently for their effects on sensitive cells. A toxin from a brewery killer strain of *S. cerevisiae* (Maule and Thomas, 1972), designated Tox_2 toxin by Rogers

and Bevan (1978), has been found to kill K19.10 cultures by a mechanism similar to that of killer toxin (Rogers, 1976; Rogers and Bevan, manuscript in preparation). However, the effects due to Tox_2 toxin were not coincident and this evidence for a definite sequence of toxin-induced alterations to cells deserves to be examined in more detail.

A toxin from a killer yeast isolated from sake mash (Inamura, Kawamoto and Takaoka, 1974) has been shown to damage *S. cerevisiae* cells by a mechanism involving inhibition of macromolecule synthesis and ATP efflux (Kotani, Shinmyo and Enatsu, 1977). Calcium chloride was found to block the ATP efflux due to sake toxin action and Kotani *et al.* suggested that the salt prevents the occurence of the damaging event in the cells. Calcium chloride at 20 mM has no effect on the action of either killer toxin or PEST (my own observations) so it appears that the sake toxin acts by a mechanism distinct in its details from that of both killer toxin and PEST.

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CLAIM TO CONTRIBUTION TO ORIGINAL KNOWLEDGE

1. Killer toxin-treated sensitive cells do not overproduce ATP. The cellular pools of ATP are depleted in the presence of toxin by hydrolysis to ADP and AMP and by leakage to the suspension fluids. ADP and AMP are also found in the fluids following toxin treatment of sensitive cells.

2. ^{42}K-loaded sensitive cells, but not toxin-producing or toxin-resistant cells, discharge their ^{42}K to the suspension fluids following killer toxin treatment. The measurement of ^{42}K-release from cells provides a useful assay for toxin action.

3. A toxic extract from cultures of the pathogenic yeast *T. glabrata*, PEST, kills sensitive cells by a mechanism which involves damage to plasma membrane permeability. The effects of PEST on sensitive yeast cells resemble those of the *S. cerevisiae* killer toxin, except that the PEST does not promote ATP leakage from cells.

4. A model for yeast toxin action is proposed in which toxin-treated cells pass from a state in which they have bound a potentially-damaging dose of toxin to a state in which they have been damaged. The lag-period characteristic of toxin action is postulated to be the minimum interval necessary for the transition between these states.

5. Killer toxin action on yeast ^{42}K pools, but not PEST action, is blocked by energy-poisons. The inhibition of killer toxin action appears to be of an event that is subsequent to toxin-binding. In terms of the model for toxin action, energy-poisons are proposed to act so as to prolong the reaction defined by the lag-period indefinitely.

6. Killer toxin action on culture absorbance and on retention by cells of arginine-derived pools, but not PEST action, is blocked by cycloheximide. A requirement by killer toxin action for cellular protein synthesis is indicated.

7. Zymolyase-5000 can be used to prepare intact spheroplasts from cell suspensions of many yeast strains.

8. In terms of the ^{42}K-release assay for toxin action, zymolyase spheroplasts from toxin-sensitive or toxin-producing (toxin-immune) whole-cells retain the response of the cells to toxins. The mechanism of action of the toxins on sensitive spheroplasts appears to be identical to that on cells: killer toxin action on the spheroplast ^{42}K-pools, but not PEST action, is blocked by energy-poisons.

9. The resistance of mutants to PEST is eliminated by the structural changes to the walls of these cells brought about by spheroplasting, a result implying that the resistance of the whole-cells is due to

cell wall-modification that blocks the access of the toxin to sensitive surface-sites. The resistance of some strain S14-derived mutants to killer toxin is similarly eliminated by converting cells to spheroplasts.

Appendix A. Potassium efflux in cells and spheroplasts.

The following figures, A1 to A3, show the results of the measurement of ^{42}K-retention by whole-cells and zymolyase-spheroplasts from various strains of *S. cerevisiae*; the data has been used in Table 7.1.

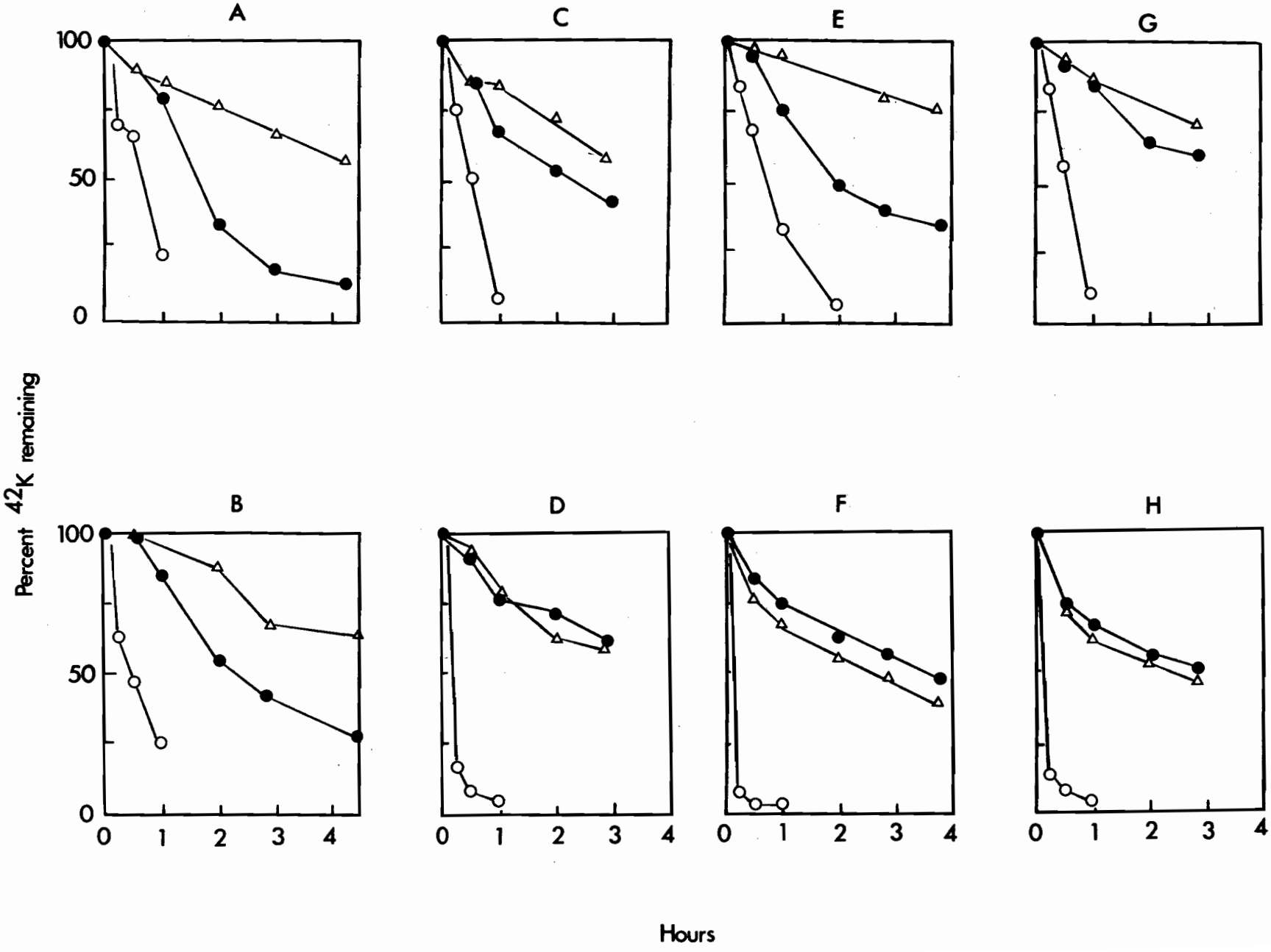
In each case the cells or spheroplasts were prepared, loaded with ^{42}K and treated with toxins as described in the text for glucose-grown cultures of strain K19.10.

A1 Plots *A*, *B*: S30 cells, S30 spheroplasts;
plots *C*, *D*: S30.5 cells, S30.5 spheroplasts;
plots *E*, *F*: S30.16 cells, S30.16 spheroplasts;
plots *G*, *H*: S30.49 cells, S30.49 spheroplasts.

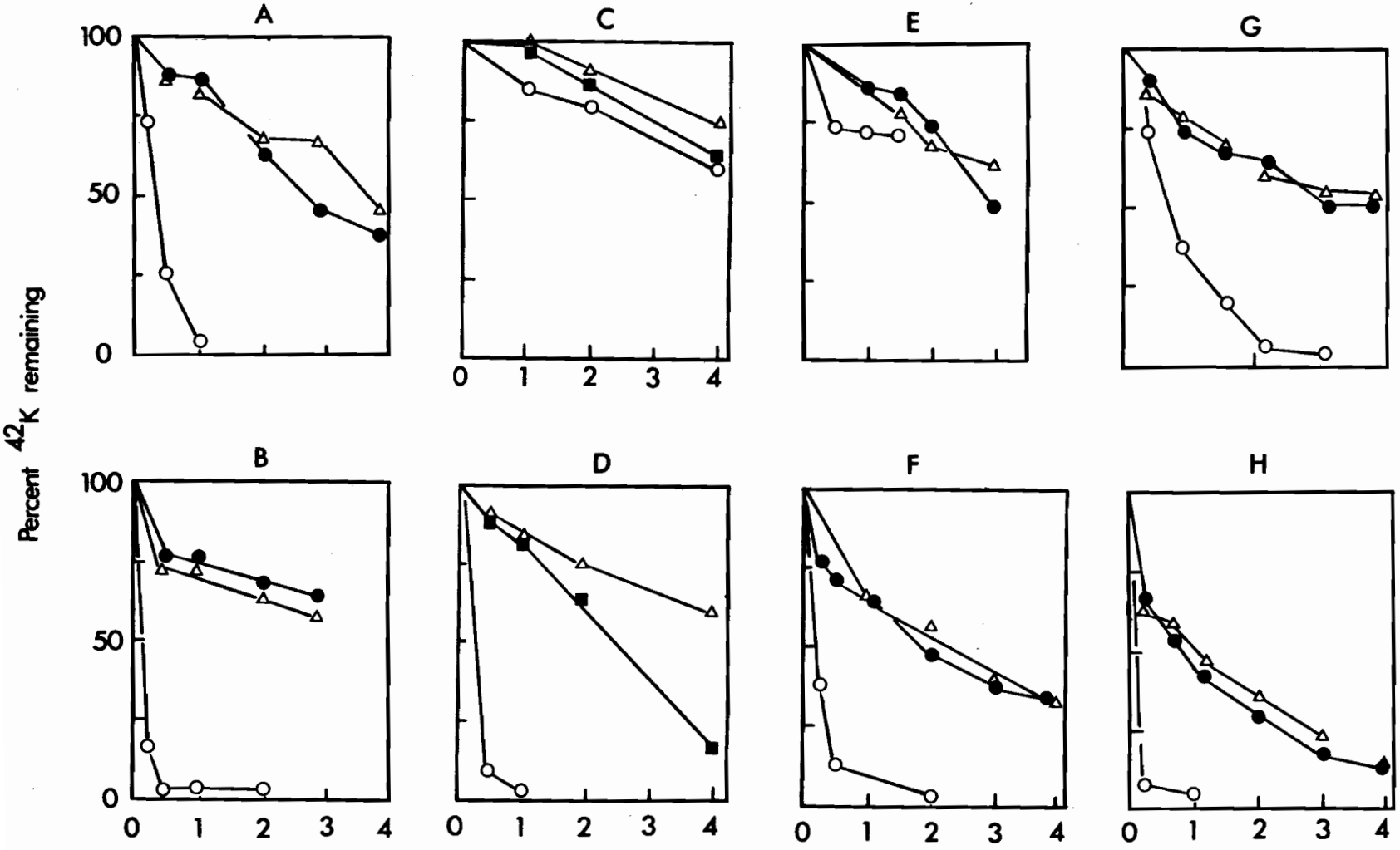
A2 Plots *A*, *B*: S30.53 cells, S30.53 spheroplasts;
plots *C*, *D*: R23 cells, R23 spheroplasts;
plots *E*, *F*: S14.94 cells, S19.94 spheroplasts;
plots *G*, *H*: S14.MB6 cells, S14.MB6 spheroplasts.

A3 Plots *A*, *B*: S14.75 cells, S14.75 spheroplasts;
plots *C*, *D*: S14.93 cells, S14.93 spheroplasts;
plots E, F: R18 cells, R18 spheroplasts.

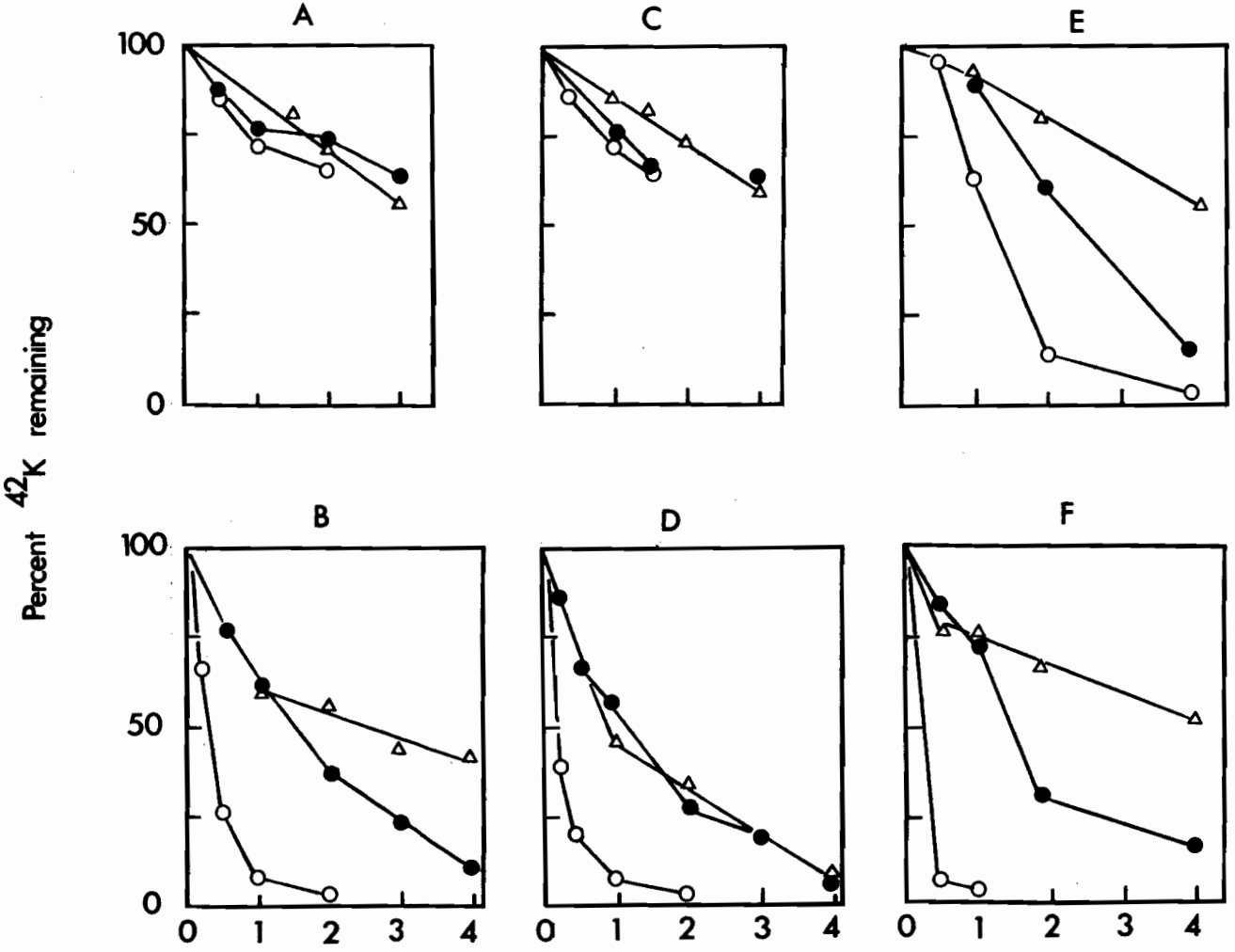
Symbols: (△), control; (●), killer toxin; (○), PEST.



A1



A2


A
C
E
B
D
F
100
50
0
0
1
2
3
4
Percent ^{42}K remaining
Hours

Appendix B. Manuscripts

Membrane-mediated killing of *Saccharomyces cerevisiae* by glycoproteins from *Torulopsis glabrata*. Bussey, H. and Skipper, N. 1975. J. Bact. 124: 476-483.

Killing of *Torulopsis glabrata* by *Saccharomyces cerevisiae* killer factor. Bussey, H. and Skipper, N. 1976. Antimicrob. Agents and Chemother. 9: 352-354.

Mode of action of yeast toxins: energy requirement for *Saccharomyces cerevisiae* killer toxin. Skipper, N. and Bussey, H. 1977. J. Bact. 129: 668-677.

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Membrane-Mediated Killing of *Saccharomyces cerevisiae* by Glycoproteins from *Torulopsis glabrata*

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Cell-free supernatants from cultures of *Torulopsis glabrata* contained glycoprotein toxins that killed sensitive and killer strains of *Saccharomyces cerevisiae* with single-hit kinetics. Growing *S. cerevisiae* treated with the toxins showed a leakage of cellular potassium, partial dissipation of the adenosine triphosphate pool, and a coordinate shutdown of macromolecular synthesis. These pool efflux-stimulating toxins have been partially purified and at least three toxic glycoproteins have been separated. Pool efflux-stimulating toxin activity was stable from pH 3 through 7, though killing was maximal close to pH 4.

The *Saccharomyces cerevisiae* killer factor kills sensitive yeast by a mechanism involving adenosine triphosphate (ATP) leakage, inhibition of macromolecular synthesis, and cell shrinkage (2, 3). We have screened other yeast species for killer factor-like activity and have found that extracellular extracts from *Torulopsis glabrata* cultures kill both killer and sensitive strains of *S. cerevisiae*. This paper describes the partial purification of the pool efflux-stimulating toxins (PEST) produced by *T. glabrata* and presents evidence that they act by interfering with the cytoplasmic membrane of sensitive cells.

MATERIALS AND METHODS

Strains and media. *T. glabrata* ATCC 15126 was obtained from the American Type Culture Collection, Rockville, Md. *S. cerevisiae* strains are shown in Table 1. Cultures of *Saccharomyces* were grown at 22 to 24 C in a yeast extract-peptone medium (1) with 2% glycose (YEPD). For experiments with ^{42}KCl, the YEPD was modified to contain 5 mM KCl, and Na_2HPO_4 replaced K_2HPO_4 (Na-YEPD). Media with pH values from 3 to 7 were prepared and contained yeast extract, 0.5%; peptone, 0.5%; citrate-K_2HPO_4 buffer, 0.1 M (5); and glucose, 2%. When required for petri plates, agar was included in media at a concentration of 2%.

Preparation of T. glabrata extracellular components. *T. glabrata* was grown in YEPD medium with the following modifications: yeast extract and peptone were each present at 0.5% and had been prefiltered through an Amicon PM-30 membrane. Batch cultures of 5 or 10 liters were grown in 1-liter flasks at 30 C overnight in a New Brunswick shaker at 200 rpm to a Klett value (blue filter) of 450 to 620. Cultures were chilled on ice and centrifuged at 10,000 × *g* for 20 min. The medium was concentrated by ultrafiltration on a PM-30 membrane to 50 to 100 ml. The concentrate was spun at 27,000 × *g* for 10 min to remove remaining cells and debris. This concentrate contained the PEST activity and was used in this crude form for most experiments. Killing activity was measured by the well test method (1, 14). Killing units were arbitrarily assigned and were obtained from a calibration curve based on a PEST dilution series. Crude extracts contained polysaccharide-to-protein ratios of 3:1 to 9:1, determined as described previously (1).

Uptake of labeled precursors into cellular pools and macromolecules. Strain S14a was grown in YEPD at 22 to 24 C to about 1.5×10^7 colony-forming units (CFU)/ml. Cytosine-[2-^{14}C]sulfate, D-[U-^{14}C]glucose, or L-[U-^{14}C]arginine was added to 2.5×10^{-4} M, 1.6 Ci/mol; 0.11 M, 3.63 mCi/mol; or 5×10^{-3} M, 0.08 Ci/mol, respectively, and the cultures were split. PEST extract was added to a final concentration of 0.2 to 0.4 mg of protein per ml to one portion and 0.1 M acetate buffer, pH 4.7, was added to the other, and the cultures were returned to incubation. At intervals each culture was assayed for radioactivity in pools and macromolecules by filtering 0.5 ml on a glass fiber disk (GF/A Reeve Angel), washing the filter with 10 ml of unlabeled growth medium, and extracting the filter with 10 ml of 60% ethanol. Counts per minute in pools (ethanol wash) and macromolecules (ethanol-extracted filter) was determined in Aquasol (NEN). Radiochemicals were from Amersham/Searle.

Measurement of the stability of the yeast potassium pool. Cultures of either K12 or S14a were grown at 22 to 24 C in Na-YEPD to about 8×10^6 CFU/ml culture, ^{42}KCl was added to about 3×10^5 counts/min per ml of culture, and incubation was continued for 2 h. During this time radioactivity in the cells, measured on culture samples that had been filtered on glass fiber disks and washed with unlabeled medium, became constant with cell mass, measured as culture turbidity. The loaded cells were harvested by filtration, washed thoroughly with unlabeled Na-YEPD, and resuspended in Na-YEPD to about 1.5×10^7

TABLE 1. *S. cerevisiae strains*

Strain	Genotype	Source or reference
K12, ATCC 28683	α, $ade_{2,8}$, M(k)	(3)
K19	a, trp_8, leu_1, M(k)	J. M. Somers
S14a, ATCC 28684	α, $ade_{2,8}$, M(o)	Clone from S14 (3)
S14a.96, ATCC 28685	α, $ade_{2,8}$, kre_1, M(o)	Killer-resistant mutant from S14a (K. Al-Aidroos)
K19.10	a, trp_8, leu_1, M(o)	Spontaneous killer-sensitive clone from K19

CFU/ml. The suspension of labeled cells was split, and each portion received either PEST to a final concentration of 0.1 mg of protein per ml or an equivalent volume of acetate buffer. Both cultures were returned to incubation and sampled at intervals for radioactivity in the cells by filtering 0.5 ml of culture on glass fiber disks, washing the filter with 10 ml of unlabeled medium, and counting the filter.

Radioactivity in ^{42}K samples was measured as described by Lauchli (9). Count rates were corrected for decay over experimental times. ^{42}KCl was from New England Nuclear Corp.

Measurement of adenylates. Cultures of S14 or K19.10 were grown at 22 to 24 C in YEPD to about 1.5 $\times$ 10^7 CFU/ml and divided. Each portion then received either PEST to a final concentration of 70 μg of protein per ml or acetate buffer (control) and was returned to incubation. Cultures were sampled at intervals for total and medium adenylates by an ethanol extraction. To extract total culture adenylates, 400 μl of culture was pipetted into 600 μl of absolute ethanol at 80 C. After 10 min at 80 C, the tube was transferred to ice, and the contents were diluted with 9 ml of cold water and filtered through a glass fiber disk. To extract adenylates in the medium, about 900 μl of culture was filtered rapidly, and 400 μl of the filtrate was processed with ethanol as described for total adenylates.

Adenosine monophosphate (AMP), ATP, and adenosine diphosphate in the samples were measured by the method of Chapman et al. (4). Pyruvate kinase (EC 2.7.1.40) and myokinase (EC 2.7.4.3) were from Sigma Chemical Co. To measure ATP in a processed sample, 100 μl of reconstituted Sigma firefly lantern extract (FLE-50) was added to a glass scintillation vial containing 1 ml of buffer (40 mM glycylglycine, 3 mM $MgCl_2$, pH 7.3) and 100 μl of the sample. After 7 s, counts per minute was recorded over 0.1 min in a Beckman LS-100 scintillation counter set at 100% gain and a wide-open window. Adenylates in yeast samples are expressed as nanomoles per milliliter of culture and are not adjusted for adenylates in sterile YEPD medium.

Survival of toxin-treated cells. Cell survival in response to a given dose of PEST is expressed as the percentage of cells able to produce colonies on YEPD agar at the end of the incubation, where 100% is the number of CFU per milliliter immediately before PEST addition.

Materials. Polyethylene glycol 6000 was from Baker. Diethylaminoethyl-Sephadex A-25 and A-50, SP-Sephadex C-25, Sephadex G-25 coarse, and concanavalin A (ConA) HTP was from Bio-Rad Laboratories.

RESULTS

Nature of toxin. The dialyzed extracellular concentrates from *T. glabrata* growth media contained all detectable killing activity. Electrophoresis of this material on 3% acrylamide-sodium dodecyl sulfate (SDS) gels gave many bands; all stained with the carbohydrate-specific periodic acid-Schiff procedure (15) and most stained weakly with Coomassie brilliant blue (12). Thus all proteins migrating on the gels appeared to be glycoproteins. The extracts were fully soluble in saturated solutions of ammonium sulfate and in 10% trichloroacetic acid and had absorbancy at 280 nm-absorbancy at 260 nm ratios of 1.2 to 0.9. These properties suggest that the extracellular material was not derived from a small fraction of lysed cells in the cultures. The PEST activity in extracts was precipitated with 40% (wt/vol) ethanol and was destroyed by boiling (5 min at 100 C). More than 99% of the activity was lost after treatment with Pronase (1 mg/ml) for 90 min in 0.1 M sodium acetate-acetic acid buffer, pH 4.7 (AB), at 30 C.

Partial purification of PEST glycoproteins. PEST was partially purified (Table 2), and at least three components having killing activity were obtained. The extracellular concentrate was precipitated with polyethylene glycol 6000 at 13.0% (wt/vol) in AB and left on ice for at least 1 h before centrifuging at 27,000 $\times$ *g* for 10 min. The pellet was washed three times with 13.0% polyethylene glycol, resuspended in 20 ml of AB, and after 30 min recentrifuged. The supernatant was applied to a double column of Sephadex A-25 (acetate form) and Sephadex C-25 (sodium form), each 2.5 by 7.5 cm, and eluted with AB. The eluted material contained most (60 to 80%) of the activity and was concentrated by ultrafiltration.

Chromatography on Sephadex C-25 columns of polyethylene glycol-precipitated material indicated that a small amount of killing activity (1 to 10%) was absorbed to the column and could be eluted with a linear 0 to 1 M NaCl gradient. This material (CII) showed a single diffuse band on SDS-gel electrophoresis (Fig. 1) and was pure by this criterion. Component CII

TABLE 2. *Summary of steps in the partial purification of PEST glycoproteins*

Purification step	Total killing units	Protein (mg)	Polysaccharide (mg)	Sp act (killing units/mg of protein)	Purification (fold)	Yield (%)
Dialyzed and concentrated culture supernatant (from 10 liters)	2,100	120	1,065	.17.5		100
Polyethylene glycol precipitate	1,900	84.4	720.4	22.5	1.29	90
Sephadex C-25, A-25 coupled column, AB eluate	1,340	37.6	334	35.6	2.03	64
CII (estimated)	100					5
Hydroxyapatite (0–0.15 M)-phosphate material (not taken further)	160					8
0.2–0.6 M phosphate material (HA-I)	660	8.81	54.2	74.9	4.28	31
Sephadex A-50 of HA-I, AB eluate (A50-I)	130	0.32	3.9	406.3	23.22	6
0.1–0.5 M sodium chloride (A50-II)	230	3.46	13.61	66.5	3.80	11

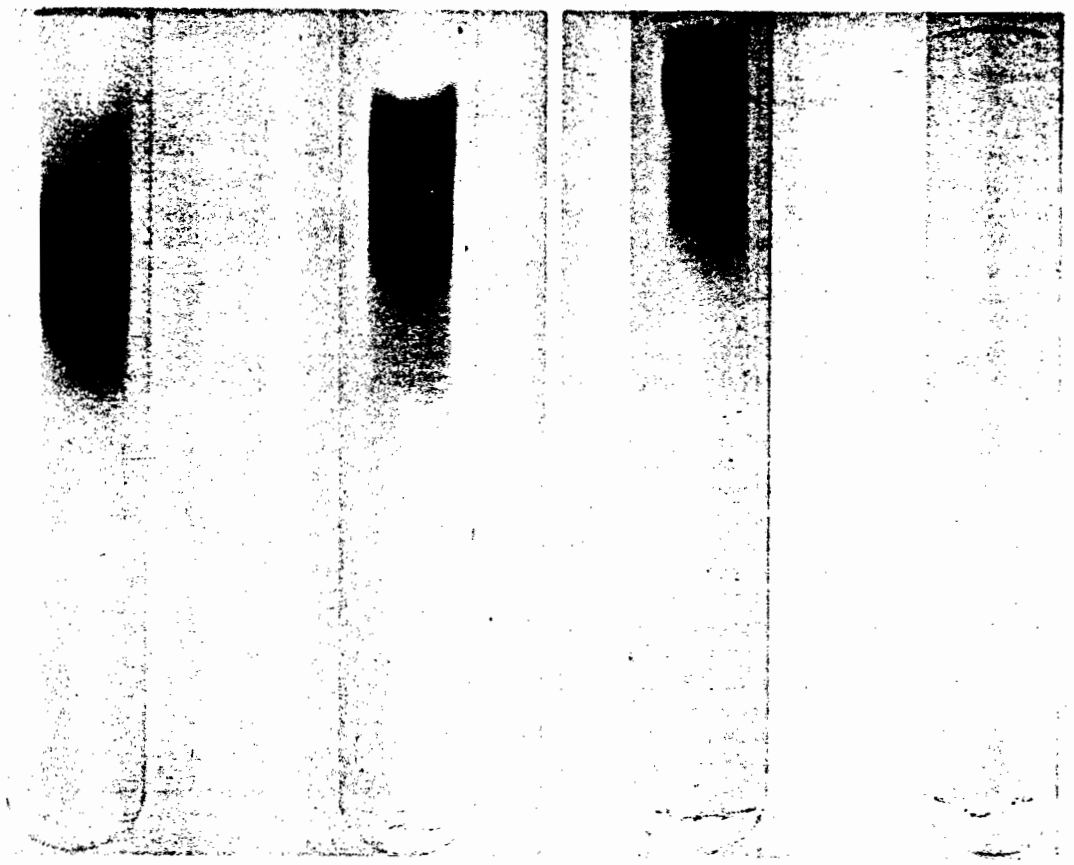

FIG. 1. *SDS-gel electrophoresis of partially purified PEST components. From left to right: CII, 100 μg of polysaccharide; A50-II, 100 μg of polysaccharide; A50-I, 50 μg of polysaccharide; control gel. Gels of 3% polyacrylamide were run at room temperature for 16 h at 4 mA/gel and stained by the periodic acid-Schiff procedure (15).*

was not routinely recovered from the double-column system.

Hydroxyapatite chromatography. A column (6 by 2.5 cm) of 75% (vol/vol) hydroxyapatite–25% Sephadex G-25 was packed over a 15-ml cushion of 50% (vol/vol) Sephadex G-25. Material from the C-25/A-25 column was applied and washed through with AB. Killing activity was absorbed and was eluted by using a linear 0 to 0.7 M sodium phosphate gradient, pH 4.7, in AB. The bulk (40 to 50%) of the applied activity eluted as a broad peak with at

least two components from 0.2 to 0.6 M phosphate, which was termed fraction HA-I. Sometimes a small amount of active material was not absorbed to the column, and a small peak of activity eluted at 0.05 to 0.15 M phosphate. All HA-I activity was absorbed to a ConA-Sepharose column when applied and eluted in AB.

Sephadex A-50. The Ha-I component was concentrated and dialyzed against AB and applied to a Sephadex A-50 column (10 by 1.5 cm). A fraction of the activity passed through the column (A50-I), and two partially resolved peaks of activity (fraction A50-II) could be eluted between 0.1 and 0.5 M NaCl by using a linear 0 to 0.6 M gradient of NaCl in AB. On re-elution some A50-I material was absorbed to an A-50 column and could be eluted with 1.0 M NaCl. A50-II material was homogeneous on rechromatography. SDS-gel electrophoresis of the A50-I material showed one major and one minor band (Fig. 1). The A50-II material of apparently lower specific activity also gave two bands on SDS-gel electrophoresis (Fig. 1).

Sensitivity of yeast strains. Extracellular extracts of *T. glabrata* contain PEST that kills killer (K12) and sensitive (S14a, K19.10) strains of *S. cerevisiae* independent of mating type (see Fig. 6 and 7). A yeast killer factor-resistant mutant (kre_1) derived from S14a was resistant to the *T. glabrata* PEST (K. Al-Aidroos, Ph.D. thesis, McGill University, Montreal, 1975). *T. glabrata* was immune to the extracellular PEST it produced but was sensitive to the action of the yeast killer factor (Table 3).

Loss of CFU of S14a on treatment with *T. glabrata* PEST is shown in Fig. 2. The kinetics of loss of CFU with PEST concentration were examined in S14a (Fig. 3), and the killing was found to be of a single-hit nature (10).

Inhibition of macromolecular synthesis. Addition of PEST to growing cells of strain S14a shut down net incorporation of radioactive precursors into carbohydrates, nucleic acids, and proteins (Fig. 4). Efflux of radioactive pools was not measured, but the variable pool depletion upon PEST addition (Fig. 5) may be accounted for by passage into macromolecules in the

TABLE 3. *Effect of killer factor and PEST on T. glabrata*

Treatment	% Survival[a]
Buffer control	100
+ Killer factor extract from K12, final concn 100 μg of protein/ml	0.4
+ Extract from sensitive strain S14a, final concn 110 μg of protein/ml	100
PEST extract from *T. glabrata*, final concn 260 μg of protein/ml (survival of K12, 3%)	100

[a] Initial CFU/ml, 2.2×10^7 to 3.3×10^7. *T. glabrata* was grown at 22 to 24 C in YEPD. Survival was measured after 3 h of incubation.

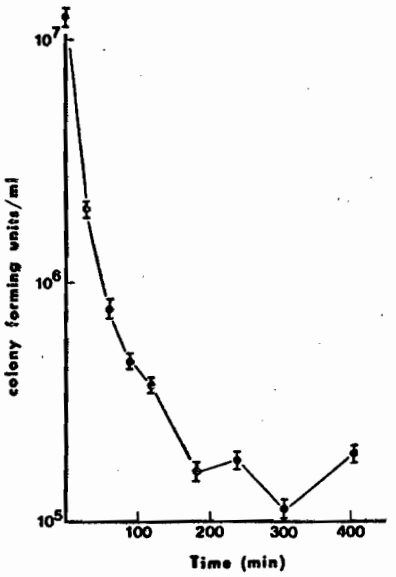

FIG. 2. *Sensitivity of S14a to PEST extract. PEST (38 μg of protein/ml, final concentration) was added at time zero to a growing culture of S14a in YEPD at 22 ± 2 C. CFU was measured at intervals by diluting and plating with YEPD. Bars represent the standard error of the colony counts.*

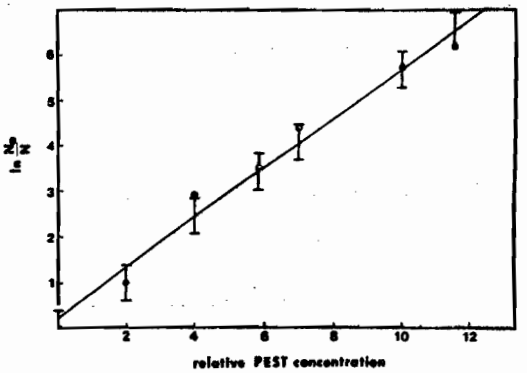

FIG. 3. *Single-hit killing of S14a with PEST. Growing cultures of S14a were treated with a range of PEST concentrations. A relative concentration of 2 contained 15 μg of protein/ml. Initial cell number, N_0, was determined before PEST addition and survival, N, was determined after killing was complete, 200 to 400 min (see Fig. 2). The line represents a least-squares fit to the points and the bars represent the standard error of the estimate of y on x for this fit. The points indicated with (■) were obtained in a separate experiment.*

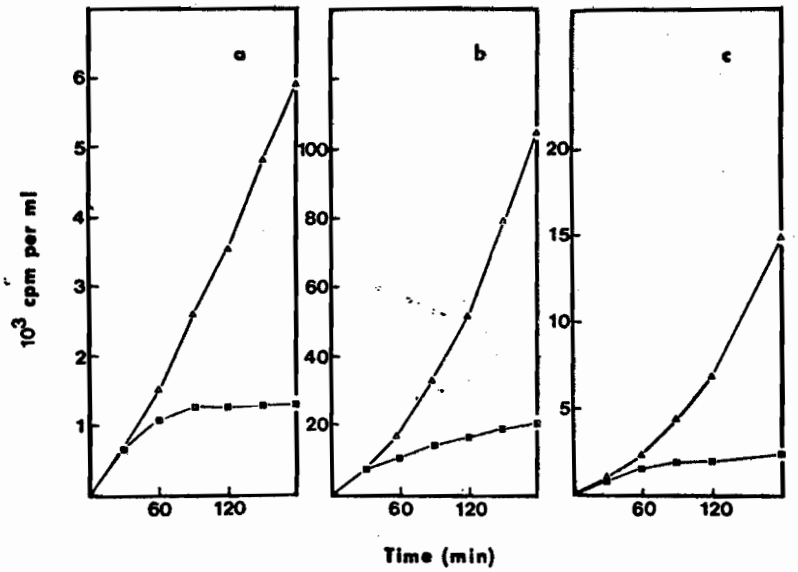

FIG. 4. *Effect of PEST on synthesis of macromolecules. PEST or buffer was added together with the labeled precursors at time zero to a culture of S14a, and the cultures were sampled at intervals for ethanol-insoluble radioactivity. (a) [^{14}C]glucose; (b) [^{14}C]cytosine; (c) [^{14}C]arginine. Culture survival was 1.1, 1.2, and 1.1%, respectively, at plus 190 min. Symbols: (■) Plus PEST; (△) plus buffer (control).*

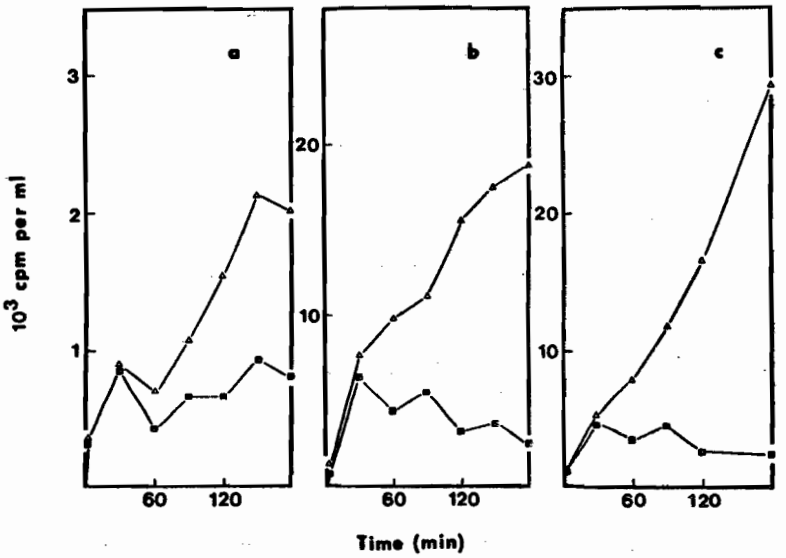

FIG. 5. *Effect of PEST on yeast pools. In the experiments described in Fig. 4, the cultures were sampled at intervals after toxin addition for ethanol-soluble radioactivity. (a) [^{14}C]glucose-derived pool; (b) [^{14}C]cytosine-derived pool; (c) [^{14}C]arginine-derived pool. Symbols: (■) Plus PEST; (△) plus buffer (control).*

absence of uptake from the medium. The inhibition pattern and the 30-min lag period before any abnormality was evident are similar to the effects of the yeast killer factor on sensitive *Saccharomyces* (3).

Leakage from the yeast potassium pool. Yeast cells maintain intracellular potassium at about 200 mM (8), and omission of the cation from the medium results in very poor culture growth. Cells of both S14a and K12 displayed massive leakage of radioactive potassium when treated with PEST (Fig. 6). Control cells, incubated without the glycoproteins, also lose potassium, but at a slow rate that probably defines exchange for ^{39}K in the medium (Fig. 6). These experiments demonstrate clearly the lag time

characteristic of PEST action. Treatment of sensitive cells with PEST in a YEPD medium containing 150 mM K^+ did not alter the cell survival. Minimizing the K^+ concentration gradient across the yeast cell membrane thus appeared insufficient to rescue the cells.

Dissipation of the ATP pool. Figure 7 shows the effects of PEST on the distribution of adenylates between ATP and AMP in a culture of K19.10. Similar data were obtained with S14a cultures. Cellular ATP was reduced to 40% of its initial level, with concomitant production of AMP and AMP leakage into the growth medium. That the AMP increase was the result of net ATP hydrolysis was supported by the fact that the sum of total culture adenylates remained constant from about 30 min to the end of the experiments (9.3 ± 0.7 nmol per ml of culture in the experiment of Fig. 7), despite the marked changes in the distribution and levels of individual adenylates during this interval. Untreated cells (Fig. 8), and PEST-treated cells for

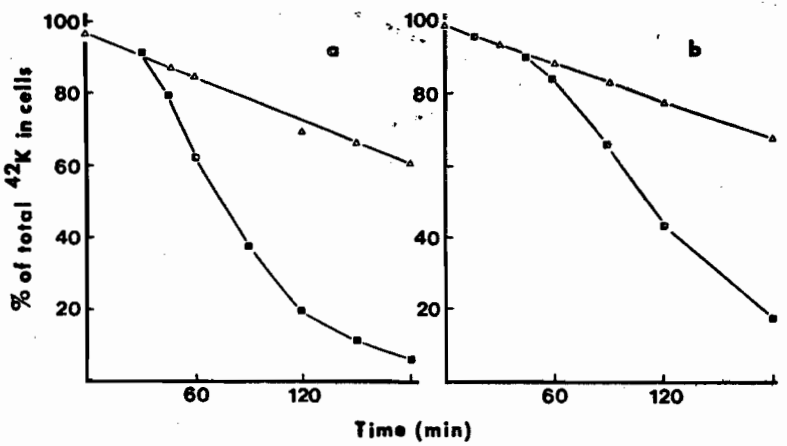

FIG. 6. *PEST-mediated leakage of ^{42}K. Cultures of K12 and S14a were loaded with ^{42}K as described in the text, incubated with or without PEST at zero time, and sampled at intervals for counts per minute retained by the cells. (a) Strain S14a; 100% = 7,320 counts/min per ml of culture; survival at 200 min, 10%. (b) Strain K12; 100% = 7,950 counts/min per ml of culture; survival, 2%. Symbols: (■) Plus PEST; (△) plus buffer.*

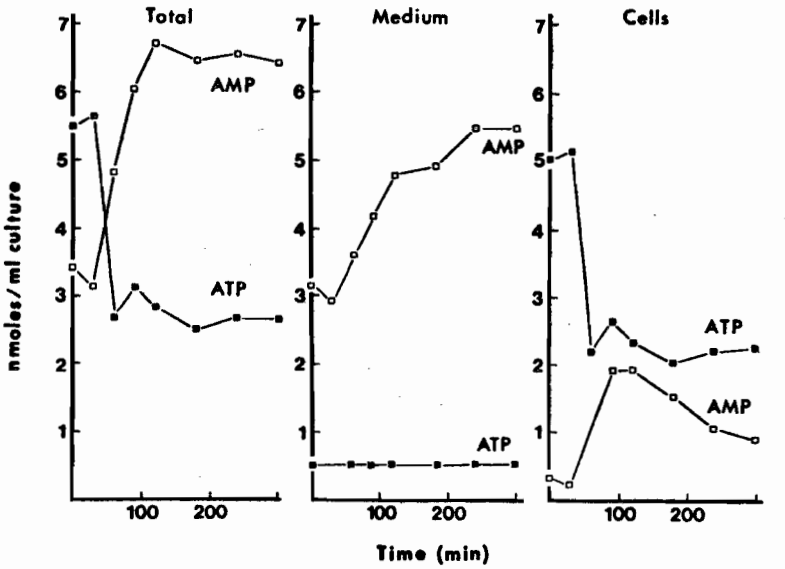

FIG. 7. *PEST-induced ATP dissipction. A culture of K19.10 (0.48 mg [dry weight] of cells/ml) was incubated with PEST at time zero and sampled at intervals for adenylates in the total culture and in the cell-free medium. Adenylates in cells are derived by subtracting medium from total. Sterile growth medium contained, in nanomoles per milliliter AMP, 1.3; adenosine diphosphate 0.25; ATP, 0.5. Cell survival was 2.6% at plus 300 min.*

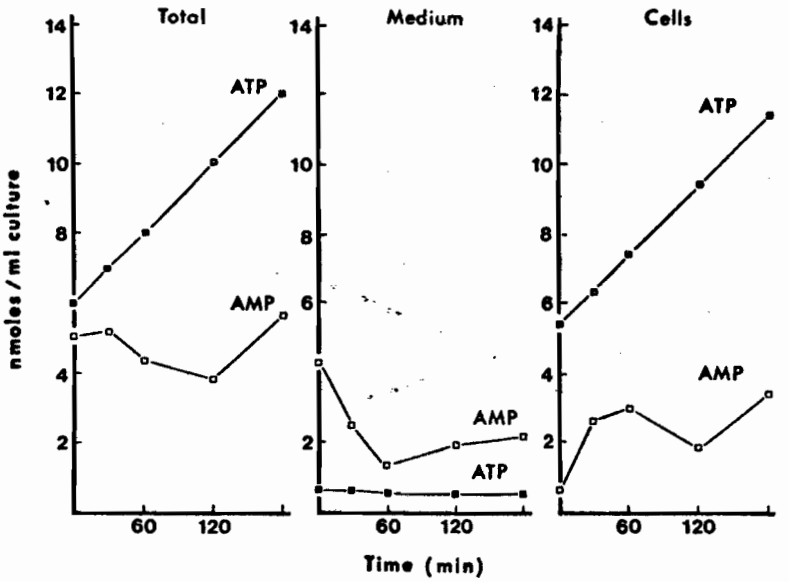

FIG. 8. *Yeast adenylate pools in an untreated culture. A K19.10 culture at the same cell density as that used in the experiment of Fig. 7 was incubated and sampled at intervals for adenylates as described in Fig. 7. Sterile growth medium contained, in nanomoles per milliliter: AMP, 4.75; adenosine diphosphate, 1.0; and ATP, 0.5. Culture doubling time was about 170 min at 22 to 24 C.*

30 min, have an energy charge [(ATP + ½ ADP)/(ATP + AMP)] of from 0.75 to 0.95, in agreement with values expected of actively growing cells and in contrast to some earlier reports for yeast (cited in reference 4).

Effect of pH on sensitivity to PEST. PEST was reasonably stable to incubation in the range pH 3 to 7 when measured by subsequent assay at pH 4.7 (Table 4). This is in marked contrast to the *Saccharomyces* killer factor, which is inactivated rapidly outside the pH range 4.6 to 4.8 (Palfree and Bussey, unpublished data). Strain K19.10, which grows at similar rates at all pH values between 3 and 7, is most sensitive to PEST near pH 4 and completely insensitive at pH 6 to 7 (Table 4). Similar results were obtained from S14a cells, grown in YEPD containing either succinate-phosphate or phosphate buffers, when challenged with PEST.

TABLE 4. *Effect of medium pH on PEST sensitivity*

pH	% Survival[a]	
	K19.10[b]	S14a[c]
3.4	57	—
4.2	1	2
5.0	7	15
6.0	100	100
7.0	100	100

[a] Cultures were grown at 22 to 24 C to about 1.5×10^7 CFU/ml and incubated with PEST at a final concentration of 45 μg of protein/ml. Survival was measured after 3 h by diluting and plating with media of appropriate pH.

[b] Media contained 0.1 M K_2HPO_4-citrate buffers.

[c] pH 4.2 media contained 0.1 M K_2HPO_4-succinate buffer and pH 6.0 and 7.0 media contained 0.1 M K_2HPO_4-KH_2PO_4 buffered media at appropriate pH from 3 to 7 for 4 h at 22 C lost 35 ± 10% of activity when assayed by well tests in 0.2 M AB buffer. All PEST samples held in above 0.1 M K_2HPO_4-citrate-pH 4.7.

DISCUSSION

The *T. glabrata*-produced PEST acts in a manner similar to the yeast killer factor and in a more general way to the membrane-acting colicins of type E1, K, and I (7). All show single-hit kinetics and alter the membrane permeability of target cells. PEST promotes leakage of cellular potassium and partial dissipation of the ATP pool with AMP accumulation in the medium of sensitive cultures. With the yeast killer factor, the shut off of macromolecular synthesis coincides with loss of several metabolites, including ATP from sensitive cells (3). Both killer and PEST show a pronounced lag after addition to sensitive cells before effects are seen; this lag is longer than that expected from binding to whole cells (2), and for PEST at 30 min (see Fig. 2 and 6) 60 to 70% of cells have bound a lethal dose without any visible metabolic change.

One difference between PEST and the yeast killer factor is that with PEST, AMP is the only adenylate found in the growth medium of

treated sensitive cells, whereas when the same sensitive strain is treated with the yeast killer factor, ATP also accumulates (3). It seems probable that the AMP released from PEST-treated sensitive cells is derived from ATP since there is a stoichiometric ATP decrease with AMP increase at constant total adenylate level. The *T. glabrata* extracellular extracts contain no ATPase activity and so activation of an ATPase by PEST but not by the killer factor seems a possible explanation.

The PEST activity remains stable over a wide pH range (at least pH 3 to 7) but shows a narrow pH range for the killing of sensitive strains (optimum close to 4; see Table 4). This distinction cannot be made with the yeast killer factor since the activity is stable only over a narrow pH range around pH 4.7. With PEST the pH 4-dependent event may be PEST binding to sensitive cells or some subsequent event, e.g., PEST stimulation of potassium efflux may be dependent upon a proton gradient.

There is specificity in the action of both PEST and the yeast killer factor. *T. glabrata* is immune to its own toxins (Table 3), as are killer strains of *S. cerevisiae* (2). PEST kills both killer and sensitive strains of *S. cerevisiae*, so the killer factor immunity component is different from that possessed by *T. glabrata*. That the immunity components are different is also indicated by the fact that the yeast killer factor kills *T. glabrata* (Table 3). Yeast killer factor-resistant mutants of type Kre_1, derived from a sensitive strain, show reduced binding of killer factor to the cell wall (K. Al-Aidroos, Ph.D. thesis). The kre_1 mutants are also resistant to PEST. This suggests that there is some common cell wall component in PEST and killer factor action. Challenging PEST with yeast killer factor to attempt to reduce killing in K12 cultures was unsuccessful.

The sensitivity of PEST activity to Pronase, the demonstration that most if not all extracellular proteins produced by *T. glabrata* are glycoproteins, and the co-purification of activity with carbohydrate-containing proteins to the one- to two-band stage on SDS-gel electrophoresis suggest strongly that at least one and probably all the PEST components are glycoproteins. The PEST components that absorb to hydroxyapatite also adsorb to ConA-Sepharose, suggesting that α-D-mannose residues are contained in their carbohydrate moiety. At least three components have killing activity; we do not yet know how they differ.

Killer factor-producing *S. cerevisiae* excrete several D-mannose-containing glycoproteins lethal to sensitive strains (R. Palfree and H. Bussey, manuscript in preparation). The production of active killer factor appears dependent upon double-stranded ribonucleic acid components in killer cells (11). We do not know whether a similar situation holds in PEST-producing *T. glabrata*, though preliminary experiments indicate that heat or cycloheximide treatments do not cure killing activity in *T. glabrata*, as they do in *S. cerevisiae* (6, 13).

The *T. glabrata*-produced PEST components should complement the yeast killer factor glycoproteins in serving as probes of surface events in the *S. cerevisiae* cell.

ACKNOWLEDGMENTS

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Killing of *Torulopsis glabrata* by *Saccharomyces cerevisiae* Killer Factor

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The *Saccharomyces cerevisiae* killer factor glycoproteins killed the pathogen *Torulopsis glabrata* by a mechanism involving membrane damage. Some other yeast species were unaffected by these glycoprotein toxins.

We have screened several yeast species for sensitivity to the *Saccharomyces cerevisiae* killer factor glycoproteins. Growing cultures of the pathogen *Torulopsis glabrata* ATCC 15126 (6, 7) were killed by the killer factor, and the treated cells showed alterations similar to those of killer factor-treated, sensitive cells of *S. cerevisiae*.

Media and methods for the measurement of cell survival, incorporation of labeled precursors into macromolecules, potassium efflux, and adenosine 5'-triphosphate, have been described previously (3). *T. glabrata* was grown at 22 to 24 C. Extracellular extracts containing killer factor glycoproteins were prepared from *S. cerevisiae* K_{12} as described (2, 4). Pure killer factor glycoproteins were obtained from these extracts by a method using polyethylene glycol precipitation, ion-exchange chromatography, and gel filtration. These glycoproteins gave single bands when electrophoresed on sodium dodecyl sulfate-polyacrylamide gels and stained using the periodic acid-Schiff stain procedure (R. Palfree and H. Bussey, submitted for publication) and (4). Table 1 shows the survival of various yeast strains in response to killer factor. *T. glabrata* was more sensitive to killer factor than sensitive *S. cerevisiae* strain S30; *Cryptococcus neoformans* ATCC 2505, *Candida albicans* ATCC 752, and *Schizosaccharomyces pombe* ATCC 2476 were unaffected.

The initial screening was performed using crude killer factor extracts; thus it was important to establish that killing was killer factor glycoprotein dependent. Both *T. glabrata* and *S. cerevisiae* S30 were insensitive to killer factor under conditions known to inactivate it, namely, incubation at pH 7.0 or with boiled killer factor at pH 4.7. In addition an extracellular extract from *S. cerevisiae* S14, an isogenic, sensitive, non-killer factor-producing strain isolated from K_{12}, had no effect on *T. glabrata* viability (Fig. 1). Pure killer factor glycoproteins (obtained from R. Palfree in our laboratory) killed *T. glabrata* on seeded plates in a well test (8).

Metabolic events associated with the loss of *T. glabrata* viability were qualitatively identical to those known to occur with killer factor-treated, sensitive strains of *S. cerevisiae* (1, 2). A culture of *T. glabrata* treated with killer factor showed a lag of approximately 40 min before efflux of the potassium pool (Fig. 2), adenosine 5'-triphosphate pool reduction, and adenosine 5'-triphosphate appearance in the medium (Fig. 3). There was also an increase in culture turbidity (Fig. 1) and, coordinate with the alterations in membrane permeability, a cessation of macromolecular synthesis (Fig. 4). The data suggest that the killer factor acts on *T. glabrata* by causing membrane damage and that both *T. glabrata* and sensitive *S. cerevisiae* are killed by a common mechanism.

The *S. cerevisiae* killer factor shows specificity of action: producing strains are immune (1) and strains of *C. albicans*, *C. neoformans*, and *S. pombe* are unaffected, but sensitive *S. cerevisiae* and *T. glabrata* are killed. The killer factor effectively acts as a specific fungal

TABLE 1. *Sensitivity of yeast to killer factor*[a]

Species	Survival (%)
Saccharomyces cerevisiae S30	8.8
Torulopsis glabrata	0.4
Schizosaccharomyces pombe	>100
Candida albicans	>100
Cryptococcus neoformans	>100

[a] Cultures were grown on yeast extract-peptone (2) at 22 to 24 C to 0.5×10^7 to 2.5×10^7 colony-forming units/ml, crude killer factor was added at 110 μg of protein per ml, and survival was estimated by plating on yeast extract-peptone agar after 300 min. Survivals are expressed as a percentage of the initial colony-forming units.

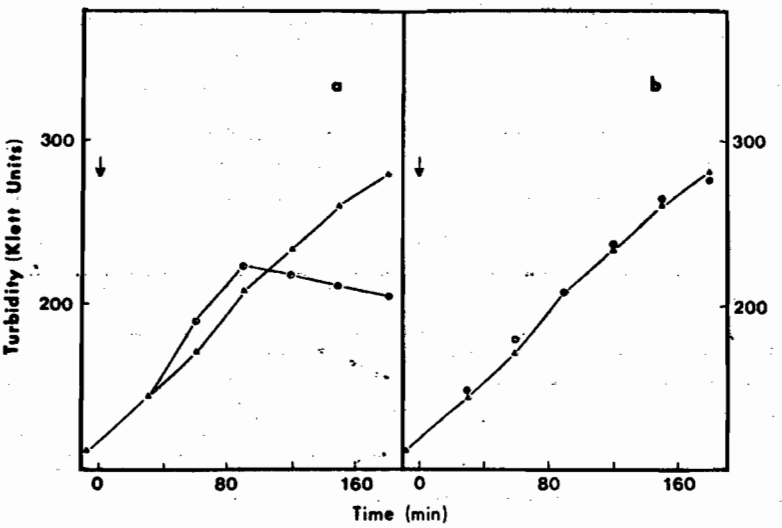
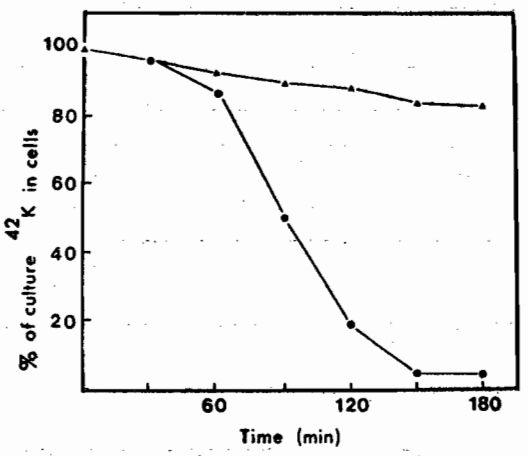


FIG. 1. *Turbidity of T. glabrata cultures on treatment with extracts from killer and sensitive yeast strains. Extracts were added at time zero (arrow) to cultures growing at 22 to 24 C. (a) Killer extract from K_{12} (100 μg of protein per ml); survival after 200 min was 0.15%. (b) Extract from S14 (110 μg of protein per ml); survival after 200 min was 100%. Symbols: (●) plus extract; (▲) plus water.*

FIG. 2. *Killer-induced potassium efflux. A culture of T. glabrata was loaded with ^{42}K, washed, and suspended in unlabeled growth medium. Killer extract at 160 μg of protein per ml or water was added at time zero, and the cultures were sampled at intervals for counts retained by the cells; 100% = 11,000 counts/min per ml. Cell survival at 200 min was 0.03%. Symbols: (●) plus killer; (▲) plus water.*



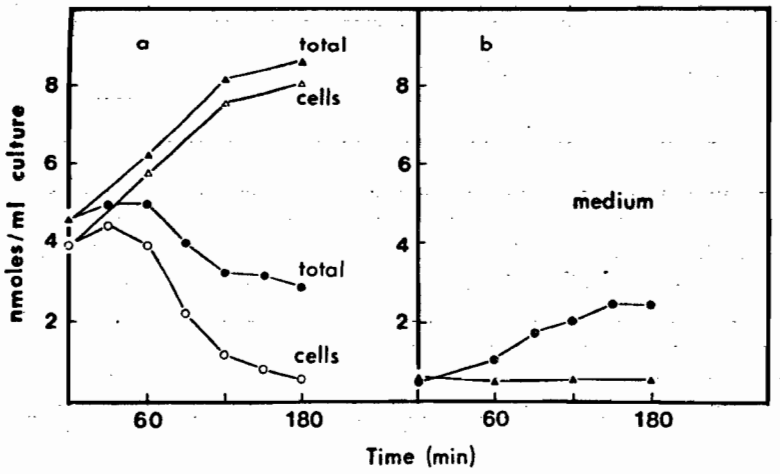


FIG. 3. *Killer-induced adenosine 5'-triphosphate (ATP) dissipation and leakage. A culture of T. glabrata was incubated with killer or water at time zero, as in Fig. 2, and sampled at intervals for ATP in the total culture and in the cell-free medium. Cell ATP = total minus medium. Sterile growth medium contained 0.53 nmol of ATP per ml. Cell survival in killer-treated culture at 200 min was 3.8%. (a) Total ATP and cell ATP; (b) ATP in medium. Symbols: (●,○) plus killer; (▲) plus water.*

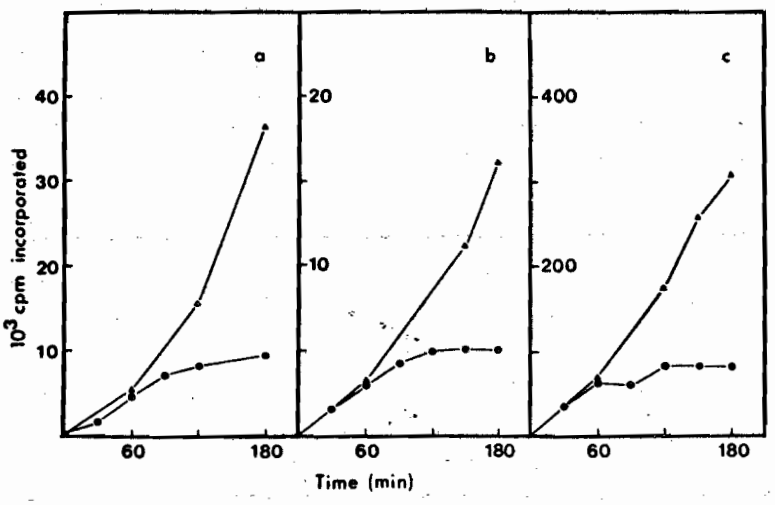


FIG. 4. *Inhibition of macromolecule synthesis by killer. Killer, as in Fig. 2, or water was added together with the labeled precursors at time zero to a culture of T. glabrata, and the cultures were sampled at intervals for ethanol-insoluble radioactivity. (a) [^{14}C]arginine; (b) [^{14}C]glucose; (c) [^{14}C]cytosine. Cell survival was 0.8, 0.2, and 0.3%, respectively. Symbols: (●) plus killer; (▲) plus water (control).*

antibiotic. This implies that fungi have specific surface components and suggests a basis for a search for other antibiotics to pathogenic strains. In *Escherichia coli* KBT001, the outer membrane sites specific for colicin E and phage BF23 are also involved in vitamin B12 transport (5). Similarly, it may be possible that the sites specific for the killer factor have other functions whose inhibitors could be antibiotic in action.

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Mode of Action of Yeast Toxins: Energy Requirement for *Saccharomyces cerevisiae* Killer Toxin

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The role of the energy status of the yeast cell in the sensitivity of cultures to two yeast toxins was examined by using ^{42}K release from cells as a measure of toxin action. The *Saccharomyces cerevisiae* killer toxin bound to sensitive cells in the presence of drugs that interfered with the generation or use of energy, but it was unable to efflux ^{42}K from the cells under these conditions. In direct contrast, the *Torulopsis glabrata* pool efflux-stimulating toxin induced efflux of the yeast ^{42}K pool was insensitive to the presence of energy poisons in cultures. The results indicate that an energized state, maintained at the expense of adenosine 5'-triphosphate from either glycolytic or mitochondrial reactions, is required for the action of the killer toxin on the yeast cell.

The *Saccharomyces cerevisiae* killer toxin and the *Torulopsis glabrata* pool efflux-stimulating toxin (PEST) kill sensitive strains of yeast by mechanisms that involve damage to the plasma membrane. Both toxins are secreted into the growth medium by producer strains, and when added to sensitive cultures, both block synthesis of macromolecules, discharge the intracellular pools of adenosine 5'-triphosphate and potassium, and increase the culture turbidity. The toxin-induced alterations are delayed and coordinate, appearing after about 40 min at 22 to 24°C (3-7).

In this paper we provide evidence that the toxins are readily distinguished by the dependence of their action on the metabolic status of the sensitive cell; killer toxin action is blocked in energy-poisoned cultures, PEST action is not. The nature of this energy requirement for the killer toxin action and the similarity of the requirement to that known for the colicin K-*Escherichia coli* interaction (21) is discussed, particularly with regard to the mechanisms of energy-coupling in the yeast plasma membrane.

MATERIALS AND METHODS

Materials. ^{42}KCl was purchased from New England Nuclear Corp., Boston, Mass.; carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), 2,4-dinitrophenol (DNP), antimycin, and bovine serum albumin were obtained from Sigma Chemical Co., St. Louis, Mo.; iodoacetic acid was from Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N.Y.; other chemicals were obtained from Fisher Scientific Co., Pittsburgh, Pa. Antimycin, DNP, and CCCP were used as solutions in ethanol; the final ethanol concentration when these reagents were used in cultures did not exceed 1%.

Strains, media, and culture conditions. Strains of *S. cerevisiae* are given in Table 1; K19.10 was the toxin-sensitive strain used in most experiments. *T. glabrata* ATCC 15126 was obtained from the American Type Culture Collection, Rockville, Md. For preparation of toxins, *T. glabrata* or *S. cerevisiae* K12 was grown in YEP medium containing 2% glucose (YEPD [8]). For experiments with toxins and inhibitors, sensitive cells were cultured at 22 to 24°C in a medium containing either glucose or ethanol as the predominant carbon and energy source. The basal medium (Na-YEP), based on the yeast minimal medium of Halvorson (12), contained yeast extract (5 g); peptone (5 g); KCl (0.37 g); $(NH_4)_2SO_4$ (4 g); $Na_2HPO_4 \cdot 7H_2O$ (13.4 g); succinic acid (5.8 g); $CaCl_2 \cdot 2H_2O$ (0.3 g); $MgSO_4 \cdot 7H_2O$ (0.5 g); and $FeSO_4$, $MnSO_4$, $ZnSO_4$, and $CuSO_4$ (0.5 mg of each) in 1,000 ml. The Na-YEP was supplemented with either 2% glucose (Na-YEPD) or 2% ethanol (Na-YEPE); the final pH was 4.7. Cultures of sensitive cells were inoculated at an absorbancy at 600 nm (A_{600}) of 0.8 and incubated to an A_{600} of 1.2 to 1.3 before use. Na-YEPE cultures were 50 ml in baffled 250-ml flasks; they were inoculated from agar slants containing YEP and 4% glycerol and were incubated with vigorous shaking. For experiments measuring ^{42}K retention, Na-YEPE cultures were transferred to a roller drum as 2- to 3-ml portions. Na-YEPD cultures were inoculated from YEP slants containing 2% glucose and were incubated throughout on the roller drum. When required for plates, media contained 2% agar.

Culture turbidity. Turbidity was measured at 600 nm in 1-cm path-length cuvettes in a Gilford 240 spectrophotometer. An A_{600} of 1.0 was equivalent to 1.4×10^7 colony-forming units (CFU) per ml and 0.41 mg (dry weight) of cells per ml when strain K19.10 was growing on glucose at 22 to 24°C;

ethanol-grown cultures at an A_{600} of 1.0 contained 1.7×10^7 CFU/ml and 0.31 mg (dry weight) of cells.

Viable counts and survival of treated cells. Cell suspensions were diluted in Na-YEP and spread on agar plates to obtain the viable count; survival is expressed as the percentage of cells able to produce colonies at the end of an experiment, where 100% is the CFU per milliliter immediately before addition of toxin or drug.

Preparation of toxins. PEST activity was partially purified from culture supernatant fluid of *T. glabrata* by concentration on Amicon PM 30 membranes and precipitation with polyethylene glycol (6). The precipitates were washed twice with 12.5% polyethylene glycol in 0.1 M sodium acetate buffer (pH 4.7) (buffer), resuspended in buffer, and precipitated with 30% ethanol. After at least 2 h in the cold, the precipitate was collected at 27,000 × *g* for 30 min, suspended in buffer, and frozen. The step with ethanol was necessary to reduce the viable count of the PEST preparation to less than 10^3/ml. Killer toxin was prepared from cultures of *S. cerevisiae* K12 by the same procedure used for PEST, with omission of ethanol precipitation. Table 2 summarizes a typical preparation for each toxin; in each case the final fraction was used in experiments with K19.10 cells. Killing units given in Table 2 were measured with *S. cerevisiae* S14a as the sensitive strain (4); protein was measured by the method of Lowry et al. (24) with serum albumin as the standard; and polysaccharide was measured by the procedure of Badin et al. (1) with D-glucose as the standard.

Retention of the yeast potassium pool. The procedure for preparing suspensions of growing cells loaded with ^{42}K and for measuring the fraction of the initial cell-bound radioactivity subsequently retained by cells was described previously (6). The data for ^{42}K retention are expressed as the percentage of ^{42}K in the cells, where 100% is the cell-bound counts per minute at zero time.

TABLE 1. *S. cerevisiae strains*

Strain	Genotype	Source or reference
K19	a trp_5 leu_1 M(k)	J. M. Somers
K19.10	a trp_5 leu_1 M(o)	J. M. Somers
K12, ATCC 28683	α $ade_{2.5}$ M(k)	6
S14a, ATCC 28684	α $ade_{2.2}$ M(o)	6
S14.96, ATCC 28685	α $ade_{2.5}$ M(o)	K. Al-Aidroos

RESULTS

Potassium release as a measure of toxin action. Yeast toxins are routinely assayed by plating out treated cultures and calculating the survival; the method effectively measures binding of toxin to the cells but cannot give information on the course of events in the culture. The procedure that measures the relative stability of the yeast ^{42}K pool allows direct observation of events in the treated cell suspension. Actively growing cells took up ^{42}K from the medium and maintained an intracellular pool of the isotope proportional to the cell density (Fig. 1A). Addition of killer or PEST to loaded, sensitive, cells resulted in almost complete release of radioactivity from the cells (Fig. 1B). The ^{42}K pool in killer-resistant (K12, K19, S14.96) and PEST-resistant (S14.96) strains was unaffected by the appropriate toxin, because it was lost to the medium at a low rate identical to that seen in untreated cultures (Fig. 1B).

Effect of energy poisons on killer-induced potassium release. The relative survival of sensitive cultures that had been treated with killer toxin, when in either active growth or stationary phase, indicated that yeasts are most susceptible to the toxin when in the expo-

TABLE 2. *Partial purification of killer and PEST*

Purification step	KU[a]	Protein (mg)	Polysaccharide (mg)	Sp act (KU/mg protein)	Purification (fold)	Yield (%)
Killer						
Concentrated culture supernatant[b]	6.16×10^{10}	334.0	143	1.8×10^8	1	100
Polyethylene-glycol precipitate	1.27×10^{10}	5.8	37	2.19×10^9	12	21
Pest						
Concentrated culture supernatant[c]	2.56×10^{10}	356.0	160	7.19×10^7	1	100
Polyethylene-glycol precipitate	1.10×10^{10}	6.9	51	1.59×10^9	22	43
Ethanol precipitate	7.56×10^9	4.7	32	1.61×10^9	22	29

[a] Killing units.
[b] From 5 liters of *S. cerevisiae* K12, grown at 19°C to 450 Klett units (blue filter).
[c] From 5 liters of *T. glabrata*, grown at 30°C to 550 Klett units.

nential phase; in addition, glycerol-grown cultures that bypassed six-carbon glycolysis were less sensitive than glucose-grown cells (3). The following experiments were designed to examine the implication that sensitivity to killer may be linked to energy metabolism.

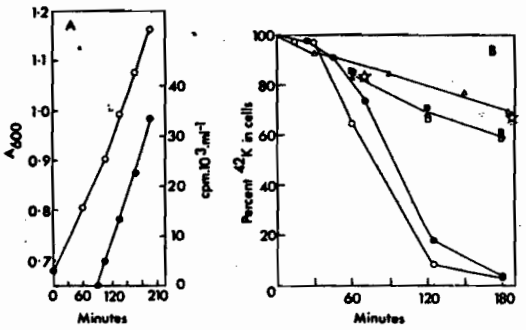

FIG. 1. *Use of the yeast potassium pool to measure toxin action. (A) Potassium loading. ^{42}KCl was added to K19.10 cells growing on glucose, and the culture was sampled at intervals for turbidity (○) and for radioactivity in the cells (●). (B) Potassium efflux in sensitive and resistant yeast. ^{42}K-loaded cultures of strains K19.10, K19, K12, and S14.96 in Na-YEPD were harvested, washed, suspended in growth medium, and incubated at 22 to 4°C. At zero time, K19.10 received either PEST to 150 μg of protein per ml (○), killer to 40 μg of protein per ml (●), or no additions (△); K12 and K19 received killer, 40 μg of protein per ml (☆), and S14.96 was treated with either PEST, 100 μg of protein per ml (□), killer, 100 μg of protein per ml (■), or no additions (▲). Counts per minute remaining in cells was measured at intervals after zero time. The control (untreated cells) data for strains K12 and K19 were identical to those shown for strain K19.10 (△).*

Glucose in appropriate concentration is known to suppress mitochondrial function in yeast (15). When strain K19.10 was cultured in the 2% glucose medium (Na-YEPD), it was sensitive to iodoacetate, an inhibitor of glyceraldehyde 3-phosphate dehydrogenase, and to the reagents DNP and CCCP; the glucose-grown cultures were not affected by cyanide or antimycin (Table 3). Killer-induced potassium efflux in glucose-grown cells was blocked by the drugs that stopped growth; the inhibitors of electron transfer in the mitochondrion did not influence the effect of killer on the ion pool (Fig. 2). When grown on ethanol, K19.10 was dependent on oxidative phosphorylation, since KCN or antimycin prevented growth (Table 3). Iodoacetate was also an effective inhibitor in these cultures, presumably due to its inactivation of yeast alcohol dehydrogenase (14). DNP or CCCP blocked the growth of the ethanol-grown cells (Table 3) as expected if these reagents uncouple phosphorylation from respiration in the yeast mitochondrion. Killer toxin discharged ^{42}K from ethanol-grown cells in a way similar to that seen in glucose-grown cells, except that the lag before ^{42}K release was extended (cf. Fig. 2A, 3A, and 5). Potassium efflux due to killer action was abolished if an uncoupler, iodoacetate, or an electron transfer inhibitor was added to ethanol-grown cells with the toxin (Fig. 3). This block of killer action by antimycin or KCN was evidently due to interference with mitochondrial function, since neither drug influenced events in glucose-grown cultures treated with killer (Fig. 2A). It can be

TABLE 3. *Survival of strain K19.10 after incubation with killer toxin and drugs*

Additions to culture[a]	Concn[b]	Survival[c] (%)	
		Glucose-grown culture	Ethanol-grown culture
None		235 ± 8 (3)	173 ± 7 (5)
Killer	50–60 μg of protein/ml	0.82 ± 0.58 (6)	3.4 ± 3.1 (5)
DNP	0.5 mM	57 ± 20 (3)	100 ± 5 (4)
DNP and killer		0.12 ± 0.06 (3)	1.6 ± 0.9 (4)
CCCP	0.5 mM	0.02 ± 0.02 (4)	37 ± 3 (3)
CCCP and killer		0.34 ± 0.05 (3)	13 ± 7 (3)
Iodoacetate	1 mM	0.040 ± 0.026 (4)	43 ± 17 (4)
Iodoacetate and killer		0.009 ± 0.007 (3)	4.2 ± 2.6 (3)
Cyanide	1 mM	217 ± 19 (4)	98 ± 4 (5)
Cyanide and killer		0.46 ± 0.28 (3)	35 ± 13 (3)
Antimycin	10 μg/ml	243 ± 10 (4)	107 ± 11 (5)
Antimycin and killer		0.27 ± 0.22 (4)	67 ± 8 (3)

[a] Additions were made to actively growing cultures (A_{600} 1.2 to 1.3).

[b] Concentrations were as given in the first instance.

[c] Cultures were incubated at 22 to 24°C for 180 min and then plated out on either Na-YEPD agar (glucose-grown cultures) or Na-YEPE agar (ethanol-grown cultures). Survival is given as the mean ± standard deviation; the number of determinations is given in parentheses.

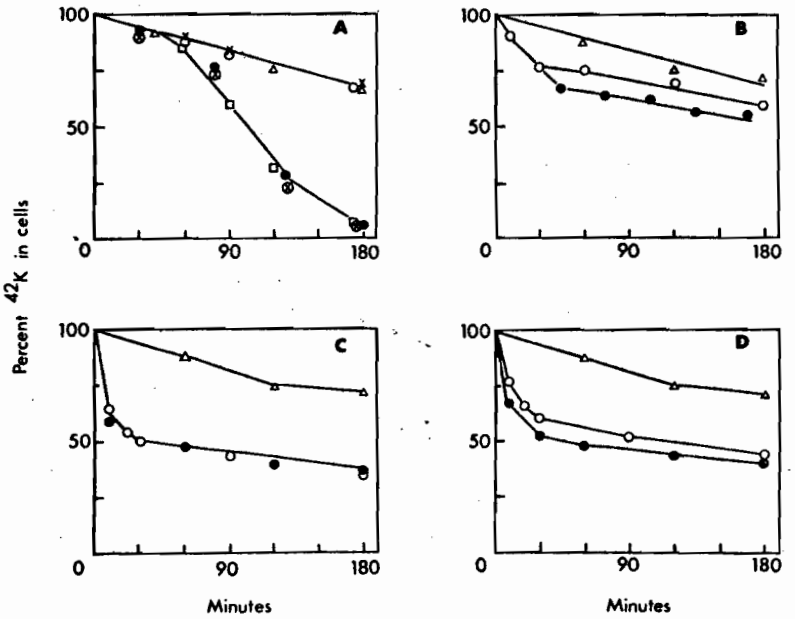

FIG. 2. *Potassium release in glucose-grown cells treated with killer toxin and drugs. ^{42}K-labeled cultures of K19.10 in Na-YEPD were incubated at zero time with killer, drug, killer and drug, or without additions, and they were sampled at intervals for counts per minute remaining in cells. Concentrations: killer, 38 μg of protein per ml; antimycin, 10 μg/ml; cyanide, 1 mM; iodoacetate, 1 mM; DNP, 0.5 mM; CCCP, 0.5 mM. (A) Control (untreated cells) (△), plus killer (□), plus killer and cyanide (●), plus killer and antimycin (⊗), cyanide only (○), antimycin only (×). (B) Control (△), iodoacetate only (○), iodoacetate plus killer (●). (C) Control (△), DNP only (○), DNP plus killer (●). (D) Control (△), CCCP only (○), CCCP plus killer (●).*

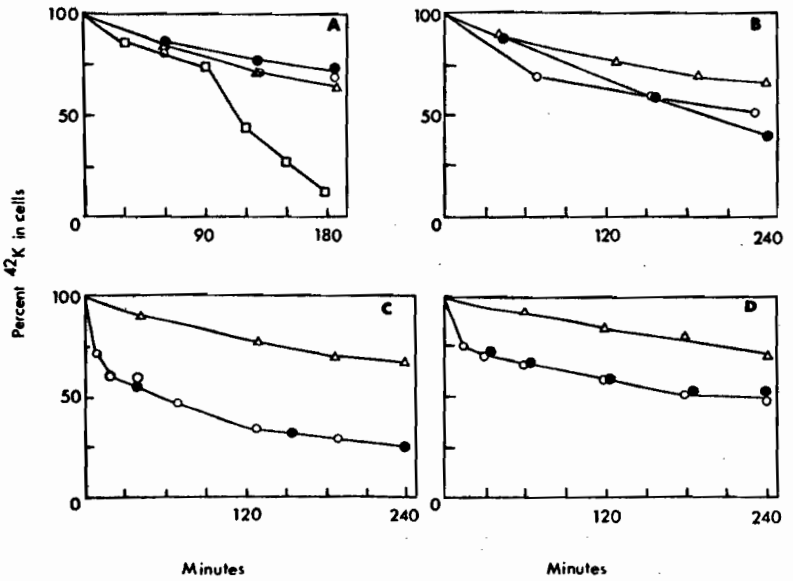

FIG. 3. *Potassium release in ethanol-grown cells treated with killer toxin and drugs. The experiments were done as for Fig. 2, except that cells were loaded with ^{42}K and incubated with additions in Na-YEPE. Drug concentrations are given in the legend to Fig. 2; killer was added at 60 μg of protein per ml (A through C), and 130 μg of protein per ml (D). (A) Control (△), plus killer (□), plus killer and antimycin (○), plus killer and cyanide (●), the data for antimycin or cyanide alone are given in the legend of Fig. 8. (B) Control (△), plus iodoacetate (○), plus iodoacetate and killer (●). (C) Control (△), plus DNP (○), plus DNP and killer (●). (D) Control (△), plus CCCP (○), plus CCCP and killer (●).*

seen that the uncouplers themselves effluxed a considerable fraction of the yeast ^{42}K pool (Fig. 2C and D and 3C and D); however, the results of experiments with PEST-treated cultures showed that such drug-stimulated ion release was not sufficient to mask the toxin-induced release (see Fig. 7, 8, and 9). Since the PEST and the killer, if added alone to sensitive cultures, produced ^{42}K efflux to a similar extent (Fig. 1B), it seemed that the uncouplers did in fact abolish killer-induced ion release and did not simply mask the killer action.

DNP arrests the killing process at a stage subsequent to killer binding. Table 3 summarizes the survival data for K19.10 cultures that had been treated either with killer, with each drug, or with combinations of killer and drugs; the cells were grown on either glucose or ethanol. When considered with the results for the stability of the yeast potassium pool in cultures treated in the same way, the survival data suggested a distinction between two stages in killer action: stage 1 is killer binding to cells and is energy independent; stage 2 is the onset of membrane alteration and is energy dependent. The clearest evidence comes from the cultures treated with DNP and killer toxin: in cultures grown on either glucose or ethanol the cells did not show toxin-specific ^{42}K release, yet they died when plated out (Fig. 2C and 3C, and Table 3). These cells had evidently bound a lethal dose of killer in the presence of DNP but were arrested in this state until the reagent was removed by dilution. Although not as easily interpreted, the effects of the other drugs on killing, under conditions where they blocked killer-specific ion efflux, were not inconsistent with a two-stage process for killer action. Antimycin or cyanide, although clearly having no effect on either killer binding (measured as death on plates) or the onset of ^{42}K efflux in glucose-grown cultures, reduced but did not abolish killing in respiring cells (Table 3, Na-YEPE cultures); either drug abolished ^{42}K efflux in killer-treated, ethanol-grown cells (Fig. 3A). CCCP and iodoacetate were themselves lethal to glucose-grown cultures to an extent that masked any differential effects they may have had on killer binding and membrane alteration (Table 3); in respiring cultures these drugs acted like antimycin or cyanide, affecting primarily the onset of ^{42}K efflux in killer-treated cells (Table 3, Fig. 3B and D).

Addition of appropriate drugs to cultures at intervals after the addition of killer toxin rapidly blocked further toxin-specific potassium release. Figures 4 and 5 show the data for the effects of adding iodoacetate or DNP to killer-treated glucose-grown cells, and for the effects of antimycin or cyanide on killer-treated ethanol-grown cells, respectively. In the case of DNP (Fig. 4B), the effect of the drug alone,

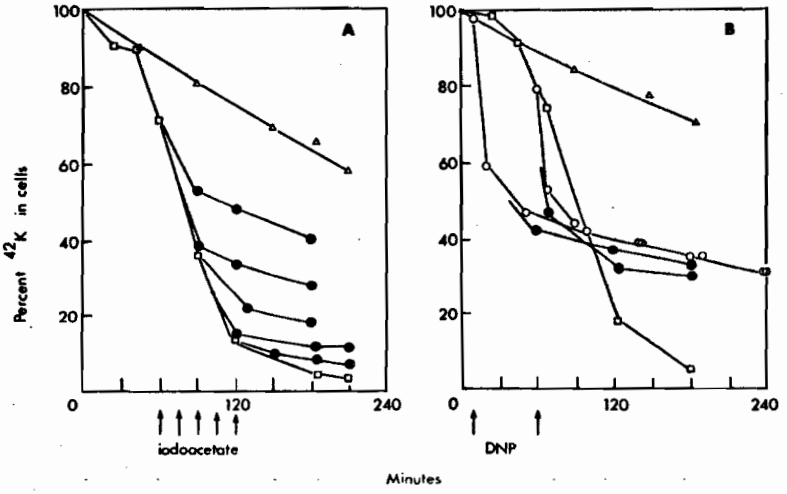

FIG. 4. *Effect of adding iodoacetate or DNP to glucose-grown cells at intervals after addition of the killer toxin. (A) A K19.10 culture, loaded with ^{42}K in Na-YEPD, was treated with 42 μg of killer protein per ml at zero time and divided into portions. At each time marked by an arrow, iodoacetate was added to a portion to 1 mM. All suspensions were sampled with time for counts per minute remaining in cells. Control (untreated cells) (△), plus killer (□), plus killer and iodoacetate (●). The effect on the ^{42}K-pool of adding iodoacetate at zero time is shown in Fig. 2B. (B) Cultures were treated with killer, as in A. At +10 and +60 min, portions received DNP to 0.5 mM. Separate suspensions received DNP only, at +10 or +60 min. Control (△), plus killer (□), plus killer and DNP at 10 min (●), plus killer and DNP at +60 min (●), DNP alone, +10 min (○), DNP alone, +60 min (○).*

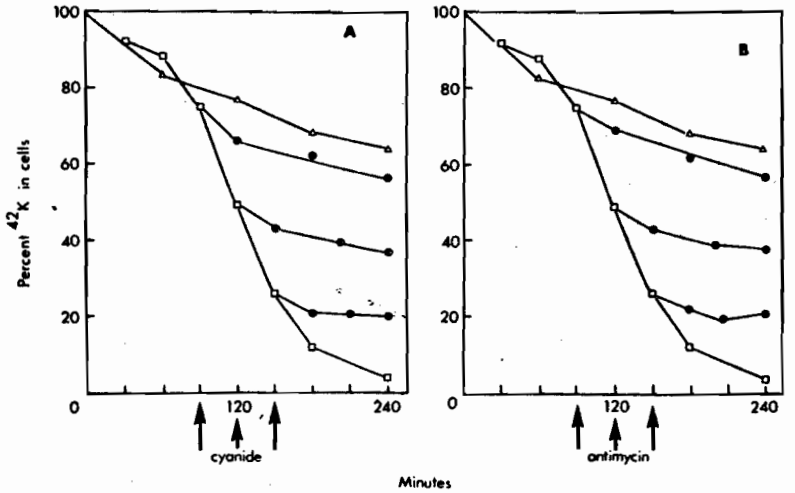

FIG. 5. *Effect of adding inhibitors of electron-transfer to respiring cells at intervals after the addition of killer toxin. (A) K19.10 cells, loaded with ^{42}K in Na-YEPE, were treated with 60 μg of killer protein per ml and incubated. At times marked by arrows, cyanide was added to a portion of the culture to 1 mM. Cultures were sampled for counts per minute retained by cells. Control (untreated cells) (△), plus killer (□), plus killer and cyanide (●). (B) The experiment was done as described in A, but antimycin was added to killer-treated cultures at the intervals marked by arrows to 10 μg/ml. Control (△), plus killer (□), plus killer and antimycin (●).*

when added at intervals to a culture, has been included to demonstrate that ^{42}K release in the killer-treated culture subsequent to DNP addition is due to the drug and not to the killer. If it is assumed that the proportion of the cellular potassium pool being lost to the medium at the control (untreated) rate is a measure of the fraction of the killer-treated population yet to reach stage 2, then the effects of energy poisons added after the toxin can be taken as evidence for a large delay between killer binding and subsequent membrane alteration. In glucose-grown cultures, survival curves showed that by 60 min about 94% of the killer-treated population had bound a lethal dose of toxin (Fig. 6, open circles); however, addition of iodoacetate at 60 min prevented about 80% of the population from entering stage 2 of killer action (Fig. 4A). The data given in Fig. 4A for the rescue of killer-treated cells from ^{42}K efflux have been plotted in Fig. 6 (solid circles) as percent rescue versus time of iodoacetate addition. Figure 6 shows that none of the toxin-treated cells had started to lose ^{42}K in the manner characteristic of killer action by that time when more than 90% of cells had bound a lethal dose (50 min). Similar treatment of the data demonstrating the block placed by cyanide or antimycin on ^{42}K efflux in respiring yeast treated with killer (Fig. 5), and a survival curve of ethanol-grown cells treated with killer (data not shown), showed that 65% of the cells had bound a lethal dose of toxin before any cell had lost its ^{42}K.

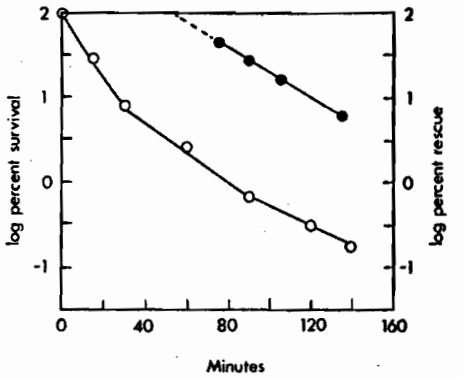

FIG. 6. *Survival on plates, and rescue by iodoacetate, of killer-treated cultures. In separate experiments, K19.10 cultures in Na-YEPD were either treated with killer and plated at intervals on Na-YEPD agar, or treated with killer and then with iodoacetate at intervals and sampled for ^{42}K retained by cells. The derivation of the percent rescue by iodoacetate is described in the text and is from the data given in the legend of Fig. 4A. Symbols: ○, log_{10} percent survival; ●, log_{10} percent rescue.*

Energy poisons have no effect on PEST-induced potassium efflux. PEST- and killer-treated cells display similar alterations, with the exception that ATP release into the medium is seen only with killer treatment (6). However, in direct contrast to the results obtained for killer-treated cultures of K19.10, the PEST-induced ion release from cells of this

strain was not affected by energy poisons. ^{42}K efflux due to PEST treatment of either glucose or ethanol-grown cultures continued in the presence of iodoacetate, DNP, CCCP, or an electron transfer inhibitor (Fig. 7–9). These results indicated that uncoupler-stimulated potassium pool discharge was insufficient to mask the discharge characteristic of toxin action, as mentioned previously.

DISCUSSION

Our results suggest that the yeast cell must be energized to be damaged by the killer toxin. The PEST, although producing effects similar to those of the killer in sensitive cells, acts by a distinct mechanism that is independent of the energized state. Comparison of the effects of energy poisons on membrane damage in killer-treated cultures, measured as ^{42}K release, and on binding of killer to cells, measured as cell death on plates, has provided evidence for a two-stage killer-cell interaction. Stage 1 is killer binding; stage 2 occurs subsequently and results in alteration to the plasma membrane, via an energy-sensitive process. The distinction between stages in killer action was most clearly suggested by the effects of DNP. The reagent abolished killer-specific ^{42}K efflux but did not block killing on plates. The effects of other reagents were consistent with the proposal that energy poisons affect primarily the transition of cells from stage 1 to stage 2.

The conditions that block killer action resemble those that interfere with colicin K action on sensitive cultures of *E. coli*. Colicin K-treated cells are maintained in a nondamaged state, stage 1, by trypsin or by conditions that prevent the cell from using energy; in stage 2 the cells have been altered metabolically and killed, and they are unresponsive to the rescue conditions of proteolysis or energy blockade (21, 27). The action of other colicins, some with distinct modes of action from colicin K, is also blocked by reagents such as DNP (18). Trypsin rescues colicin K-treated cells in that it maintains the viability of those cells in the population that have yet to receive metabolic damage (27), suggesting that the colicin is effectively displaced from the receptor in these cells. In the present situation we have used "rescue" to mean the block imposed on the stage 1 to stage 2 transition, as measured by the suppression of the onset of ^{42}K release in yeasts that have bound a lethal dose of killer toxin. The large interval between killer binding and ^{42}K release (about 50 min in glucose-grown cells and about 80 min

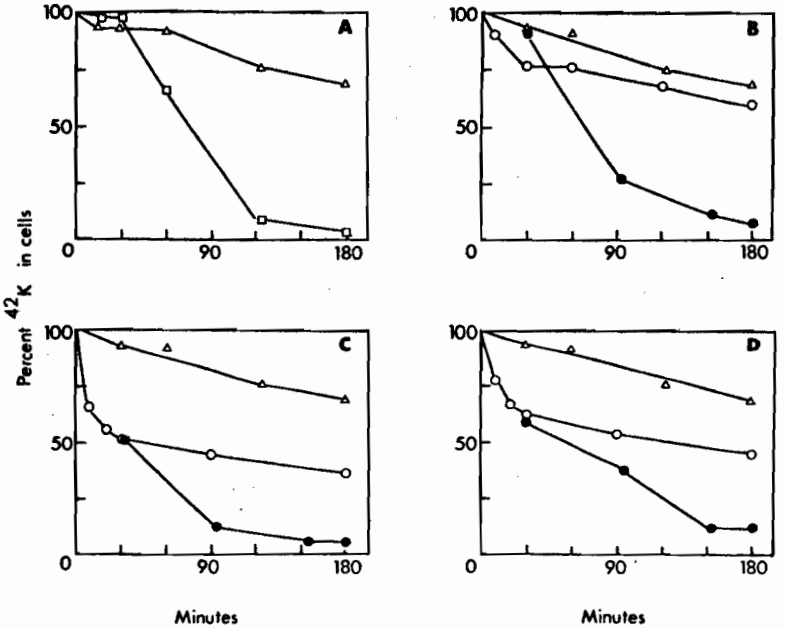

FIG. 7. *Potassium release in glucose-grown cultures treated with PEST and energy poisons. The experiments were done as described in the legend to Fig. 2, except that PEST was the toxin added, to 150 μg of protein per ml. Survival in the culture treated with toxin only was about 1%. Drug concentrations are given in the legend to Fig. 2. (A) Control (untreated cells)* (△), *plus PEST* (□). *(B) Control* (△), *plus iodoacetate* (○), *plus iodoacetate and PEST* (●). *(C) Control* (△), *plus DNP* (○), *plus DNP and PEST* (●). *(D) Control* (△), *plus CCCP* (○), *plus CCCP and PEST* (●).

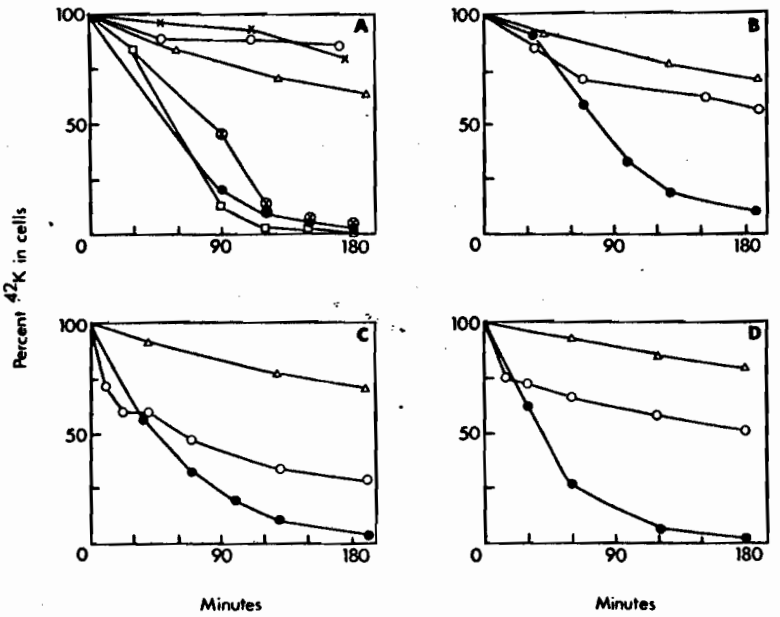

FIG. 8. *Potassium release in ethanol-grown cultures exposed to PEST and drugs. ^{42}K-loaded cultures of K19.10 in Na-YEPE were incubated at zero time wth either a drug, PEST, or PEST plus a drug; the suspensions were then sampled at intervals for radioactivity in the cells. PEST was at 115 μg of protein per ml (A through C), and at 100 μg of protein per ml (D). (A) Control (untreated cells)* (△), *plus PEST* (□), *plus PEST and cyanide* (⊗), *plus PEST and antimycin* (●), *cyanide only* (×), *antimycin only* (○). *(B) Control* (△), *plus iodoacetate* (○), *plus iodoacetate and PEST* (●). *(C) Control* (△), *plus DNP* (○), *plus DNP and PEST* (●). *(D) Control* (△), *plus CCCP* (○), *plus CCCP and PEST* (●).

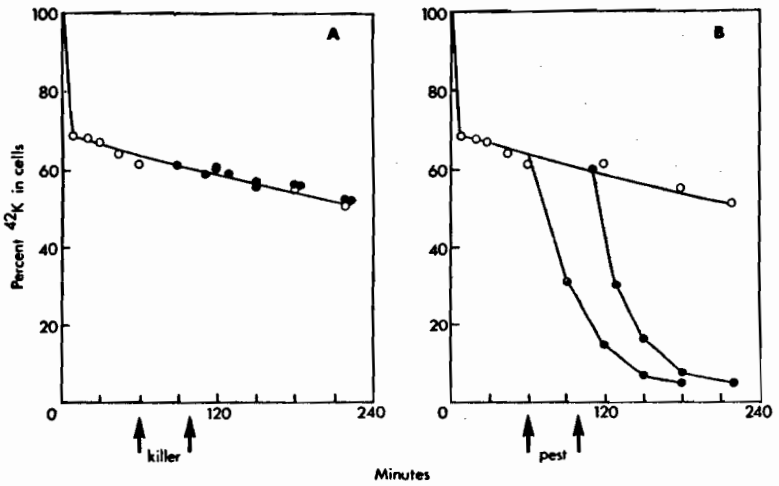

FIG. 9. *Potassium release in DNP-treated cultures treated with either killer toxin or PEST at intervals. A Na-YEPD culture of K19.10 was loaded with ^{42}K, treated with 0.5 mM DNP, and incubated. At the times marked by arrows, portions of the culture received either killer toxin to 40 μg of protein per ml or PEST to 150 μg of protein per ml, and were sampled for ^{42}K remaining in cells. (A) Cells plus DNP* (○), *plus killer at 60 min or plus killer at 100 min* (●). *(B) Cells plus DNP* (○), *plus PEST at 60 min or plus PEST at 100 min* (●).

in ethanol-grown cells) should allow selective digestion of the bound toxin from the yeast surface. Some maintenance of viability in killer-treated cultures is achieved with the wall-digesting preparation gluculase; trypsin has no such effect (3).

We interpret the inhibition of killer action by the various drugs to mean that ATP, or some

form of energy derived from it, is required for the maintenance of a state necessary for membrane damage to occur, a state analogous to the energized plasma membrane in bacteria (13, 31). By analogy to the situation in prokaryotes, it seems likely that a yeast plasma membrane adenosine triphosphatase (ATPase) couples ATP generated from glycolysis or from mitochondrial reactions to membrane-linked work, perhaps via the proton-motive force in the chemiosmotic hypothesis of Mitchell (26). This energy-coupling state is required for, and altered by, the action of the yeast killer toxin; its modification leads to the spectrum of damage to the cell. A similar argument has been advocated to account for the effects of cytochalasin A on the yeast cell (22). The effects of iodoacetate, antimycin, and cyanide on killer action may be attributed to their blocking ATP synthesis, due to inhibition of glyceraldehyde 3-phosphate dehydrogenase and alcohol dehydrogenase (14, 30), cytochrome *c* to b_1 electron transfer (11), and cytochrome *c* oxidase (23), respectively. The effect of iodoacetate reported here may have been nonspecific since we could not arrange a situation in which the alkylating reagent did not block the ^{42}K efflux in killer-treated cells. Iodoacetate is known to produce grossly altered membranes in yeast (25), albeit at concentrations higher than the 1 mM used in our experiments. ATP discharge due to uncoupling and to stimulation of ATPase in mitochondria (32) may account for the inhibition of killer action by DNP or CCCP in the ethanol-grown cultures, but the block of killer action by these drugs in glucose-grown cells is not easy to explain. Inhibition by DNP of energy-linked functions in organisms generating energy in a nonrespiratory mode is well known (13), and many examples have been reported in yeast growing either anaerobically or in high glucose concentrations (2, 9, 28, 29, 33). It has been suggested that DNP dissipates glycolytic ATP in yeast by stimulating a promitochondrial ATPase (28). However, oligomycin or dicyclohexylcarbodiimide, specific inhibitors of the mitochondrial ATPase, do not block the effects of DNP on energy-linked systems in fermenting yeast (10, 19). An alternative view is that reagents such as DNP and CCCP block any membrane-associated and work-requiring systems by eliminating proton gradients (13). CCCP is considered to act by a similar mechanism to DNP (16, 17), and, in our experiments, the two drugs had similar effects on both the stability of the yeast ^{42}K pool in untreated cultures and on the ion efflux in killer-treated cultures. These effects of DNP and CCCP were not dependent on the mode of energy generation in the cells, suggesting that the reagents disrupted energy-linked processes in either glycolyzing or respiring yeast by interacting with the plasma membrane.

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