Our experiments indicate that GPAG is an adequate macromolecular serum factor for attachment of the majority of the brain cells to the collagen-coated surface and for enabling growth and morphological and biochemical differentiation of neural tissue in vitro. The attachment of cells is followed by histiotypic organization and prompt formation of neurites which are characteristic signs of neural tissue differentiation as well as the development of AChE activity. These findings have important implications, because a serum-free medium will facilitate physiological and biochemical studies of freshly explanted neuronal cells, for example neurotransmitter synthesis, synaptogenesis and the development of electrical excitability. Furthermore, in our previous publications we reported that GPAG pinocytosis plays an essential role in transmembrane substrate transport[13] and in the uptake of exogenous DNA by mammalian cells in vitro[14]. It has been demonstrated that GPAG associates with inorganic phosphate, thymidine, uridine, and lysine during incubation at 37 °C and transports the bound precursors into the cells in a compartmentalized manner. The pinocytic carrier function of GPAG is connected with the growth promotion of continually replicating metazoan cells. In this regard, it is interesting to note that since 1953 it is known that the probable cause of the growth of an embryonic neurite is the intake of fluid[15]. Vacuole formation in ganglionic neurites has already been observed by Lewis[16] who first described pinocytosis[17]. For this reason one of the potential uses of GPAG-supplemented medium is to study the mechanisms by which macromolecular serum factor(s) promote the maintenance of neurons in vitro. Since GPAG is present in the blood of metazoan organisms these studies might indicate simultaneously the role of specific serum macromolecules in the maturation of the brain.

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Flow microcalorimetric bioassay of polyene antibiotics: Interaction with growing *Saccharomyces cerevisiae*[1]

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Summary. Microcalorimetric investigation of the interaction of polyene antibiotics with mid-exponential cells of a growing culture of *Saccharomyces cerevisiae* has been used as the basis of a bioassay procedure. The assay is rapid, sensitive and reproducible. The results are compared to classical assays and potency ranking orders.

Calorimetric methods of analysis have been extensively reviewed[3-5]. The application of microcalorimetry to the study of drugs has also been reviewed recently[6]. This review suggests that there are only a few reports of quantitative bioassay procedures for drugs. Given the improvements in sensitivity, reproducibility and speed[7] offered by calorimetric techniques relative to the classical microbiological procedures this is somewhat surprising. The many and varied reports of a qualitative nature on the interaction of drugs with micro-organisms are adequately reviewed by Beezer and Chowdhry[6].

The results of experiments reported previously[7-9] employing isothermal flow microcalorimetry in the bioassay of polyene antibiotics with yeast cells *(Saccharomyces cerevisiae)* used buffered glucose as the only source of nutrient for the organism. Use of this medium produced simple power-time curves (p-t curves; previously termed thermogram, IUPAC, IUB, IUPAB draft recommendations, 1980) which could be analyzed to yield dose/response relationships. The results were used to give an antibiotic biopotency ranking order and this ranking order was compared with the order produced employing minimum inhibitory concentration (MIC) data. The 2 potency rankings were significantly different[10].

The evaluation of the bioassay system depended upon comparison of the appropriate bioassay parameters for the classical and microcalorimetric systems. However, as noted previously there were substantial differences in the experimental conditions. The fundamental difference being that the microcalorimetric system employed nongrowing cells whereas the classical bioassay systems (agar plate diffusion, turbidimetry[11]) employ growing organisms. Thus, the development of a microcalorimetric bioassay involving growing organisms would more nearly mimic the classical technique of bioassay.

Lucensomycin has been shown previously[9] to occupy a borderline position in the response observed with respiring (i.e. nongrowing) cells. Interaction with growing cells may support the view that lucensomycin has 2 modes of action and thus has 2 differing bioassay ranges.

Methods. Frozen inocula of *Saccharomyces cerevisiae* (NCYC 239 Food Research Inst., Colney Lane, Norwich) were prepared, stored and assayed as described previously[12]. The calorimeter (LKB type 10700-1, LKB Produkter AB, Sweden) its design and operation are as described previously[12] except that organism growth regimes rather than respiration regimes were employed. Thus the following variations to the reported[12] procedure were made. Nitrogen

Potency order of drugs

Antibiotic	MIC (μg ml^{-1}) for *Saccharomyces cerevisae*[17]	Microcalorimetric potency ranking Antibiotics added ½ MIC[b] to respiring stationary phase cells[9]	Antibiotics added as equimolar solutions to[a]: Respiring stationary phase cells[8]	Mid-exponential phase cells (this work)[c]
Nystatin	0.8–3.1	4	5	4
Amphotericin B	0.09–0.5	2	1	2
Candicidin	0.03	1	2	3
Pimaracin	0.9–15.0	3	3	1
Lucensomycin	–	–	4	5

[a] The most potent drug being that one which, at equimolar concentrations, inhibits the respiration or growth of the yeast cells in the shortest time. [b] ½ MIC concentration taken was half the lowest value in the quoted range. [c] This order was confirmed by parallel flow nephelometric experiments.

saturated sterilised medium (containing, g l^{-1}, in glass distilled water: Lab-Lemco powder, 1.5; yeast extract 1.5; mycological peptone, 5.0; NaCl, 20.0; glucose, 11.0; K_2HPO_4, 1.32; KH_2PO_4, 3.68; the pH was adjusted to pH 6.0) was pumped (52 ml h^{-1}) through the calorimeter to establish a baseline. 1 ml (10^7 cells ml^{-1}) of stationary phase yeast cells[13] was added to an incubation vessel containing 150 ml of the sterilised medium maintained at 303 K and the contents of this incubation vessel were pumped through the calorimeter. The effluent from the calorimeter was returned to the incubation vessel. The response of the microcalorimeter was amplified and recorded on a chart recorder in the form of a control p-t curve (fig. 1a). The calorimeter was housed in a room maintained at 298 ± 0.5 K and operated at 303 ± 0.005 K.

For antibiotic treated incubations the following procedure was adopted. Known concentrations of the polyene antibiotics were dissolved in 0.3% dimethyl formamide (DMF)/phosphate buffer at pH 6.0 and added in 1.0 ml aliquots to mid-exponential phase yeast cells (point A in fig. 1a). Simultaneous experiments by flow nephelometry[14](MSE Vitatron) indicated that the yeast cells in the incubation vessel underwent growth from a lag phase through an exponential phase to the stationary phase. This demonstrated that exponential increase in power output accompanied exponential growth (i.e. that point A (fig. 1a) was indeed at the mid-point of exponential growth. The addition of 1.0 ml of 0.3% DMF/buffer at the mid-point of the exponential phase served as a control. This addition had no observable effect upon the derived p-t curve. Replicate p-t curves for a fresh yeast culture were identical with those for frozen inocula except for a slight extension of the log phase (20 min). This finding confirms that microcalorimetrically fresh and frozen yeasts are indeed comparable in all important respects.

Materials. Nystatin and Amphotericin-B (E.R. Squibb and

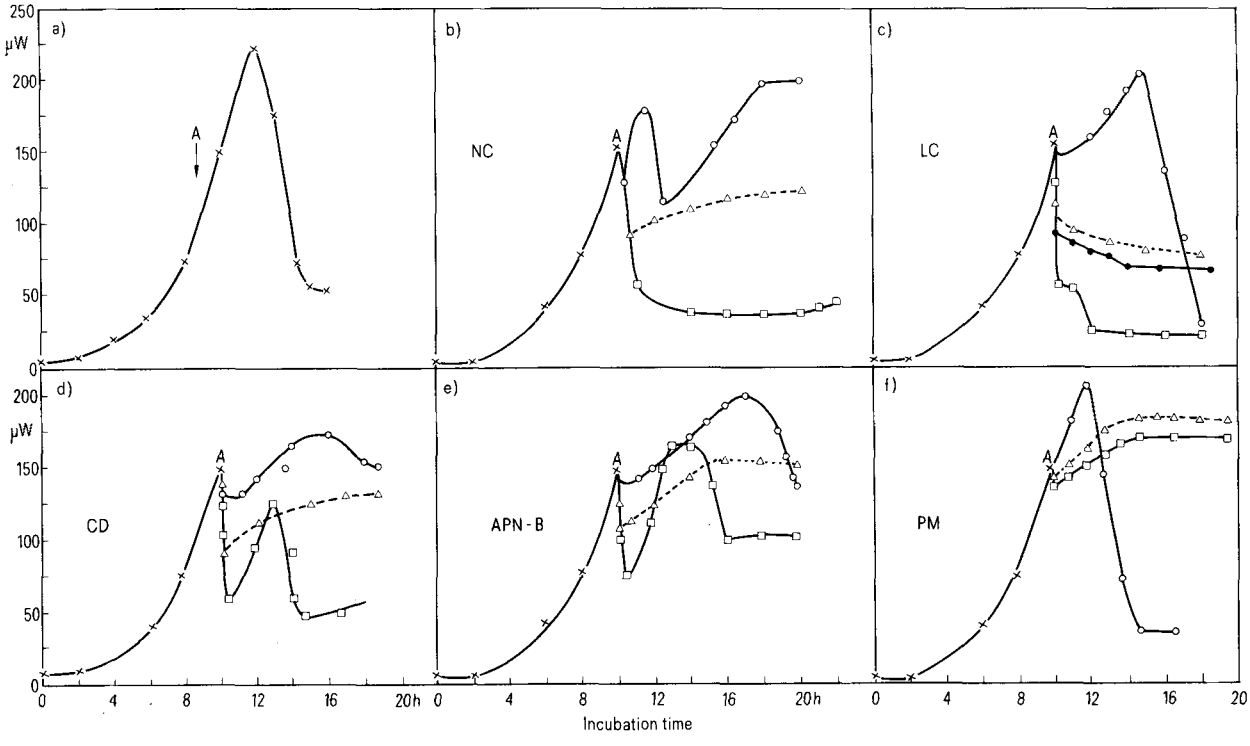

Figure 1. *a* represents control – no antibiotic; (A) marks point at which antibiotics were added in interaction experiments. *b* Nystatin; *c* Lucensomycin; *d* Candicidin; *e* Amphotericin B; *f* Pimaricin; ○ 1×10^{-6} M; △ 5×10^{-6} M; ● 6×10^{-6} M; □ 1×10^{-5} M.

Sons Ltd, Merseyside, U.K.), Pimaricin (Gist Brocades N.V., Delft, Holland), Lucensomycin (Farmitalia, Milan, Italy) and Candicidin (Pharmax, Crayford, U.K.) were stored under nitrogen at 250 K until required. UV spectroscopic investigation on receipt of the antibiotics showed λ_{max}-values in accord with literature values[13].

Results and discussion. Stationary phase yeast cells added to growth medium in the absence of antibiotics (fig. 1a) showed a lag phase indicated by a low power followed by exponential growth which was accompanied by exponential increase in power. When the cells entered the stationary phase there was a decrease in power to the baseline level. The onset of this stationary level coincided with a rapid fall in the glucose concentration. The baseline level represented exhaustion of glucose and an enormous decrease in metabolic rate of the yeast cells[6]. The single peak in the p-t curve appeared because the glucose present in the medium was the only major carbon source present. Under the conditions employed no second phase of growth on a produced metabolite was observed. Metabolic modifiers when present in the medium can significantly affect the appearance of such p-t curves[6]. Thus the addition of polyene antibiotics to growing yeast cells at the mid-point of exponential growth resulted in p-t curves which differed from the control curve in a manner which was dependent upon the nature and concentration of the antibiotic applied (fig. 1b–f). However, all the p-t curves showed a rapid initial decrease in heat output rate. At certain antibiotic concentrations yeast growth was resumed (as measured both microcalorimetrically and by nephelometry[14]) but not to the final level achieved in the absence of antibiotics.

The processes which contribute to the yeast cell-antibiotic p-t curves are complex and ill-understood. Nevertheless the kinetics of cell-antibiotic interactions are faithfully recorded by the microcalorimeter and interesting analytical data can be obtained from the p-t curves which cannot be obtained by other analytical methods. This is because the microcalorimeter results give a continuous record of the effect of polyene antibiotics on the total metabolism of the yeast cells. Other physico-chemical (i.e. non-microcalorimetric) studies of cell-polyene antibiotic interactions have not employed the range of polyene antibiotics used in this study. Moreover in these reports of yeast cell-polyene antibiotic activity only component reactions (e.g. K^+ efflux[15] or amino acid efflux[16] from cells) have been used to monitor bioactivity.

The polyene antibiotic lucensomycin produced a total inhibition of growth of yeast cells at a concentration of 10^{-5} M. At concentrations between 6×10^{-6} M -10^{-5} M the heat output rate of yeast cells showed a 'biphasic' response indicating that 2 separate processes or groups of processes occurred in the presence of lucensomycin (fig. 1c). Thus at 1×10^{-6} M the p-t curve resembled, in form, those observed for the other antibiotics but at 5×10^{-6} M and 6×10^{-6} M there was no 2nd peak or subsequent rise in the p-t curves. The reasons for this 'biphasic' response are not understood at present but merit further investigation.

Bioassay of the antibiotics can be achieved by empirical analysis of the p-t curves. There exists a linear relationship between antibiotic concentration and the value of the decrease in power over the initial part of the curve (see fig. 2). This bioassay procedure may permit analysis of the active drug content of clinical samples (e.g. serum or other biological fluids) following antibiotic administration to patients. This is so since calorimetry makes no demand for optical clarity, colourless solutions etc. It only requires a reaction.

The reproducibility of the control and treated p-t curves is $\pm2.4\%$ which compares favourably with the 5–10% expected for classical agar diffusion disc bioassays[7]. The time per assay (i.e. the time from the point of addition (A in fig. 1a) to the minimum in the curve) is nowhere greater than 30 min. Thus the time to reach point A in figure 1a is 9 h and total assay time is therefore 9 h 30 min. However since the control p-t curve is so reproducible it is possible to arrange a 'train' of suitably treated incubations flowing through the flow midrocalorimeter at pre-determined intervals. These intervals could be so judged as to only record the relevant portion of the p-t curve. This practice is not uncommon in other assay systems e.g. automatic amino acid analyzers. However there is no doubt that the availability of a multichannel flow microcalorimeter would radically improve sample throughput rate. The concentration range accessible is lower (i.e. the assay is more sensitive, than the classical technique allows (lowest determinable concentration by agar diffusion is 20 units $ml^{-1}\equiv 5\times10^{-6}$ M).

The final level of power output of yeast cells in the presence of antibiotics indicates the biopotency of the antibiotics since this parameter represents the metabolic activity of yeast cells after complete interaction with antibiotics. The order of biopotency of antibiotics, on a molar basis, measured for mid-stationary phase respiring yeast cells[8] and mid-exponential phase cells (this study) differ from each other and from the order obtained by measurement of MIC using classical microbiological assay methods[17] (table 2).

Scepticism over the significance of MIC measurements of antibiotics has been expressed by Greenwood[18]. Variation occurring not only with the bioavailability of the drug but also with the size of the inoculum, the incubation period and the medium used. The biopotency results obtained in this study for antifungal antibiotics support other microcalorimetric studies with anti-bacterial antibiotics which question the usefulness of comparisons of MIC's of related drugs. It is to be noted that the MIC's of polyene antibiotics show a wide variation[17].

Interestingly the 'generally accepted order for membrane activity of polyene antibiotics is roughly amphotericin B > (lucensomycin, pimaricin) > nystatin and the order of antiyeast activity amphotericin B > nystatin > pimaricin'[16]. Both these ranking orders differ from those obtained in the present study.

Microcalorimetry has been shown to be useful in the study of blood and its fractions[6] and polyene antibiotics are

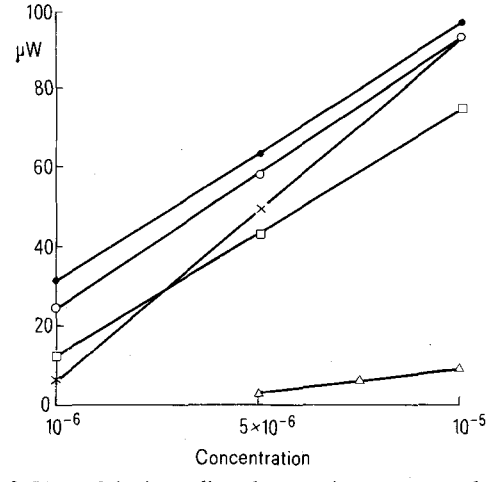

Figure 2. Plots of the immediate decrease in power over the initial part of the p-t curves and concentration of antibiotics. Each point represents the average of at least 3 determinations. ● nystatin; ○ candicidin; □ amphotenicin B; × lucensomycin; △ pimaricin.

known to bind to erythrocytes. Thus it would be interesting to investigate the bioactivity and establish a bioassay of polyene antibiotics against yeasts in the presence of blood. The polyenes are thought to interact with sterols in sensitive cell membranes[19]. The sterol in membranes of erythrocytes is cholesterol and that in yeast cells is largely ergosterol. Such studies may indicate a more in vivo bioactivity and could contribute to our understanding of the rather severe side effects of polyene antibiotics (amphotericin B for example is nephrotoxic[20]).

Flow microcalorimetry in conjunction with standardized liquid nitrogen stored inocula may also prove useful for examining the effects of polyene antibiotics on yeast cells at different stages of growth. The effect of pharmaceutical formulations of the antibiotics may reveal the effects, if any, of pharmaceutical excipients on bioactivity of polyene antibiotics.

Finally the effects of combinations of antifungal compounds on yeast cells could be examined for possible inhibitory or potentiation effects by microcalorimetry.

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Influence of adult food on the reproduction of virgin females of an *Acanthoscelides obtectus* strain originating from Colombian altiplanos

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Summary. The reproductive activity of virgin females of an *Acanthoscelides obtectus* strain originating from the high Colombian plateau was variable. Some females did not synthesize vitellogenin during imaginal life while others produced mature oocytes. In the case of virgin females without reproductive activity, the supply of pollen in the adult diet did not stimulate synthesis and incorporation of vitellogenin. On the other hand, the supply of pollen always induced a higher vitellogenin titre in the haemolymph of females which produced oocytes, and stimulated ovarian production.

Previous research on *Acanthoscelides obtectus* has shown variations between the reproductive activity of virgin females from strains of different geographic origins.

In a population obtained from *Phaseolus vulgaris* seeds harvested on the Colombian altiplanos in the insect's zone of origin, 90% of the first generation females did not produce any mature oocytes when kept as virgin females in the absence of their host plant and fed with honeyed water[1,2]. Laboratory experiments showed that the introduction of *Phaseolus vulgaris* seeds and copulation can remove this reproductive quiescence after a latency period. This type of regulation would make possible a synchronization between the insect's and the host plant's reproductive cycles in nature. Production and prolonged oocyte retention in the female genital tract would thus be avoided outside the host plant's maturation period. This would be advantageous to the insect since prolonged retention of oocytes affects their subsequent developmental capacity after fertilization[3].

However, in another Bruchidae (*Bruchus pisorum*) which also shows a reproductive quiescence outside the host plant's maturation period, Pajni et al.[4] have shown that a diet containing pea-flower pollen is one of the factors inducing reproductive activity. We have therefore tried to determine whether a pollen diet does indeed induce the reproductive activity of *Acanthoscelides obtectus* females, or favour the action of other stimulating factors, and might thus be involved in the synchronisation of the insect and host plant's reproductive cycles.

Materials and methods. The *A. obtectus* strain was collected in the Buesaco area (Province of Nariño) on the Colombian altiplano. The insects were reared in climatic conditions approximating those found in their biotope (2° N latitude, altitude 1800 m); photoperiod 12 h D:12 h L; thermoperiod 21 °C (L)–13 °C (D); relative humidity 70%. Experiments were carried out on insects reared for 15 generations under these conditions. Only 20–30% of the virgin

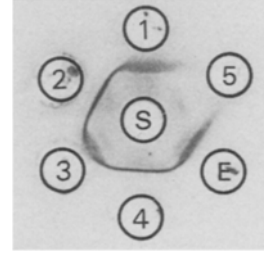

Figure 1. Demonstration of vitellogenin present in the haemolymph by the Ouchterlony technique in *A. obtectus* females. S, antiserum; E, egg; aqueous extracts of females 1, 2, 3, 4 contain vitellogenin, female 5 has not synthesized vitellogenin.