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An assay is described to measure sterol uptake in **Saccharomyces cerevisiae.** This assay involves specific binding of radioactively labelled sterol to actively growing yeast cells.

It was found that uptake does not occur in stationary cultures and that some growth is necessary for FY3, a sterol auxotroph, to take up exogenous sterol. Uptake is maximized at pH 5.5 and in the absence of Tween 80 in short term (4 hour) assays. The optimal temperature for uptake was found to be about 31° C. Most uptake studies were carried out at 28° C for convenience.

The sterol uptake system exhibits Michaelis-Menten kinetics and shows a K_m of 62.9 µg/ml cholesterol under the conditions described. The activation energy is 94000 cal/mole.

Sterol Uptake in
Saccharomyces cerevisiae

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STEROL UPTAKE IN **SACCHAROMYCES CEREVISIAE**

INTRODUCTION

The obligate nature of sterol in eukaryotic membranes is well-established. In **Saccharomyces cerevisiae** up to 10% of the cell's dry weight may be sterol, and as much as 90% of that is ergosterol (Karst and Lacroute, 1977). Under conditions which do not allow the cell to synthesize its own sterol, **S. cerevisiae** continues to grow by means of a system in which sterol is taken up from the growth medium. A variety of sterols may fill this requirement although preference is given to those sterols most similar to ergosterol (Taylor and Parks, 1981).

Few studies have been done on sterol uptake in living systems. In the past the utility of this system lay in the enrichment of yeast membranes with unusual sterols by supplementation of an anaerobic culture. Recently, an interrelationship between heme biosynthesis and sterol biosynthesis has been noted. Heme-containing cytochromes have been found to be specifically involved at several points along the sterol biosynthetic pathway. By combining a heme mutation with a sterol biosynthesis mutation it is possible to get tight sterol auxotrophs so that sterol uptake may be studied more easily in aerobic cultures than in anaerobic systems.

FY3 is a sterol auxotroph with concomitant requirements for unsaturated fatty acid and methionine.

It was necessary to develop a simple, repeatable method for examining sterol uptake in which conditions (sterol species, growth conditions, etc.) might be varied.

^{14}C-Cholesterol was chosen as the sterol supplement in these experiments because it provides for good growth in FY3. In addition the molecule is very stable and was readily available. This assay is devised so that any radioactively labelled sterol may be used. This paper also reports various physical parameters and kinetics of the sterol uptake system.

LITERATURE REVIEW

The function of sterol in biological systems is not well understood. A wealth of material has been written on the role of sterols in membranes. Recently new technology has made possible the experiments to reveal specific actions of sterols in yeast cells.

As early as 1925, Leathes recognized the condensing effect of cholesterol on lecithin membranes and noted that the magnitude of this effect was determined by the type of fatty acid moieties in the lecithin molecule as well as by the molar ratio of cholesterol to lecithin. This lead scientists to predict a bulk structural role for sterol in biological membranes. This has been supported by more recent studies with differential scanning calorimetry. This method measures the temperature at which a phospholipid bilayer membrane undergoes a phase transition from gel to liquid-crystalline state. Addition of sterol to the phospholipid bilayer may change the transition temperature or reduce or eliminate the phase transition altogether (Parks et al., in press). This does, in fact, indicate a structural function for sterol in maintaining fluidity over a temperature range.

In experiments carried out by Rodriguez et al. (1982) sterol auxotrophs were examined for growth on various sterols and stanols. It was found that cholestanol was unable to support growth, however the addition of a very small amount of ergosterol was enough to allow the culture

to grow well. The authors refer to this as a "sparking" effect. This data indicate that ergosterol is fulfilling some obligate function for which only a small amount of sterol is required. The structure of the sterol in this function being more critical than that function which is fulfilled by the cholestanol structure.

In addition, experiments in which plasma membrane enzymes, such as Mg-ATPase and chitin synthetase, were examined for their sensitivity to nystatin (Bottema et al., 1982) have been reported. Nystatin is a polyene antibiotic. The mode of action involves binding of the antibiotic to the sterol component of the membrane (Norman et al., 1976). It was found that Mg-ATPase was unaffected by the addition of nystatin, while chitin synthetase was very sensitive, indicating that the environments of the two enzymes differ greatly in sterol content and that sterol must exhibit asymmetry in the membrane resulting in sterol-rich and sterol-poor regions.

Nystatin has also been used to generate yeast sterol mutants. Yeast which are resistant to nystatin accumulate intermediates of the ergosterol biosynthetic pathway, and it is through the study of these mutants as well as that of specific inhibitors, which has enabled scientists to outline the biosynthetic pathway of ergosterol in yeast (Figure 1).

Maguigan and Walker (1940) were among the first to detect a correlation between sterol synthesis and

aerobiosis in yeast. Since that time specific requirements for molecular oxygen have been analyzed. Among the requirements which have been studied are 2,3-oxidosqualene cyclization, desaturations and demethylations (Aries and Kirsop, 1978; Hata et al., 1981; Osumi et al., 1978; Reddy et al., 1977).

In the absence of O_2, sterol synthesis proceeds only as far as squalene. Since squalene is unsuitable in the role of sterol, an exogenous source of sterol is required under anaerobic conditions (Andreasen and Stier, 1953a; Buttke and Bloch, 1980; Nes et al., 1976). This requirement does not stringently call for ergosterol. A variety of other sterols, including cholesterol, have been found to support the growth of **S. cerevisiae** to varying degrees. Anaerobic cultures also have concomitant requirements for fatty acid and methionine due to an absence of cytochromes involved in the biosynthetic pathways of each (Andreasen and Stier, 1953b).

This requirement for sterol has been exploited to enrich yeast membranes in unusual sterols to study their effects in biological systems. It was found useful to develop a mutant strain of **S. cerevisiae** which had an absolute requirement for exogenous sterol under all conditions so that the total sterol content of the membrane might be manipulated more easily. After many attempts it has not been possible to develop a viable, non-leaky mutation in sterol synthesis resulting in

Figure 1. **Transformation sequences from squalene to ergosterol.** The compounds described with Roman numerals are: I, squalene; II, squalene epoxide; III, lanosterol; IV, 4,4-dimethylcholesta-8,14,24-trienol; V, 14-demethyllanosterol; VI, 4 -methylzymosterol; VII, zymosterol; VIII, fecosterol, ergosta-8,24(28)- dienol; IX, episterol, ergosta-7,24(28)-dienol; X, ergosta-5,7,24(28)-trienol; XI, ergosta-5,7,22,24(28)-tetraenol; XII, ergosterol, ergosta-5,7,22-trienol; XIII, 4 -methylfecosterol, 4 -methylergosta-8,24(28)-dienol; XIV, cholesta-7,24-dienol; XV, ergosta-8,22-dienol; XVI, ergosta-8-enol; XVII, ergosta-7,22,24(28)-trienol; XIX, ergosta-7-enol; XX, ergosta-7,22-dienol; and XXI, ergosta-5,7-dienol. (Parks, 1978).

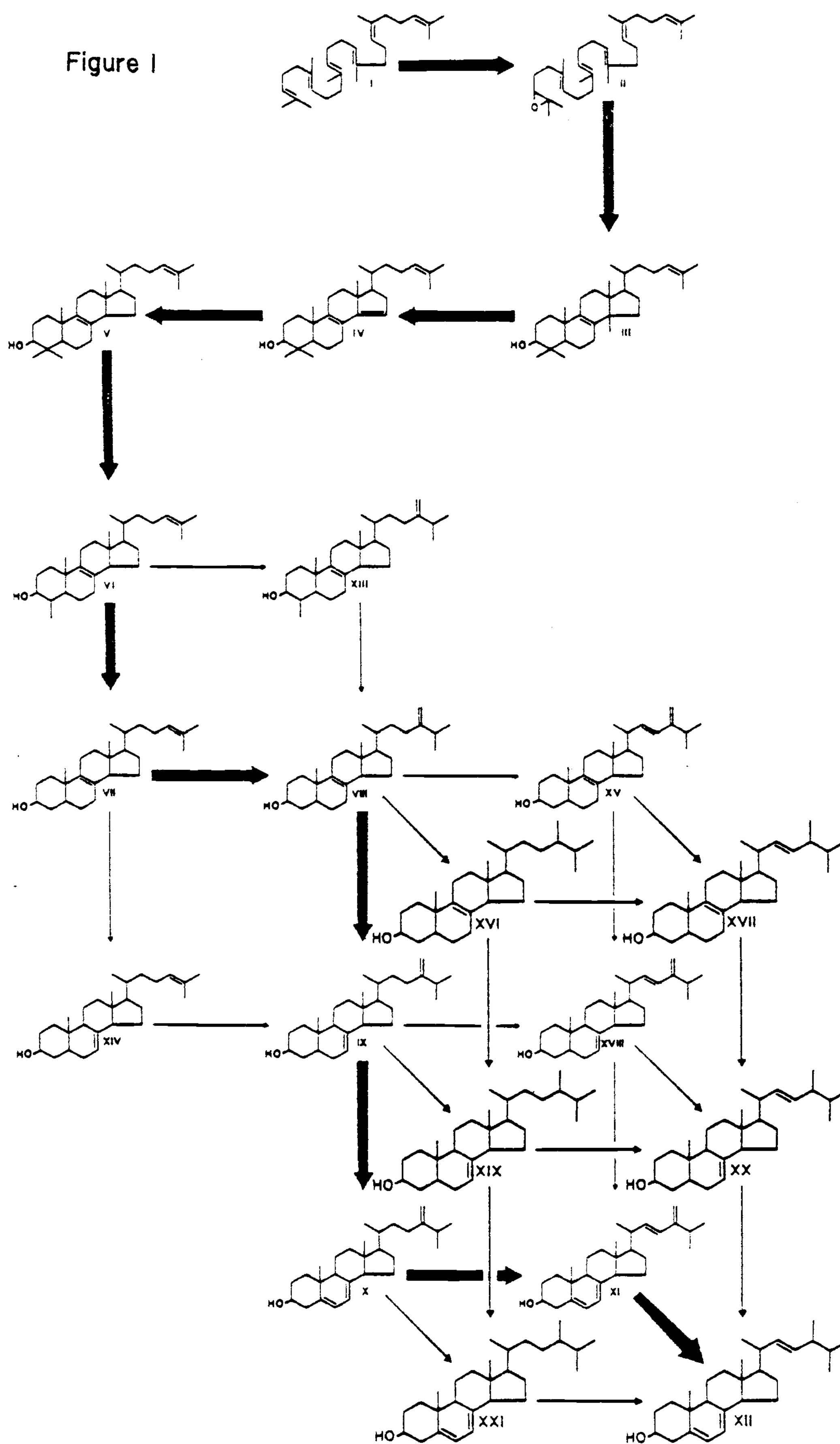


Figure I

auxotrophy without a concurrent mutation early in the heme biosynthetic pathway (Buttke and Bloch, 1980; Gollub et al., 1977; Karst and Lacroute, 1973). Therefore, sterol auxotrophs available are also auxotrophic for fatty acid and methionine. In a yeast auxotroph which only carries a mutation in the heme pathway, supplementation with one or another of the heme biosynthetic intermediates will, in most cases, restore wild-type sterol and fatty acid composition and inhibit sterol uptake (Bard et al., 1974).

There is very little information available on the uptake of sterol by cells. Work with mycoplasma cells and membranes in **Acholeplasma laidlawii** (Razin, 1978) indicates that sterol uptake depends on membrane fluidity as determined by temperature and fatty acid composition of the membrane. This uptake is independent of energy metabolism. Pronase digestion of the isolated membrane has no effect on the incorporation of sterol (Razin et al., 1979). Experiments with **Mycoplasma capricolum** did show enhanced uptake in growing cells over membranes (Razin et al., 1980), however this may be stimulated by concurrent phospholipid uptake which is dependent on growth.

In **S. cerevisiae** sterol uptake appears to be much more specifically regulated. This is expected because the plasma membrane in yeast is isolated from the environment by a cell wall, unlike mycoplasmas in which the membrane

is in direct contact with the medium. Also, yeast normally synthesize their own preferred sterol and do not depend upon exogenous sources except under specialized conditions. It has been shown that sterol uptake appears to be under enzyme action for which substrate specificity and metabolic energy dependence has been demonstrated (Taylor and Parks, 1981). By measuring total sterol taken up in a culture grown in the presence of two sterols differing in a single structural change, it was possible to identify some of the structural characteristics which resulted in preferential uptake by the cell. These were the Δ^5, Δ^7, and Δ^{22}-bonds and the 24β-methyl group.

MATERIALS AND METHODS

Saccharomyces cerevisiae strains. Strain FY3 was the organism of choice for this study (Taylor and Parks, 1980). FY3 is blocked in sterol biosynthesis at 2,3-oxidosqualene cyclase and also in heme biosynthesis at δ-aminolevulinic acid synthase (Figures 1 and 2). In a defined medium, FY3 requires exogenous sources of sterol, fatty acid and methionine.

Strain S288C, a wild-type haploid which accumulates ergosterol as its predominant sterol, was used in various experiments as a negative control for sterol uptake.

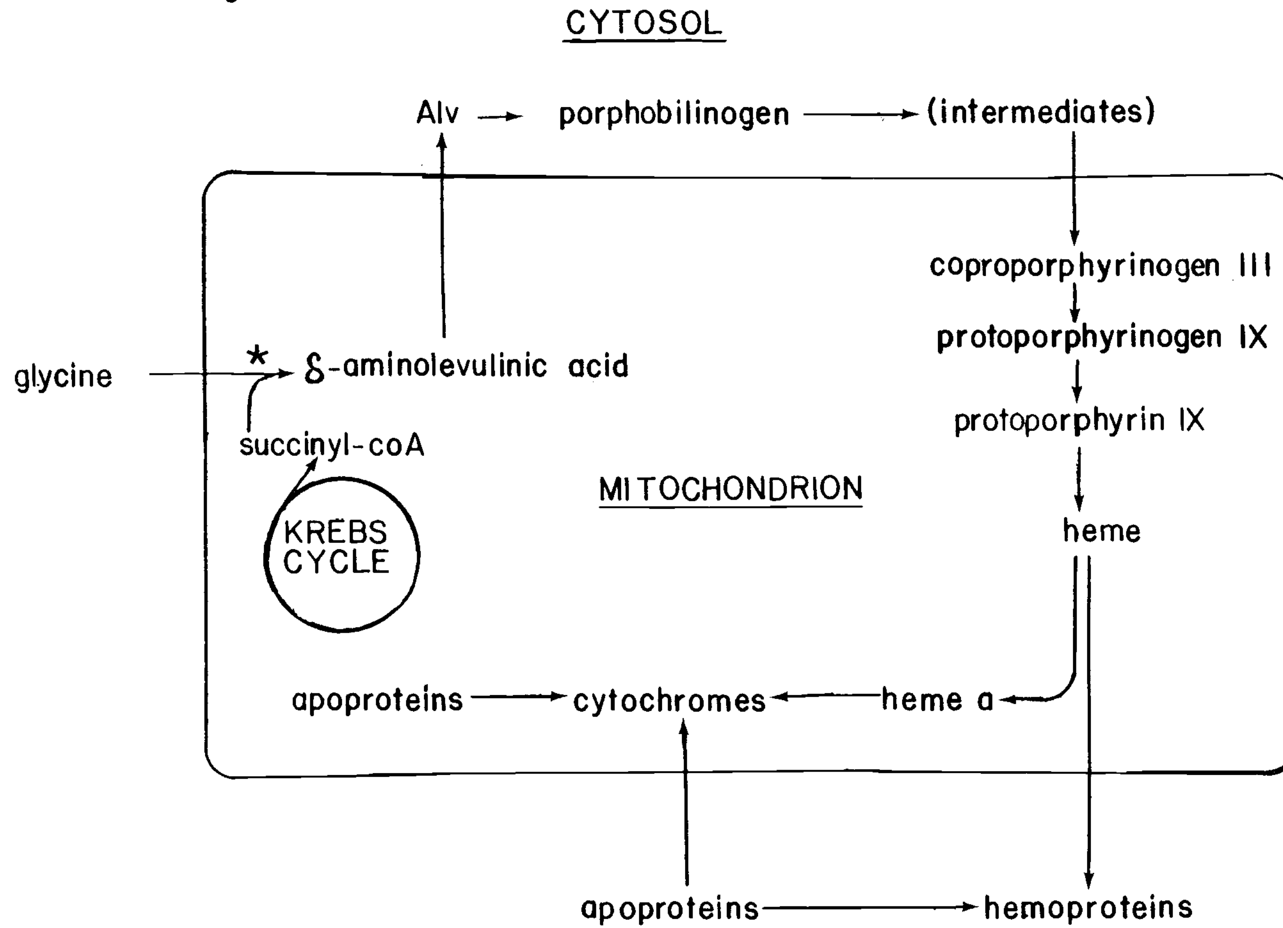
Media and Culture Conditions. Minimal medium was 0.67% Yeast Nitrogen Base without amino acids (Difco), 2.0% dextrose, 1.0% polyoxyethylene sorbitan mono-oleate (Tween 80), 0.1 M KH_2PO_4, 0.01% methionine, and 0.001% of each of the following: adenine, histidine, leucine, lysine, phenylalanine, tryptophan, tyrosine, and uracil, adjusted to pH 5.5 with 6% KOH. After autoclaving, cholesterol was added to a final concentration of 10 μg/ml from a solution of 4 mg/ml cholesterol in tergitol:ethanol (1:1, v/v).

Cultures were inoculated from stationary phase cultures and incubated at $28 \pm 1^{\circ}$ C with shaking to mid-logarithmic growth phase. Cells were harvested by centrifugation at 4080 x g in a Sorvall RC-2B centrifuge equipped with a GSA rotor, and washed twice in sterile distilled water.

Assay Conditions. Minimal medium as described above,

Figure 2. **Heme biosynthetic pathway.** ★ indicates site of hem 1 mutation (Alv synthase).

Figure 2



without Tween 80 unless otherwise stated, was added with ^{14}C-Cholesterol solution (see below) to a total of 9.9 ml and allowed to equilibrate to temperature for 90 minutes with shaking. A Scientific Industries temperature gradient incubator set at 28 $\pm$ 1° C was used. Cells were then added in 0.1 ml distilled H_2O to a concentration of 100 to 300 Klett units as measured on a Klett-Somerson spectrophotometer (approximately 1 x 10^7 to 3 x 10^7 cells/ml measured with a Coulter Counter). Aliquots of 250 µl were removed in triplicate immediately after adding cells and Klett absorbance measurements were taken when cells were added. This procedure was repeated at one hour intervals for a period of 4 hours. The reaction was stopped by placing tubes containing the cells in an ice bath.

Samples were then filtered through 0.45 µm HAWP millipore filters and washed with six, 1 ml aliquots of a solution of 2.5 ml tergitol:ethanol (1:1,v/v) per liter of distilled H_2O. After air drying, filters were resuspended in 0.1 g/L 1,4-bis[2(5-phenyloxazolyl)]- benzene, (POPOP), 3.0 g/L 2,5-diphenyloxazole (PPO) in toluene, and counted for radioactive decay in a Beckman Model LS 8000 scintillation counter for 5 minutes.

Preparation of 4-^{14}C-Cholesterol Solution. Reagent grade cholesterol was resuspended in redistilled isopropyl alcohol and the solution run in 250 µl aliquots through a Beckman high performance liquid chromatograph (HPLC)

equipped with an Altex Ultrasphere ODS column in a solution of 85.5% methanol, 10% ethanol, and 4.5% H_2O at a flow rate of 6 ml/min. Methanol and ethanol were redistilled and the H_2O was filtered for purity for HPLC work. Sterol was detected by monitoring absorption at 210 nm on a Hitachi Model 100-40 spectrophotometer. The fraction containing sterol was collected from the column and dried with low heat under N_2. HPLC-purified cholesterol was quantitated by the Liebermann-Burchard colorimetric assay as described (Taylor and Parks, 1980).

4-^{14}C-Cholesterol (50 µCi/0.33 mg) was added to the HPLC purified cholesterol to a final concentration of 8 mg/ml in tergitol:ethanol (1:1,v/v) and a specific activity of 23900 cpm/µg.

Calculations. The rate of cholesterol uptake was determined as follows. The average radioactive decay for three samples at each time point was determined. The decay at initial time point (t_o) was considered the control for non-specific binding and subtracted from each of the following data points. The number of Klett units is directly proportional to the concentration of cells in a sample. The corrected average cpm for each time point was divided by the number of Klett units for that time point (Klett measurements during logarithmic growth corrected with line of best fit calculation; Snedecor and Cochran, 1980). Rate of uptake is then expressed as:

$$\frac{\dfrac{(cpm_n - cpm_o)}{(\#\ Klett_n)(0.250\ ml)} - \dfrac{(cpm_{n-1} - cpm_o)}{(\#\ Klett_{n-1})(0.250\ ml)}}{t_n - t_{n-1}\ (hours)} = cpm/ml/Klett/hour$$

The data confirmed Taylor and Parks' (1980) observation that sterol uptake is not always linear for approximately the first 30 minutes. Therefore, the final rate of uptake was considered as the average of the rates observed after one hour.

Chemicals and Materials. 4-^{14}C-Cholesterol was obtained from New England Nuclear Laboratories and was of 99% purity. Amino acids were from Sigma Chemical Company. Other chemicals were of reagent grade unless otherwise specified.

RESULTS AND DISCUSSION

Growth Experiments. During growth experiments mid-logarithmic and stationary phase cells were monitored for cholesterol uptake. Cholesterol uptake was observed to continue in a linear fashion until cells entered stationary phase. The uptake was then negligible (Figure 3). From this data it was concluded that measurable uptake be monitored only when cells are growing and/or dividing. Therefore rates of uptake were always measured for cultures exhibiting logarithmic growth.

Tween 80. A source of fatty acid is required when a stationary phase inoculum is used for growth studies, however actively growing cultures will continue to grow in the absence of exogenous fatty acid for several generations, until the fatty acid content of the cell is diluted to about 25% of the original concentration (Gordon and Stewart, 1972). To determine the effect of Tween 80 on sterol uptake, the concentration of this compound was varied from 0 to 1.25% (Figure 4). A difference is not noticeable at 10 µg/ml cholesterol, in contrast with 36 µg/ml, where there is a dramatic decrease in uptake from 0 to 0.25% Tween 80. Further increase in Tween 80 concentration did not result in further decrease in uptake. The decrease is thought to be due to the solubility of cholesterol in Tween 80 and the resulting availability of cholesterol to the cell. Since it was necessary to observe small differences in uptake, Tween 80

Figure 3. **Sterol uptake of a culture entering stationary phase.** Tween 80 concentration is 1.0%. Circles indicate sterol taken up in cpm/ml; triangles indicate growth in log Klett units. A-FY3. B-S288C.

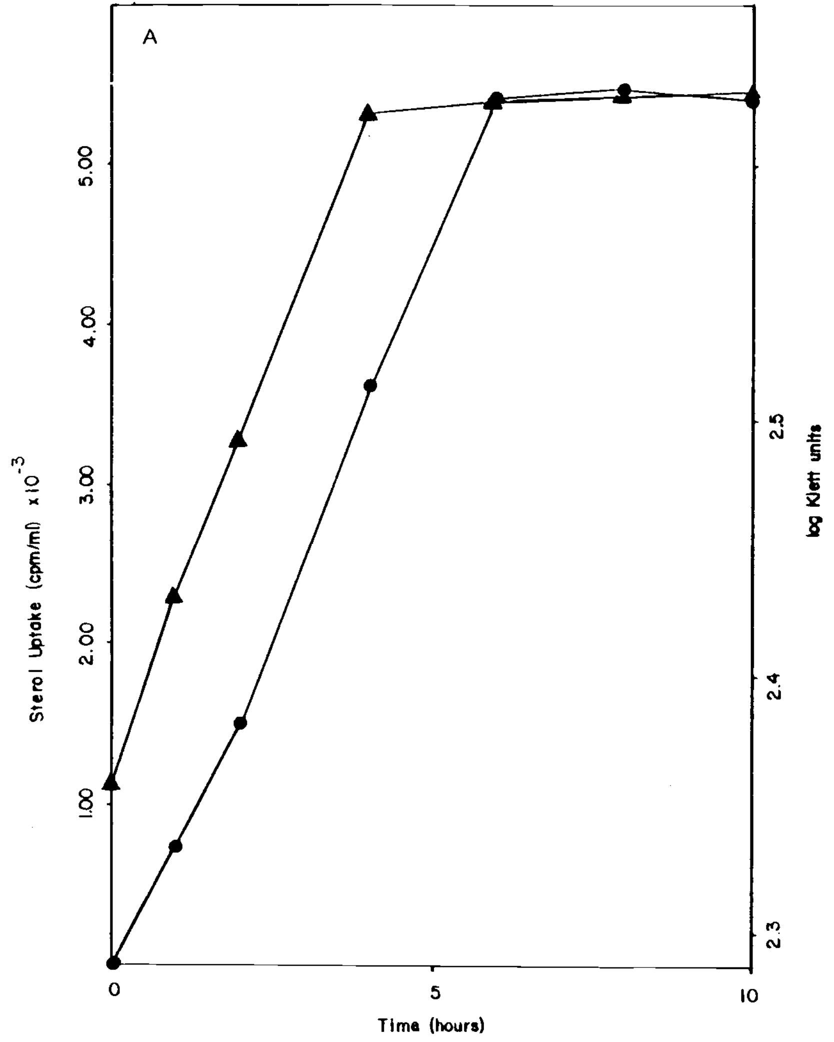


Figure 3

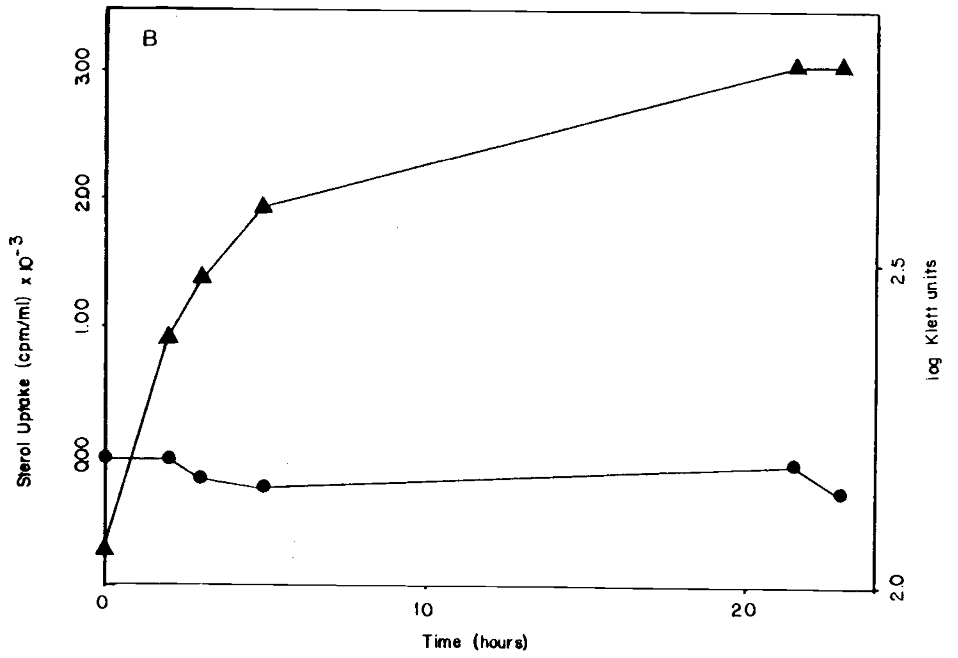


Figure 4. **Effect of Tween 80 on sterol uptake in FY3.** Closed circles indicate uptake at 10 µg/ml cholesterol; open circles indicate uptake at 36 µg/ml cholesterol.

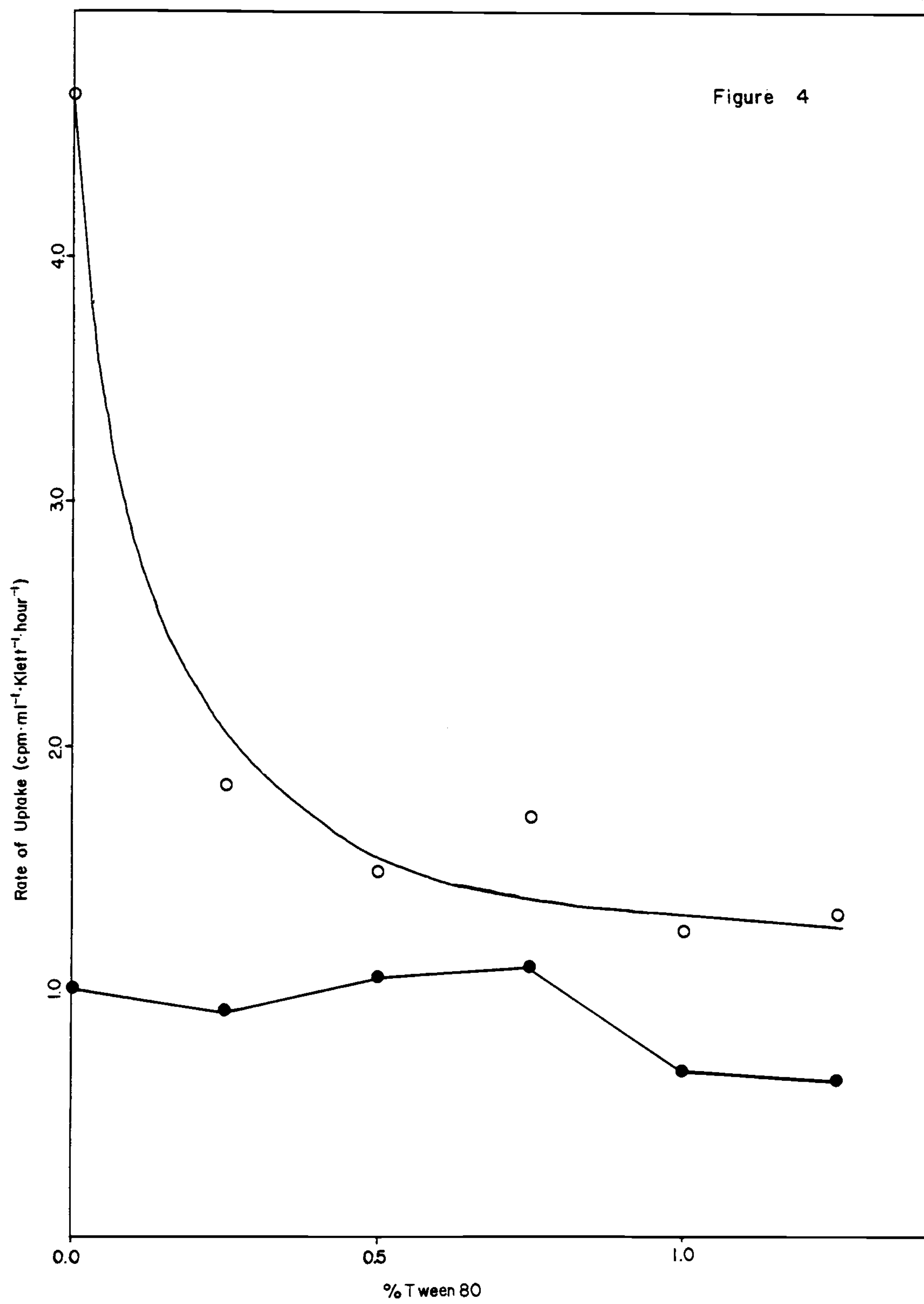


Figure 4
Rate of Uptake (cpm·ml⁻¹·Klett⁻¹·hour⁻¹)
4.0
3.0
2.0
1.0
0.0
0.5
1.0
% Tween 80

was omitted from the assay medium in most experiments.

pH Studies. The effect of pH on sterol uptake medium was examined. A Corning digital pH meter, model 125 was used to determine the pH of the medium before and after autoclaving. A comparison of pH versus rate of cholesterol uptake shows that optimal activity occurs at about pH 5.5 (see Figure 5). This optimum is coincident with optimal growth rate.

Kinetics. In order to vary the sterol concentration the amount of ^{14}C-cholesterol stock solution added to the assay mixture was varied, and tergitol:ethanol (1:1, v/v) was added to make the same total volume as was added in the assay for the highest concentration of ^{14}C-cholesterol.

Sterol uptake exhibits Michaelis-Menten kinetics at higher sterol concentrations (Figure 6). The K_m of cholesterol uptake at pH 5.5 and 28 $\pm$ 1° C is calculated to be about 62.8 μg/ml and the V_{max} is 9.94 cpm/ml/Klett/hour. Since the solubility of cholesterol is limited, it was necessary to work with concentrations below K_m. However with some attention to accuracy comparisons can be made between reaction conditions, and low levels of uptake can be detected.

Temperature Experiments. Sterol uptake is greatly affected by temperature (Figure 7). The optimal rate of uptake occurred at about 31° C which is also the optimal temperature for growth. The activation energy derived

Figure 5. **Effect of pH on sterol uptake.** Cholesterol concentration is 16 µg/ml.

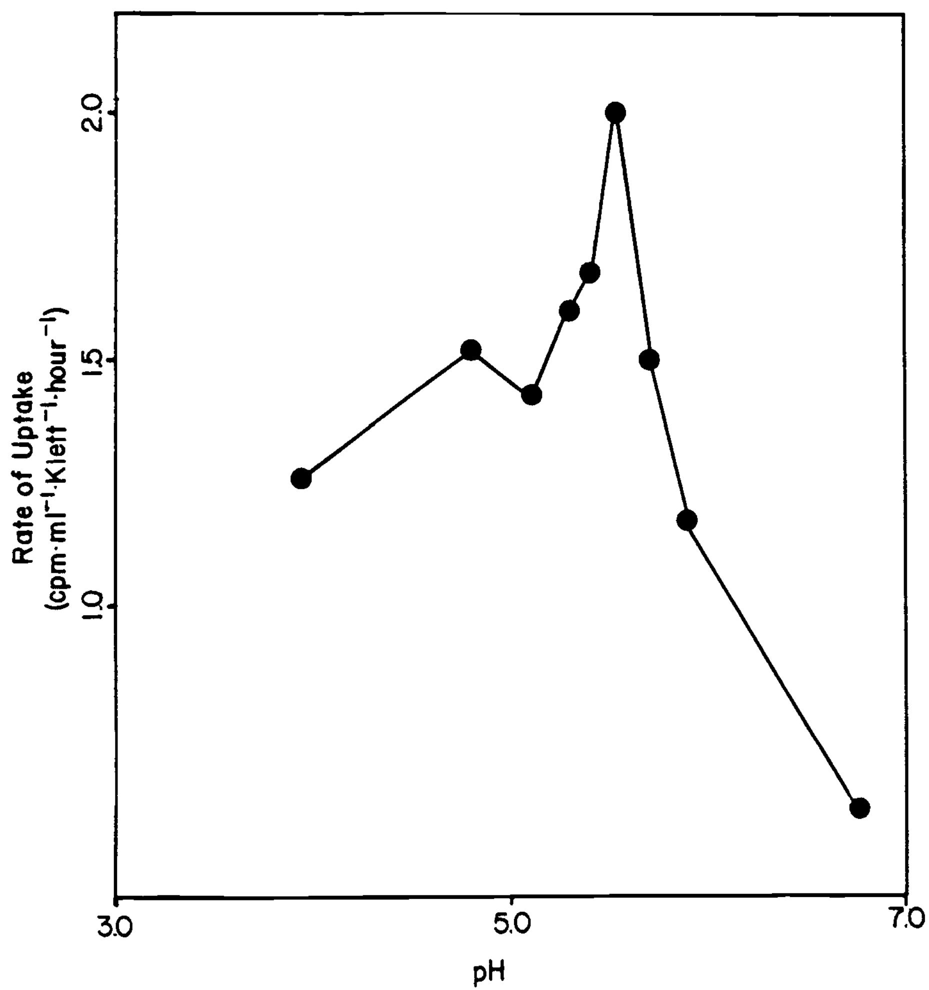


Figure 5

Figure 6. **Lineweaver-Burke plot of sterol uptake.** Temperature is 28 ± 1° C; pH 5.5.

Figure 6

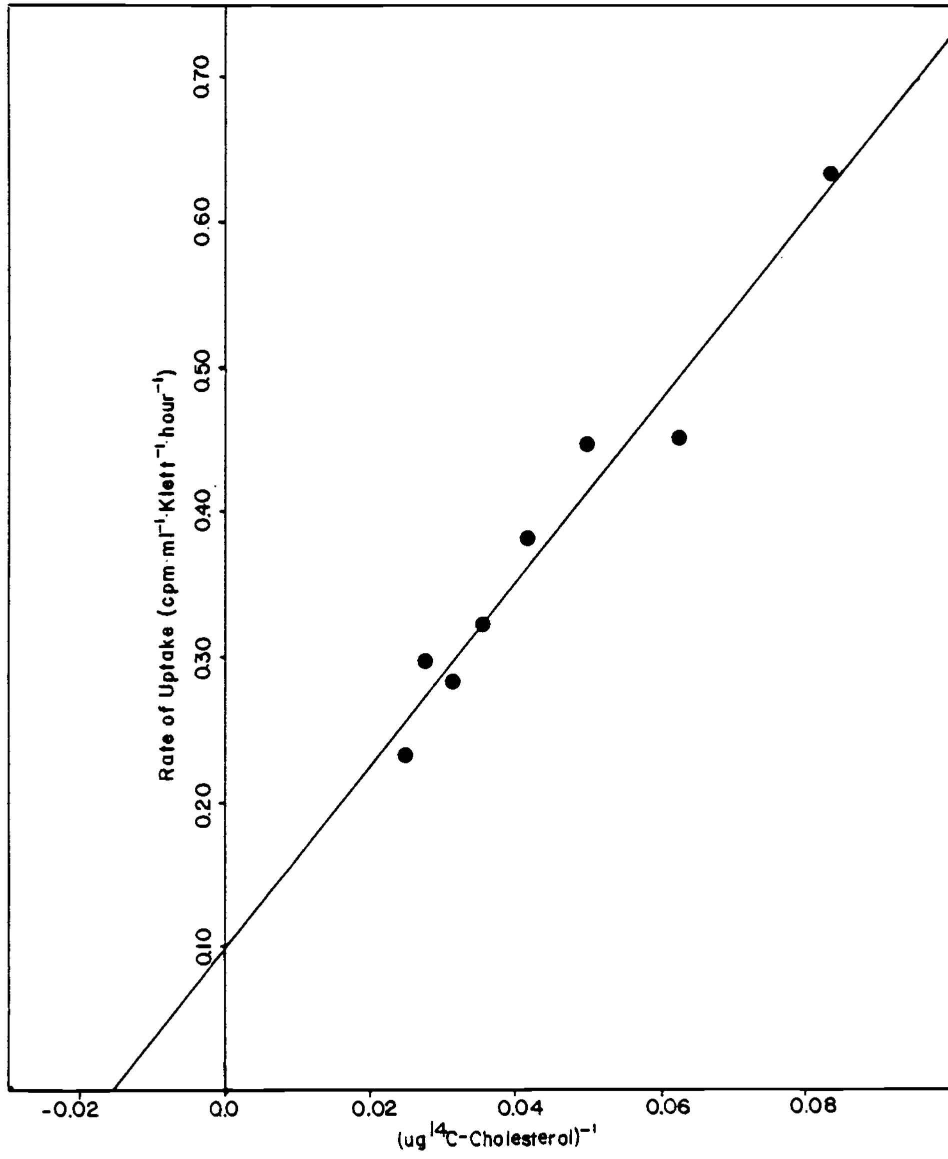


Figure 7. **Temperature profile of sterol uptake in FY3.** ^{14}C-Cholesterol concentration is 16 μg/ml.

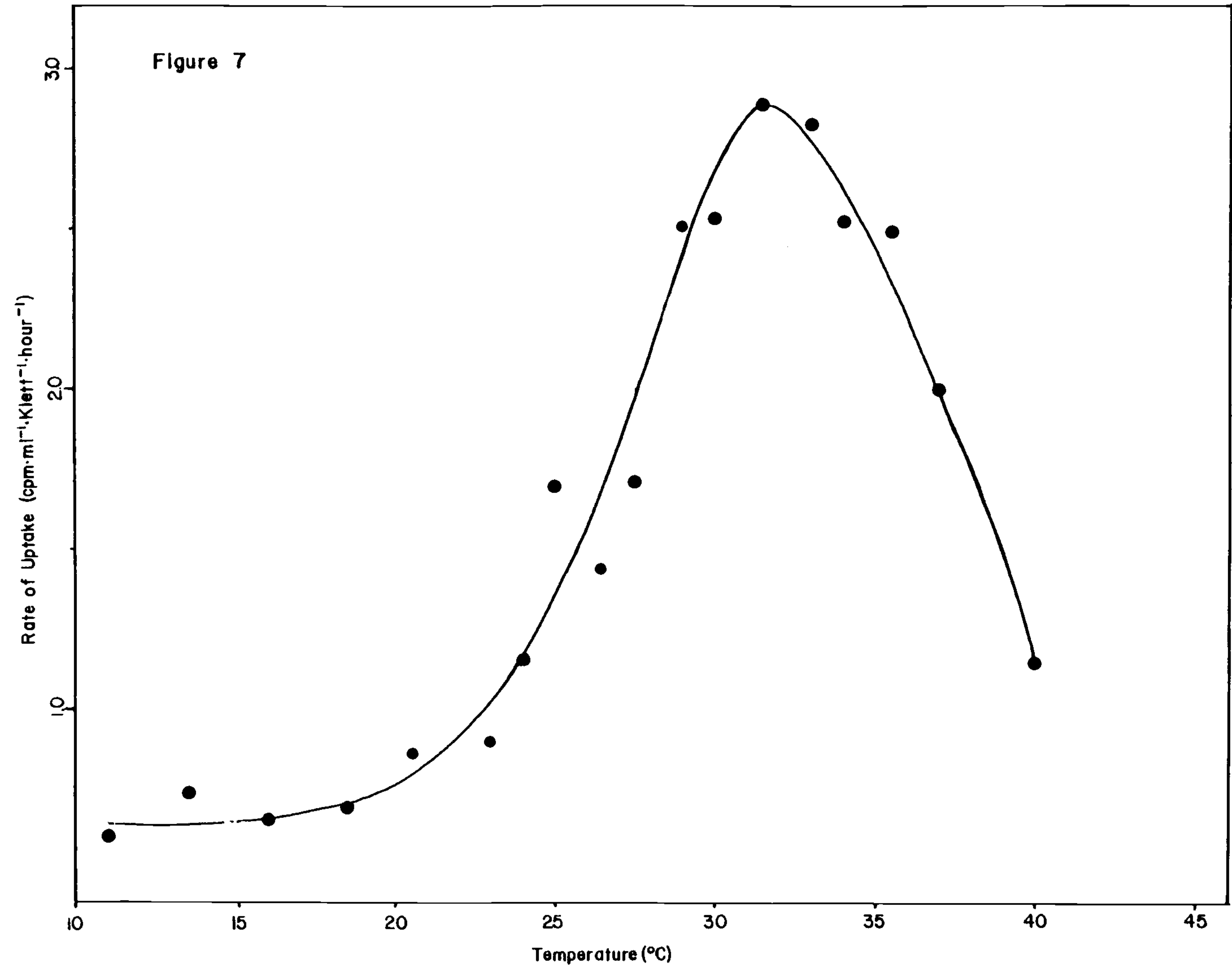
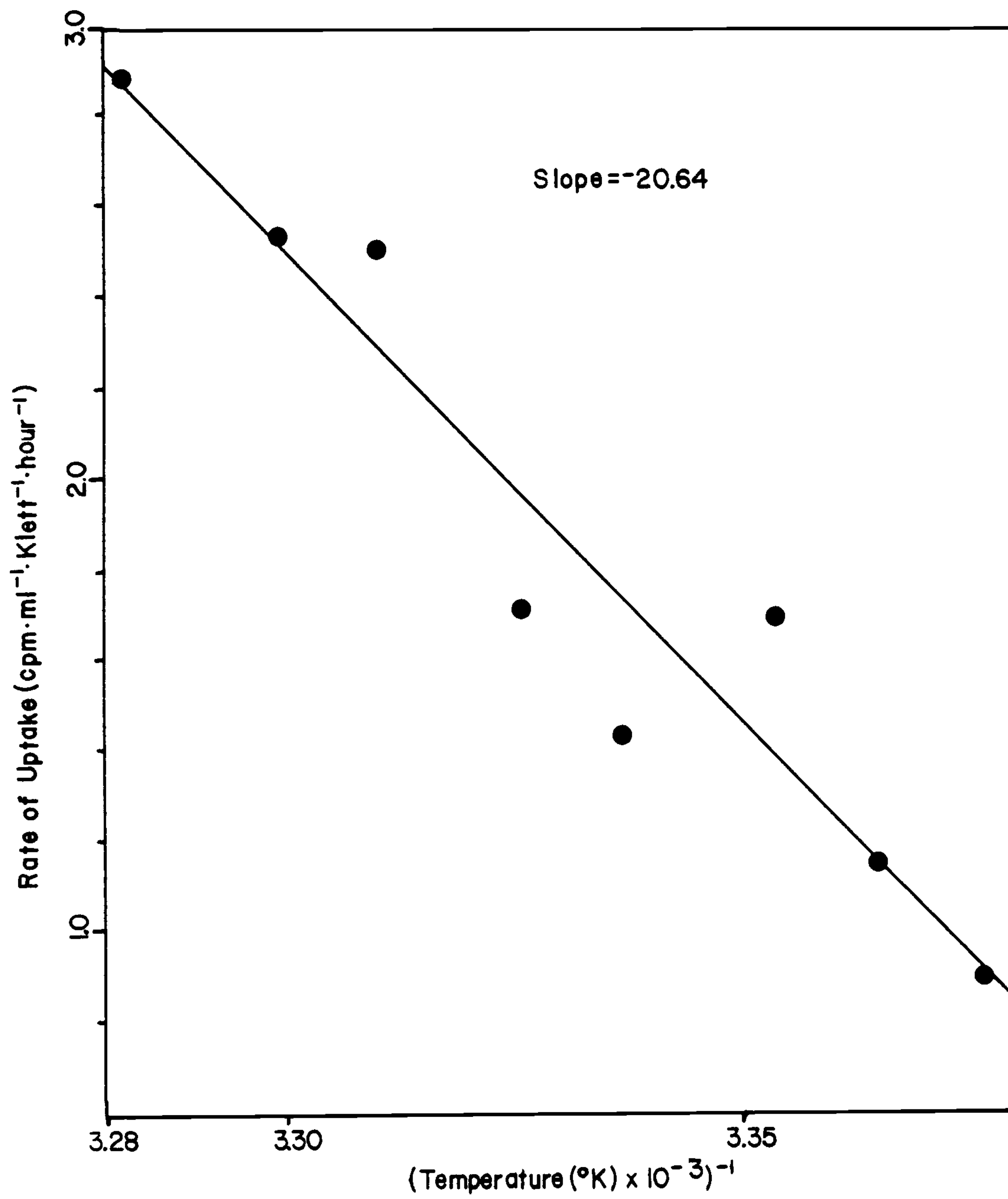


Figure 7
Rate of Uptake (cpm·ml⁻¹·Klett⁻¹·hour⁻¹)
3.0
2.0
1.0
10
15
20
25
30
35
40
45
Temperature (°C)

Figure 8. **Arrhenius plot of cholesterol uptake in FY3.**

Figure 8



from an Arrhenius plot (Figure 8) is 94000 cal/mole.

In Figure 9, growth rate and sterol uptake are compared. The tight coupling of uptake to growth is apparent and the question becomes, is growth enhanced by increased sterol uptake, or is sterol uptake augmented by faster growth? The answer to this question is beyond the scope of this study but it serves to show how critical it is that conditions be consistent between assays.

Temperatures above 42° C prevent or inhibit yeast growth. These cultures exhibit an initial increase in sterol uptake, maximal at about 45° C, and then a decrease (Figure 10). This is thought to be due to the exposure of other sterol binding components to the medium as the cell wall loses its integrity and consequent sterol bound thereof. At extremely high temperatures these proteins are either denatured or released into solution as cells lyse, or both, and bound sterol is no longer detected by the filtration assay.

Figure 9. **The dependence of sterol uptake on growth.** Closed circles refer to growth rate varied by temperature; open circles refer to growth rate varied by pH. Cholesterol concentration is 16 µg/ml.

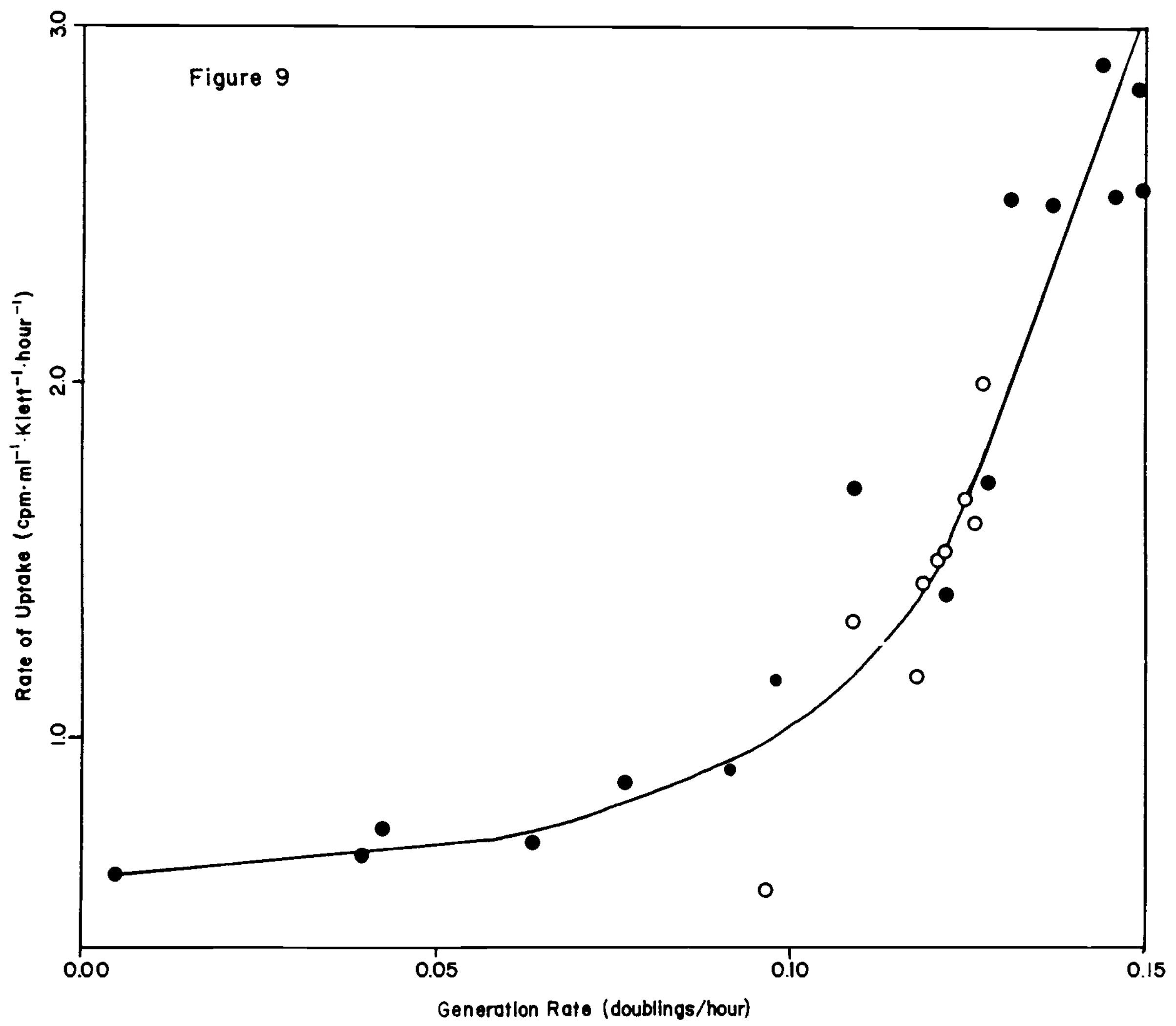


Figure 9
Rate of Uptake (cpm·ml⁻¹·Klett⁻¹·hour⁻¹)
3.0
2.0
1.0
0.00
0.05
0.10
0.15
Generation Rate (doublings/hour)

Figure 10. **Sterol uptake at higher temperatures.** Cholesterol concentration is 16 µg/ml.

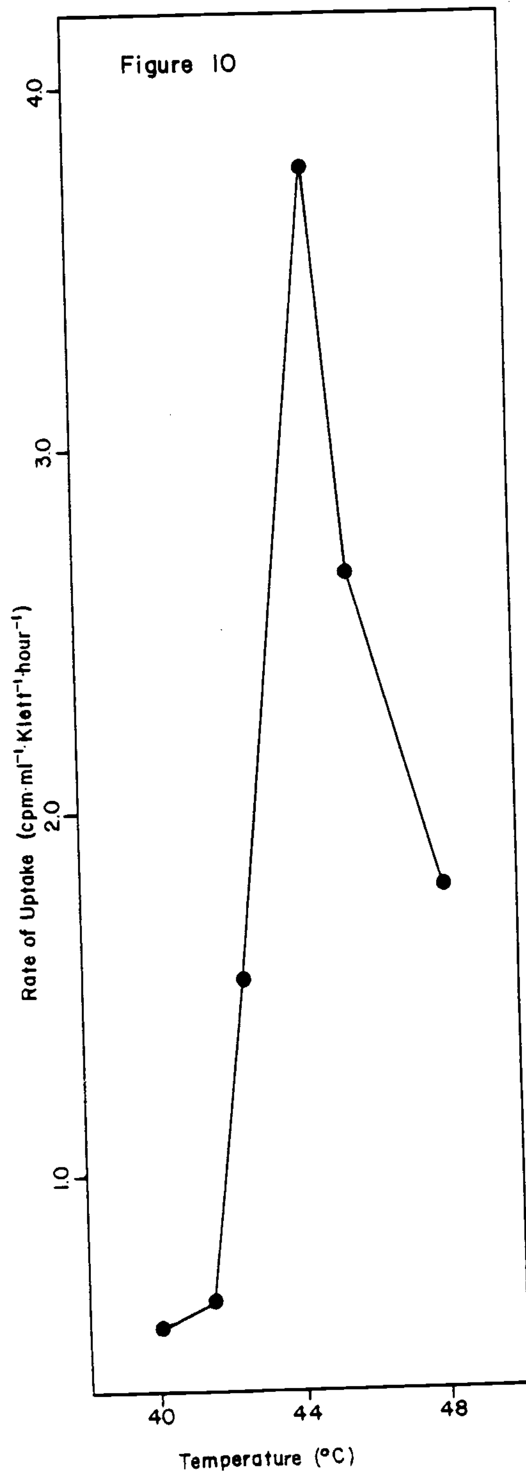


Figure 10
Rate of Uptake (cpm·ml⁻¹·Klett⁻¹·hour⁻¹)
4.0
3.0
2.0
1.0
40
44
48
Temperature (°C)

SUMMARY

Sterol uptake in FY3 exhibits all the characteristics of a typical enzyme catalyzed reaction, e.g. Michaelis-Menten kinetics, temperature dependence, pH affects and suppression by endogenous sterol. Also, sterol uptake appears to be highly dependent upon energy metabolism and growth rate. The rate of sterol uptake reflects the rate of growth though just how the two are related is yet to be discovered.

Sterol uptake was not affected by glucose concentration from 0.5 to 2.0%, although uptake terminated upon cessation of growth in cultures which were denied a carbon source, a similar occurrence to that observed when cultures were denied a source of fatty acid or methionine (unpublished results).

From the Arrhenius plot of temperature data, the energy of activation was found to be 94000 cal/mole and there are no breaks. The absence of breaks in the Arrhenius kinetics may indicate that the enzyme which catalyzes uptake could be located in the periplasm where effects of the membrane fluidity would not be influential on the enzyme's activity. Therefore the enzyme could have greater mobility, and would be more available to exogenous sterol for binding than would the same protein imbedded in the bilayer.

It is known that there is a relationship between hemes and sterol biosynthesis (Aries and Kirsop, 1978;

Hata et al., 1981; Osumi et al., 1978). It is thought that there may be a point of regulation of sterol uptake as well which involves hemes. This hypothesis is supported by the fact that a non-leaky mutation blocking sterol biosynthesis appears to be lethal to the cell, while a second mutation blocking heme biosynthesis allows growth of the cell. This is presumably due to a lack of interaction of hemes or cytochromes at some point regulating sterol uptake so that exogenous sterol may then be incorporated as under anaerobic conditions.

By the use of various heme mutants and specific inhibitors, the effects of heme production on uptake in yeast with a wild-type sterol phenotype may be examined. The results presented here outline a simple method for measuring sterol uptake in a continuous fashion. This assay is especially useful since it allows for changes in growth conditions at any point along the growth curve and concurrent sterol uptake may be followed.

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