Biochemical Pharmacology, Vol. 37, No. 21, pp. 4217–4224, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

PRIMARY MITOCHONDRIAL ACTIVITY OF GOSSYPOL IN YEAST AND MAMMALIAN CELLS

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(*Received* 8 *August* 1987; *accepted* 15 *June* 1988)

Abstract—Gossypol showed primary antimitochondrial activity in yeast cells in that the drug (1) inhibited growth of cells utilizing mitochondrial substrates as carbon and energy sources, and (2) selectively inhibited mitochondrial protein synthesis. Primary antimitochondrial activity was demonstrated in guinea-pig keratinocytes (GPK) by early arrest of growth and loss of viability in medium with glutamine (a mitochondrial substrate) as carbon and energy source compared with cells utilizing glucose. Gossypol depressed oxygen uptake directly in respiring cells.

Gossypol interacted with the known antimitochondrial agents ethidium bromide and 5-fluorouracil (FU), potentiating the activity of FU but reversing that of ethidium bromide in yeast and GPK. Also, the activity of the mitochondrial inhibitor oligomycin was reversed by the presence of gossypol in yeast cells but not tested in GPK. The uptake and retention of the mitochondria-specific dye rhodamine 123 were much depressed by gossypol in GPK.

Gossypol showed little or no inhibitory effects in yeast or GPK in the presence of ethanol (0.2–0.5%). The drug was not mutagenic with respect to the yeast mitochondrial system.

It was tentatively suggested that mitochondrial perturbation could explain the antifertility effect of gossypol if it is assumed that mitochondria have a special role to play in spermatogenesis and sperm motility, making these tissues more sensitive to mitochondrial inhibitors than somatic cells.

Gossypol is a phenolic bisesquiterpene isolated from cotton seeds which causes reversible male sterility in mammals including man [1]. A number of studies have shown that the compound acts mainly at the later stages of development of spermatids and spermatocytes and degeneration of Sertoli cells has also been observed. The mechanism of action of the drug is unknown but results from a variety of *in vitro* systems show that it is able to inhibit certain enzymes including lactate dehydrogenase, ATPase, and to depress both DNA and protein synthesis [2, 3]. However, most attention has been focused on the antimitochondrial activity of gossypol and among the effects reported are uncoupling of oxidative phosphorylation [4], inhibition of the mitochondrial respiratory chain [5], induction of structural aberrations in organellar membranes [6] and alteration in membrane potential which can be detected in an alteration in the uptake and retention of the mitochondria-specific dye rhodamine 123 [7]. These studies were carried out using both *in vitro* and *in vivo* systems as well as isolated mitochondria and have led to the theory that the antifertility effect of gossypol is due to a disturbance of energy metabolism following selective reactivity with mitochondria [8].

We have developed a system using cultures of yeast and mammalian cells for detecting primary antimitochondrial activity of drugs [9, 10]. Results with our system provide more definitive evidence in support of the claims that gossypol primarily effects mitochondrial function.

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MATERIALS AND METHODS

Yeast culture. Media contained 1% yeast extract with either 2% glucose (YED) or 4% glycerol (YEG) as fermentable and non-fermentable carbon and energy sources respectively. Other non-fermentable substrates used in tests were lactate (2%), succinate (10 mM), pyruvate (1%), acetate (1%) and ethanol (2%). The first criterion for primary antimitochondrial activity of a drug is the arrest of growth in YEG medium while growth continues normally in fermentable medium in the presence of the mitochondrial inhibitor [9]. This is possible in yeast since the organism is a facultative anaerobe. Agar (Difco, 2%) was added to obtain solid medium. Twenty-one strains of *Saccharomyces cerevisiae* from the collection of this laboratory were used and were inoculated onto agar plates by a multiple inoculation procedure [11] in tests of growth inhibition by gossypol and other drugs. Growth curves were obtained from shake cultures of individual yeast strains by sampling and counting cells in a haemocytometer at intervals during growth.

Cytochrome spectra. Absorption spectra were obtained from whole cells (approx. 10^9/ml) suspended in a cuvette and scanned in a Uvispek SP1800 recording spectrophotometer using filter paper as blank as previously described [9].

Oxygen uptake. Rates of oxygen uptake into cells and protoplasts were measured in a Clark-type oxygen electrode at 30°. Cells were harvested in the exponential phase, washed and resuspended in phosphate buffer (pH 7.4). Two millilitre samples (10^8 cells/ml) were introduced into the electrode

chamber and saturated with oxygen. On attaining a steady rate of oxygen uptake, 8 μl gossypol from a stock solution of 50 mg/ml was added to the chamber and at appropriate time intervals a further 8 μl, 16 μl and 32 μl were added respectively. The effect on respiration rate was recorded polarographically using a pen recorder.

Where protoplasts were used, these were suspended in buffer stabilised with 0.6 M KCl in the electrode chamber.

Protoplasts were obtained by incubating cells for 30 min in 5% β-glucuronidase, mercaptoethanol (1%), 12.5 mM Tris–HCl (pH 7.5), 15 mM $MgSO_4$, 600 mM KCl.

Petite *mutation*. Induction of the mitochondrial mutation to respiratory deficiency *(petite)* was assessed by plating cells on YED medium. *Petite* colonies were identified as described in previous publications [9]. They were small and white and were unable to grow when subcultured onto YEG medium. They also failed to develop cytochromes aa_3 and *b* in cytochrome spectra.

Drugs. Gossypol acetic acid was obtained from the Sigma Chemical Company (St Louis, MO) and the tetraacetic acid derivative was a gift from the Rockefeller Foundation. Stock solutions were made up in 0.5 N NaOH and the pH adjusted to 6.0 with HCl.

Ethidium bromide, 5-fluorouracil and oligomycin were obtained from Sigma and stock solutions were made up in water.

Mammalian cell culture. Guinea-pig keratinocytes (GPK) were routinely cultured in Eagle's Minimal Essential Medium (MEM) containing Earle's salts supplemented with 0.03% sodium bicarbonate, 10% foetal bovine serum, 20 nm Hepes buffer, 2 mM L-glutamine, 100 units% penicillin and 100 μg% streptomycin. This medium contained 5.55 mM glucose.

Experiments were carried out in MEM and glucose-free MEM (Flow Laboratories) supplemented in the same way but with glutamine (mitochondrial substrate) replacing glucose as the carbon and energy source.

GPK were cultured as a monolayer in flasks (725 cm^2 polystyrene flasks, Beckton, Dickinson Ltd., U.K.) in 10 ml standard medium to near confluence and subcultured by trypsinization into either flasks containing 10 ml medium or 24-well culture dishes (3.5 ml capacity, Flow Laboratories) containing glass coverslips and 1 ml of medium per well. Seeding densities were approximately 5×10^4 cells/ml per well and 1.5×10^4 cells/ml in flasks. Cells were grown overnight in the appropriate medium to allow attachment before addition of various drugs. The effect of the drugs on cell proliferation and viability was examined. Loss of viability of cells was indicated by change in morphology to a small, rounded form, detachment and trypan blue (0.4%) staining.

Rhodamine 123 (Sigma), the mitochondria-specific fluorescent probe [12] was added to cultures at a concentration of 50 μg/ml. Cells were exposed for 2 hr, rinsed twice and viewed with a fluorescent microscope using an excitation wavelength of 485 nm.

Table 1. Minimum inhibitory concentration (MIC) of gossypol required to arrest growth of 21 haploid yeast strains utilizing the non-fermentable substrate glycerol as carbon and energy source*

Yeast strain	MIC gossypol (mg/ml)
A30	1.0
188	0.5
B21	0.4
D22	0.4
D18	0.75
B/B	0.4
6-81	0.4
D11	0.4
B41	0.5
D4	0.2
B27	0.4
2180	0.4
41/161	1.0
D6	0.5
B/A	0.4
A285	0.2
5178B	0.4
5178B-1-C	2.0
D26	0.4
D75	0.5
22-701	0.4

Concentrations of drug used in tests: 0.2, 0.4, 0.5, 0.75, 1.00 and 2.0 mg/ml. Further details in Materials and Methods.

* At the highest concentration used (2 mg/ml), growth proceded in glucose-containing medium in all strains.

RESULTS

Effects on growth

Yeast studies. Gossypol seriously inhibited the growth of all 21 strains multiple-inoculated onto agar medium but only in YEG where the energy source is glycerol (Table 1). In glucose-containing medium, equivalent and higher amounts of the drug did not arrest the growth of yeast cells. As with all drugs, the extent of the inhibitory effect was strain dependent and the amounts of gossypol required to inhibit growth in non-fermentable medium varied from 0.2 to 2.0 mg/ml depending on the strain. This dependence on genetic background is an important aspect in the study of drug action irrespective of the organism. These tests were repeated several times with similar results and provided the first indication of a primary effect of gossypol on mitochondria. The results were obtained using the monoacetic acid derivative. A comparative study using the tetraacetic acid derivative showed the latter to be marginally more potent in the inhibition of growth. However, we were able to conclude that these two derivatives of gossypol were identical in mode of action since a close cross-correlation between strains was seen with respect to their relative sensitivities to two drugs.

The effects on growth were further studied in liquid culture using strain D4 which was relatively sensitive to gossypol in the plate test. Results are shown in Fig. 1 from which it can be seen that growth is severely restricted in YEG medium by the drug but there is little or no effect on growth in YED during the glycolytic phase. These results were repeatable.

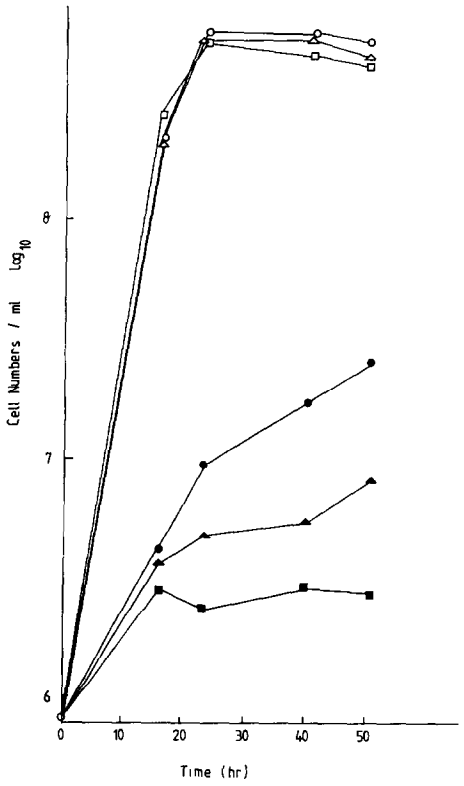

Fig. 1. Growth curves of *S. cerevisiae* strain D4 in glycerol medium (●, ▲, ■) and glucose medium (○, △, □) in the presence and absence of gossypol. ●, ○, 0 μg/ml; ▲, △, 200 μg/ml; ■, □, 400 μg/ml.

Reversibility of gossypol inhibition. The inhibition of growth in nonfermentable medium was readily reversible and cells resumed growth on transferring to fresh YEG medium. These results were obtained in test strains D4 and 6-81 in which gossypol concentrations up to 2 mg/ml were used. The drug had little or no detectable toxicity as measured by cell death: the numbers of colonies that came up from platings of treated cells (up to 2 mg/ml gossypol) were not significantly different from those of control cells.

Tests with various non-fermentable substrates. Other mitochondrial substrates were tested in place of glycerol in the yeast system and included lactate, succinic acid, pyruvate, acetate and ethanol. All gave similar results to those obtained with glycerol with the notable exception of ethanol. In the presence of the alcohol, gossypol had little or no effect on yeast cells, an effect also seen in animal cells (see below).

The relatively high concentrations of gossypol required to inhibit the mitochondrial system in yeast cells could be ascribed to the presence of a thick cell wall affecting permeation. This was shown to be the case by digesting the cell wall to form protoplasts in strain D4 and plating on drug-containing YED medium. About 1% of protoplasts were capable of regeneration and growing into colonies on drug-free medium but gossypol at a concentration of 20 μg/ml totally inhibited the regeneration of protoplasts. In these experiments, a minimum of 10^5 protoplasts were plated onto control and drug-containing petridishes respectively.

These results indicated that gossypol could show inhibitory activity in protoplasts at a concentration about 1/10 that required to inhibit intact cells. These findings are substantiated by those obtained in oxygen uptake studies described below.

Interaction of gossypol with known mitochondrial inhibitors

Further evidence of mitochondrial activity of gossypol was looked for in its possible interaction with known antimitochondrial agents. The first of these was oligomycin, a highly selective inhibitor of mitochondrial ATPase [13]. In strain D4 for example, this compound inhibited growth in YEG medium at a concentration of 1 μg/ml but was ineffective in preventing growth in glucose medium at a concentration of 200 μg/ml. The inhibitory effects of each of these drugs are shown in Fig. 2 and their interaction when present together in the culture medium. Interaction leads to the restoration of growth to a large extent, a result consistently found in repeat experiments with strain D4 and strain 6-81, leading to the conclusion that gossypol reacts with the mitochondrial inner membrane in a way that is antagonistic to oligomycin. The same is true of oligomycin with respect to the activity of gossypol, that is, there is mutual antagonism, each reversing the antimitochondrial action of the other. An alternative interpretation that the interaction takes place at the plasma membrane and that each impedes the permeation of the other into the cell is unlikely since it was found that the growth of cultures inhibited by gossypol was largely restored by addition of oligomycin to the culture medium.

The petite *mutation.* Other antimitochondrial drugs used in this series of experiments were ethidium bromide (EB) [14] and 5-fluorouracil (FU) [15]. These are mitochondrial mutagens and induce the mitochondrial mutation known as *petite* with high

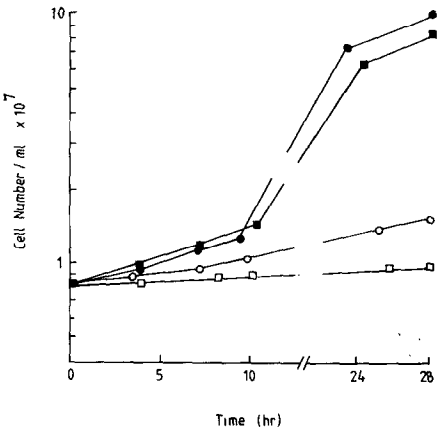

Fig. 2. Inhibition of mitochondrial function by oligomycin (2 μg/ml) and its suppression by gossypol (400 μg/ml) in glycerol grown cultures of *S. cerevisiae* strain D4. ●, control; ■, oligomycin + gossypol; ○, gossypol; □, oligomycin.

efficiency. The mutation seems to arise from a flaw in the replicative integrity of the mitochondrial chromosome of *Saccharomyces* species leading to extensive deletions in the DNA molecule [16]. The mechanism of the deletion is not clearly understood but the mutation is selectively induced by most mutagens/carcinogens [17]. The mutation arises with a high spontaneous rate and about 1 cell in 100 in most strains will show the condition and give rise to a *petite* colony on plating on YED medium. The high frequency and well-defined characteristics of the respiratory deficient mutant allow a convenient and unequivocal identification of these colonies to be made.

Gossypol did not induce the *petite* mutation in five strains tested, a finding which is in agreement with the report that the drug was non-mutagenic in the Ames test (see [3]). However, the presence of gossypol affected the *petite* inducing capacity of EB (Fig. 3) and FU (Fig. 4). In strain D4, the presence of 10 μg/ml EB in the culture medium transformed 100% of cells to the *petite* condition. The simultaneous presence of gossypol reversed this mutagenic activity in a dose-dependent manner (Fig. 3). On the other hand, the mitochondrial mutagenicity of FU was significantly enhanced by the presence of gossypol (Fig. 4). These results were repeated in two other strains and, taken together with the results of the experiments with oligomycin, provide good evidence of a mitochondrial site of action of gossypol. Fuller accounts of the antimitochondrial activity of oligomycin and *petite* induction by EB and FU in our yeast strains are given in previous publications [18, 19].

Effect of gossypol on oxygen uptake

The effect of introducing gossypol into the oxygen electrode system containing respiring cells is recorded in Table 2. The drug significantly decreased the rate of oxygen uptake at a concentration of 400 μg/ml and maximum reduction of about 50% was seen at 800 μg/ml. The recorded results were obtained with strain D4 but similar results were obtained using strain 6-81. These findings indicated direct reactivity of gossypol with the mitochondrial inner membrane assembly.

Protoplasts of D4 and 6-81 were much more sensitive to respiratory inhibition by gossypol than intact cells and significant decrease in the rate of oxygen uptake was seen at concentrations of less than 100 μg/ml. At a concentration of 200 μg/ml of the drug, pronounced lysis of protoplasts was observed in both strains.

Effect of gossypol on mitochondrial protein synthesis

Absorption spectra of whole cells reveal the presence of the haem-containing enzymes of the mitochondrial respiratory chain. Absorption peaks of cytochromes aa_3, *b* and *c* are shown in Fig. 5 in cells of strain D4 grown in the presence and absence of gossypol. It is apparent that cytochromes aa_3 and *b* are much depressed while the synthesis of cytochrome *c* is marginally affected by the presence of the drug in the culture medium. Since mitochondrial genes code for cytochrome *b* and for three of the components of the polymeric cytochrome aa_3 and

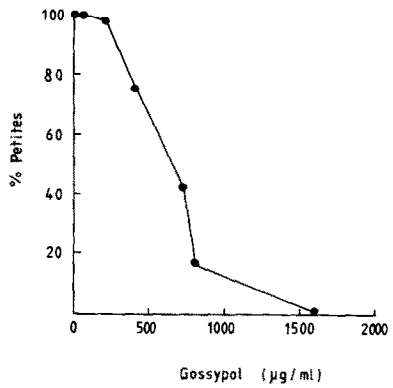

Fig. 3. Effects of gossypol on ethidium bromide (10 μg/ml) induced *petite* mutation in *S. cerevisiae* strain D4.

are translated on mitochondrial ribosomes while cytochrome *c* is coded by a nuclear gene which is translated on cytoplasmic ribosomes, it may be concluded that gossypol is selective in the inhibition of mitochondrial protein synthesis. The mechanism of this effect is not known but there is a claim, already cited, that the drug can have an adverse effect on protein synthesis in animal cells.

Studies with guinea pig keratinocytes (GPK)

Animal cells, unlike yeast cells, are not facultative anaerobes so that blockage of mitochondrial respiration would lead to arrest of cell growth and eventual cell death. However, in standard culture medium which is supplemented by 2% glucose, glycolysis can supply energy requirements for a limited period to maintain cellular integrity. In our cultures we have been able to substitute glutamine for glucose as carbon and energy source and since glutamine is a mitochondrial substrate, inhibitors of the organelle would have, predictably, a more immediate effect on cells in glutamine medium than in glucose medium [10]. The system is analogous to that of yeast in which the non-fermentable energy source was glycerol.

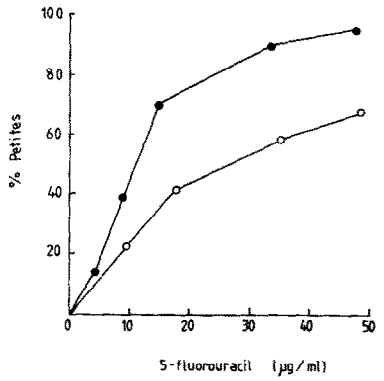

Fig. 4. Induction of the mitochondrial mutation *petite* by 5-fluorouracil (○) and its potentiation by gossypol (400 μg/ml) (●).

Table 2. Effect of gossypol on oxygen uptake into yeast cells (strain D4) measured polarographically in a Clark-type oxygen electrode

Gossypol (μg/ml)*	Oxygen uptake: nmoles O_2/min	Oxygen uptake: Inhibition (%)
0	129	0
200	122	5.5
400	83	36.0
800	68	47.5
1600	67	49.0

* Drug added at intervals throughout the recording.

Gossypol was more toxic to GPK in the glutamine medium compared with glucose cultures and at a concentration of 5 μg/ml, cells metabolizing glutamine showed changes in morphology after only 5 hr incubation (Fig. 6). This concentration seriously hindered growth and caused extensive cell death after 48 hr (Plate 1) mainly in the glutamine medium. Selective inhibition by gossypol under conditions requiring mitochondrial function was in agreement with the results obtained with yeast cells and provided further evidence of primary antimitochondrial activity of the drug.

Interaction with other inhibitors

(1) *Ethanol.* As in the yeast experiments, ethanol reversed the inhibitory effects of gossypol. The range of ethanol concentrations efficient in this respect was 0.2–0.5% above which the alcohol itself was toxic. Again it is not clear how ethanol interferes with the reaction site(s) of gossypol but membranes are likely to be involved.

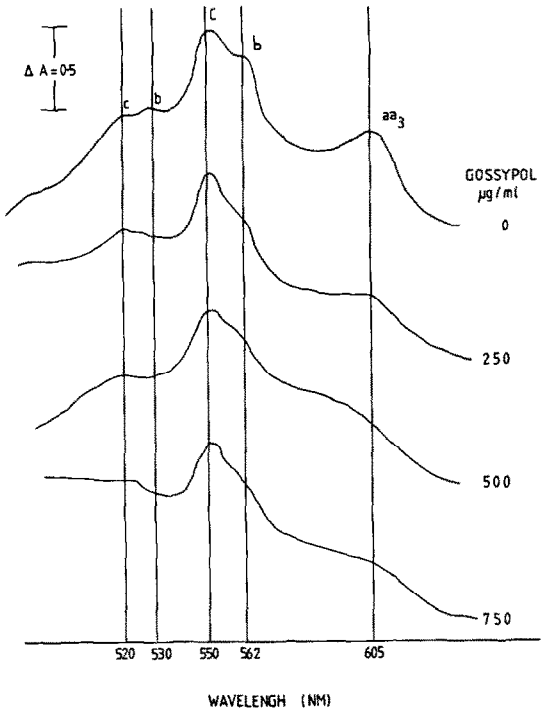

Fig. 5. Effects of gossypol on the absorption spectra of whole yeast cells grown in glucose medium for 48 hr. α peaks of cytochromes aa_3, *b* and *c* appear at 605,562 and 550 nm respectively; β peaks of *b* and *c* at 530 and 520 nm respectively.

(2) *Ethidium bromide.* At a concentration of 30 μg/ml, EB had a marked inhibitory effect on GPK growing in the glutamine medium in the short term compared with glucose cultures. The inhibitory effect was reversed in large measure in the presence of gossypol (Fig. 6, Plate 2) and, once again, this was in agreement with the results obtained with yeast cells further indicating a mitochondrial site of gossypol action.

(3) *FU.* The anticancer drug selectively inhibited the growth of GPK in glutamine medium compared with glucose-supplemented cultures. This inhibitory effect and loss of viability was much increased by the presence of gossypol, yet again supporting the conclusion drawn from the studies with yeast cells of a mitochondrial target of the antifertility agent (Fig. 6, Plate 2).

(4) *Rhodamine* 123. This dye is used as a fluorescent probe which is taken up specifically by mitochondria. It has been shown by Chen and associates [7] that gossypol impedes the transport of the dye into the mitochondria of rat tumour cells. GPK treated with rhodamine at a concentration of 50 μg/ml, rapidly accumulated the dye in cytoplasmic particles presumed to be mitochondria when viewed in the fluorescence microscope. Cells pregrown for 16 hr in 5 μg/ml gossypol were greatly reduced in their ability to take up the dye which appeared more diffuse in the cell cytoplasm. Also, gossypol-treated cells lost this fluorescence in 1 to 2 hr whereas control cells took up to 6 hours to lose the fluorescence. These results are roughly along the same lines as those obtained by other workers.

Rhodamine is a cationic dye so that its accumulation in mitochondria is probably due to the high electric potential across the organellar membrane. Reduction in potential by drugs reacting with the inner membrane assembly (such as uncouplers and inhibitors of the respiratory chain) would disturb permeation of rhodamine and its retention in the organelle. Apparently, gossypol comes into this category of membrane reacting drugs.

DISCUSSION

The findings in this paper of the interaction of gossypol with known antimitochondrial agents such as oligomycin, ethidium bromide and fluorouracil have provided further evidence for the claims in the literature that gossypol perturbs mitochondrial function. Although the mechanism of interaction of these mitochondrial agents is not clear, the site of action appears to be the inner membrane which has special physico-chemical characteristics which distinguish it from other cell membrane systems. This reactivity affects both the respiratory process and organellar biogenesis. If mitochondrial perturbation is the mechanism of the antifertility effect of gossypol it would be necessary to conclude that mitochondria have a special role to play in spermatogenesis and sperm motility since mitochondrial

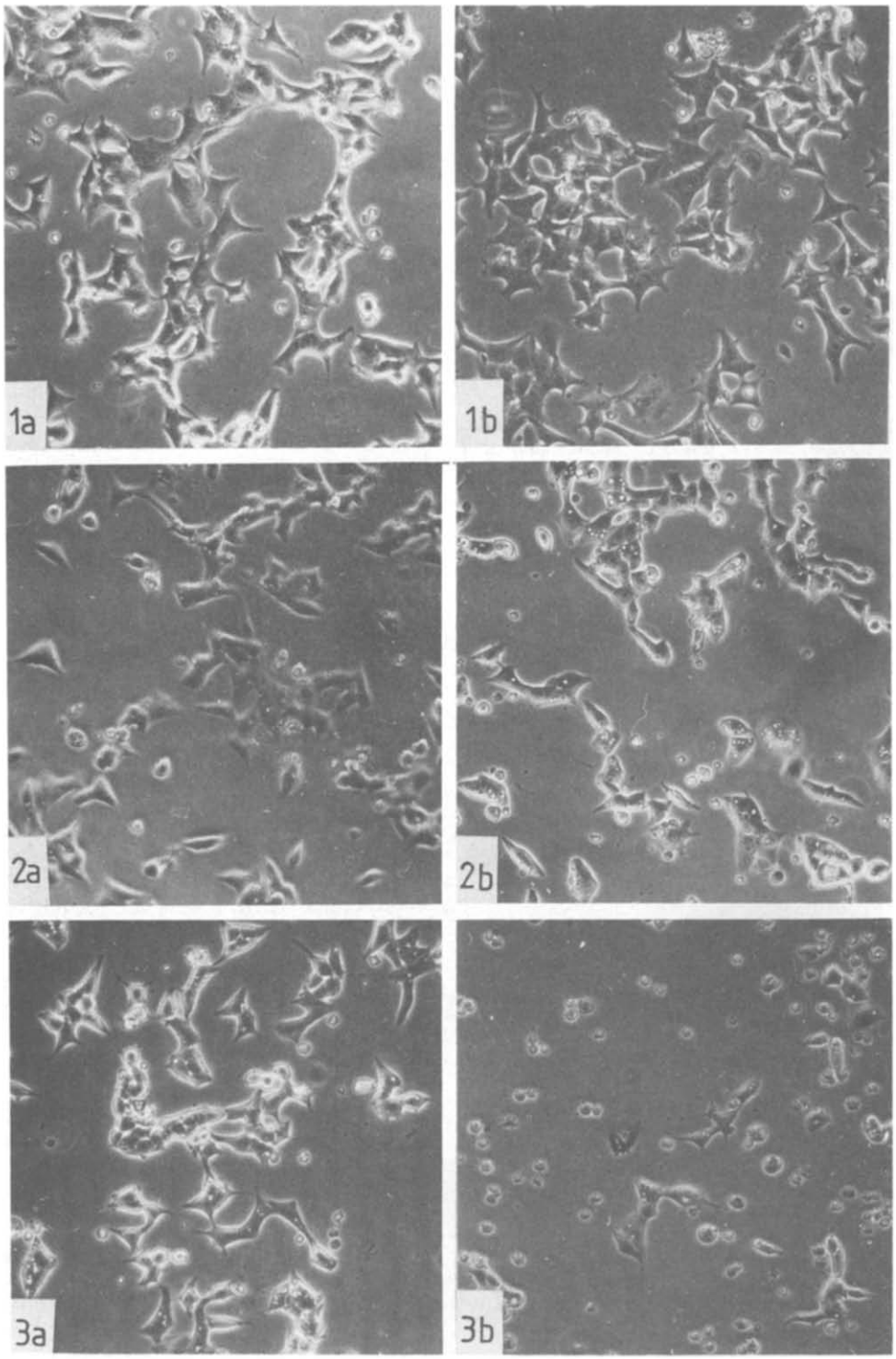

Fig. 6. Plate 1.

inhibition apparently does not significantly affect the biogenesis and function of other (somatic) tissues. Stringent energy requirements in sperm motility may be a factor while mitochondrial functions other than oxidative phosphorylation in ATP production may be critical during spermatogenesis. In this connection, the effects of mitochondrial aberration on cell surface characteristics may be relevant [20].

Whatever the mechanism of infertility, mitochondria seem to be involved and should be the

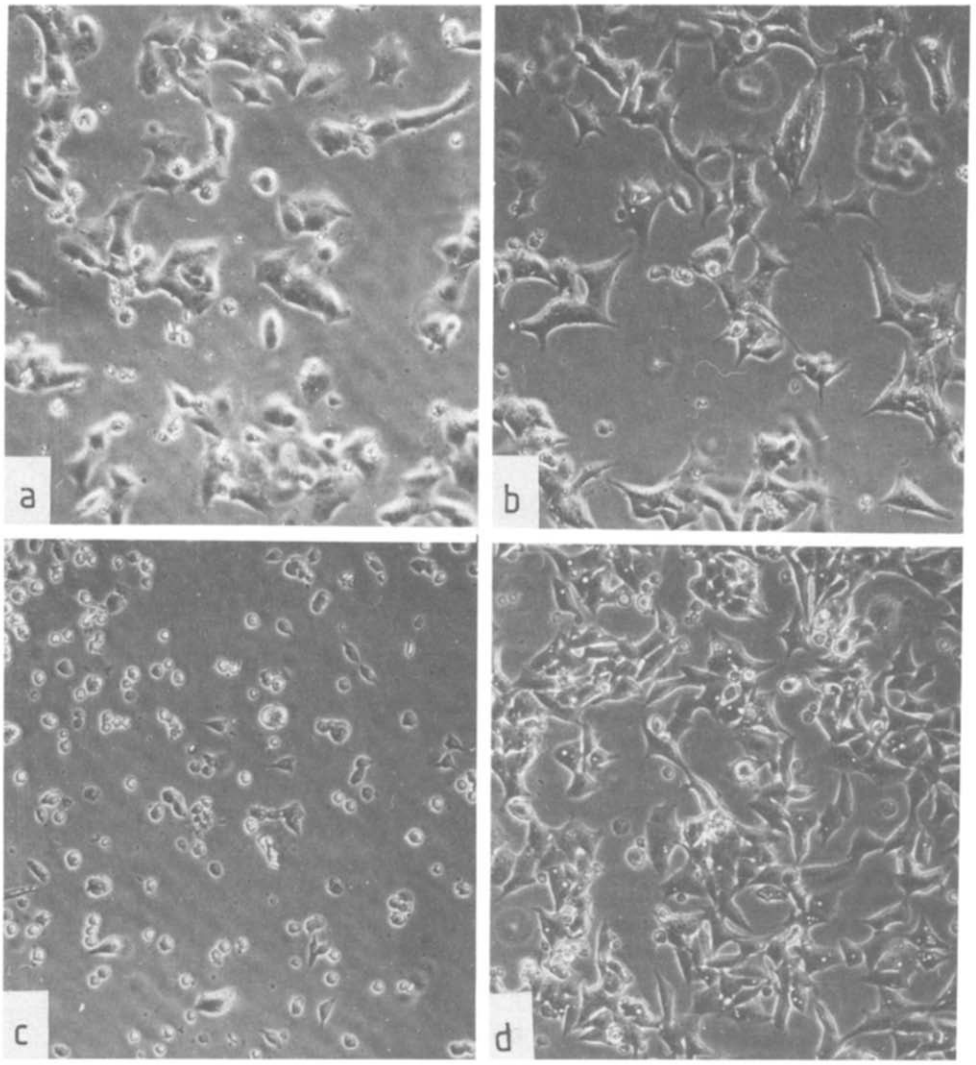

Fig. 6. Plate 2.

Fig. 6. Effect of gossypol on growth, morphology and viability of guinea pig keratinocytes (GPK) under various cultural conditions. Microscope magnification × 70. Plate 1. Effect of the anti-fertility drug gossypol on 2-day old GPK cultured in wells and utilizing respectively glucose (series a) and glutamine (series b) as carbon and energy source. 1a, control (90%)*; 2a, +5 μg/ml gossypol (74%); 3a, +7 μg/ml gossypol (38%); 1b, control (89%); 2b, +5 μg/ml gossypol (41%); 3b +7 μg/ml gossypol (1%). *Per cent viable cells estimated by trypan blue exclusion test. Plate 2. Two-day-old GPK growing in the presence of the mitochondrial inhibitors 5-fluorouracil (FU) and ethidium bromide (EB) in non-fermentable glutamine medium, with and without gossypol. a, +8.75 μg/ml FU (73%); b, +30 μg/ml EB (55%); c, +8.75 μg/ml FU + 5 μg/ml gossypol (0%); d, +30 μg/ml EB + 5 μg/ml gossypol (93%).

focal point of further study. The yeast cell system described in this report provides a convenient means for elucidating the antimitochondrial activity of gossypol, particularly since it appears that results obtained can be extrapolated to mammalian cells. This was predictable in view of the fact that the mitochondria of eukaryotic organisms from yeast to man are similar in all fundamental respects.

It may be mentioned that selective inhibition by gossypol of GPK cultures utilizing glutamine as carbon and energy source could be due to blockage of one or other of the several steps in gluconeogenesis rather than blockage of mitochondrial metabolism of the amino acid. In view of all the evidence supporting the latter mode of action, particularly interaction with known antimitochondrial agents, interference with glucose production is considered unlikely.

Acknowledgement—The authors are grateful to the Rockefeller Foundation for a grant in support of these studies.

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