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ANTIMITOCHONDRIAL ACTIVITY OF LAMPREN IN *SACCHAROMYCES CEREVISIAE*

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Abstract—Twenty-four haploid strains of *Saccharomyces cerevisiae* were totally inhibited in growth in the presence of 0·5 μg/ml Lampren in conditions of obligatory mitochondrial function. In a fermentable medium on the other hand, 10 μg/ml of the drug permitted growth but with extended lag period. The drug stimulated oxygen uptake 2- to 3-fold when added to respiring cells and to rat liver mitochondria respectively, and restored oxygen uptake in cyanide-inhibited cells by about 35 per cent. It was concluded that Lampren acts as a hydrogen acceptor using substrates of the respiratory chain and by-passing cytochrome oxidase. Supporting evidence was seen in a correlation between Lampren and phenazine methosulphate which is chemically related to the drug.

Yeast strains and their Lampren-resistant mutants showed a good cross-resistance relationship with oligomycin, triethyl tin and chlorimipramine, known inhibitors of mitochondrial function. The antimitochondrial action of Lampren (the first to be reported) results in depressed protein and RNA synthesis in cells metabolizing glycerol at low concentrations of the drug (about 5 μg/ml inhibits 50 per cent biosynthesis in a sensitive strain), but 20 μg/ml are required to inhibit these processes to a significant degree in a glucose medium.

LAMPREN (CIBA-Geigy) is a rimino phenazine with the chemical structure as shown in Fig. 1. It is used clinically in the treatment of tuberculosis and leprosy. As well as being a potent inhibitor of mycobacterial growth in culture, this red, crystalline compound has a wide antibacterial spectrum. The mode of action of Lampren is unknown, but Barry *et al.*[1] who synthesized the drug, briefly reported an effect on respiration of *Mycobacterium smagmatis* in culture experiments.

In screening the drug for activity against the eukaryotic yeast cell, it was found to

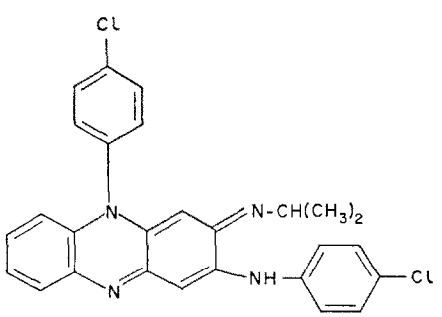

FIG. 1. Chemical structure of Lampren, 2-(4-chloranilino)-3-isopropylimino-5-(4′-chlorophenyl)-3,5-dihydrophenazine.

have a very potent and highly selective action against mitochondria in intact cells and some aspects of the nature of this activity are reported in this paper.

MATERIALS AND METHODS

The demonstration of selective inhibition of the mitochondrial system in intact yeast cells by drugs, employs the simple procedure of replica-inoculating a number of genetically different haploid strains of *S. cerevisiae* onto agar media containing the drug in various concentrations. Two Petri dish series were used, one with a fermentable carbon source, usually 2% glucose, and the other containing nonfermentable substrate (glycerol). All media are supplemented with 1% Difco yeast extract. Respiratory active cells are used in all tests.

S. cerevisiae is a facultative anaerobe in which cell growth and division can proceed in the absence of oxidative phosphorylation, the cellular supply of ATP being provided by glycolysis (fermentation) alone. Thus in a situation where mitochondrial synthesis or function is primarily inhibited by a drug, cell growth will proceed only in fermentable medium. For further details of the yeast test system for primary antimitochondrial effects see Wilkie.[2]

Cytochrome spectra of cells were made in the Unicam SP800 recording spectrophotometer. Treated and untreated cells in a glucose medium were used after reaching stationary phase. Cells were washed and used in thick suspension in distilled water.

Oxygen uptake of cells and isolated mitochondria was measured in the Rank oxygen electrode coupled to a pen recorder.

Lampren, kindly supplied by CIBA-Geigy U.K. Ltd., was either added directly to the medium or from stock solutions. The solvent in the latter case was either ethanol or dimethyl formamide (DMF).

In testing for respiratory deficiency, cells were sampled from the individual colonies under test, plated on glucose medium and resulting colonies scored, firstly on the basis of colony size and secondly by velvet pad replication onto fermentable and nonfermentable medium (glycerol 4%), respectively. Failure to grow on the latter medium was scored as respiratory deficiency (petite phenotype[3]), with small colony as supporting evidence.

Rat liver mitochondria were prepared essentially by the method of Widnell and Tata.[4]

In measuring cellular RNA synthesis, approx. 10^6 cells from the log phase of glucose culture were suspended in 1 ml liquid medium to which 0·5 μCi [^{14}C]uracil was added. After 30 min incubation at 30° cells were collected on a filter, washed five-times with water and their radioactivity measured by scintillation counter.

RESULTS

Effects on cell growth. A total of 27 haploid strains of *S. cerevisiae* were tested by replica-inoculation from individual cell suspensions onto the two agar series, respectively fermentable and nonfermentable. Lampren was added to give concentrations of the drug ranging from 0·25 to 25 μg/ml. In no case was growth arrested on glucose medium even at the highest concentration of the drug, but 22 of the strains were inhibited on the glycerol medium at a concentration of 0·5 μg/ml and in the majority of

these, 0·25 μg/ml was sufficient to arrest growth. A more detailed account of the selective inhibition on nonfermentable medium was obtained from liquid culture experiments and typical growth curves are shown in Fig. 2. The remaining 5 strains which showed little or no inhibition of growth in the presence of 25 μg/ml of Lampren in the glycerol series, appeared to have a permeability barrier to the drug, since the colonies that came up on the drug medium were comparatively white in colour: the glycerol-sensitive strains growing on glucose-drug plates became red, due to cellular uptake of Lampren.

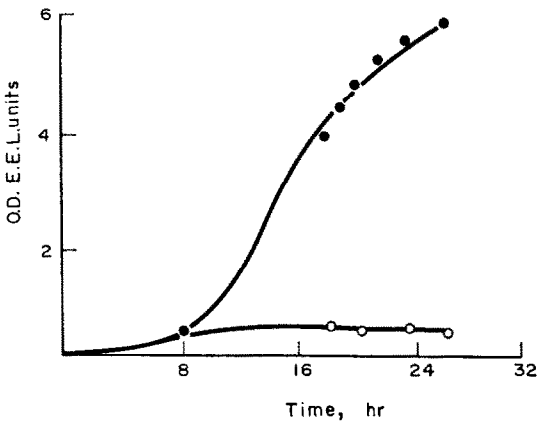

FIG. 2. Growth curves of yeast strain D6 in the presence of 10 μg/ml Lampren. ●——●, Glucose medium; ○——○, glycerol medium.

From the sensitive strains, a number of spontaneous resistant mutants were isolated as small colonies that came up, presumably from individual resistant cells in the inoculum, against a background of inhibited parental cells on glycerol-drug plates. All mutants studied were found to be stable in their resistance and they fell into two categories, those that took up the drug and stained red, like the sensitive parental cells, and those that did not take up the drug. These findings indicated that both intracellular and permeation mechanisms could operate in conferring resistance. A number of mutants with intracellular resistance were selected for cross-resistance testing (Table 2). In view of the highly selective activity of the drug on the respiratory system, it was expected, that in some at least of these mutants, resistance resulted from an alteration in a mitochondrial component involved in drug reactivity.

At the concentration of 25 μg/ml in the medium (added from a stock solution in ethanol of 1 mg/ml), the drug imparts a pink colouration to the agar. It was seen that zones around the colonies were a darker colour than the rest of the medium and acidification resulting from cell growth seemed the most likely cause of this. This led to an investigation of the influence of pH on the spectrum of aqueous solutions of Lampren. Solutions containing 10 μg/ml in 0·1 M sodium phosphate buffer were made up at pH values of 5·8, 6·2, 6·6, 7·0, 7·4 and 7·8. Absorbance was measured with a Unicam SP800 spectrophotometer in the range 200–850 nm. The spectra obtained were typical of those seen with a basic indicator at various proton concentrations, consisting of a mixture of ionized and nonionized forms in equilibrium

$$B + H^+ \rightleftharpoons BH^+.$$

Each spectrum consisted of two broad peaks at wavelengths varying with pH and isobestic points were observed at 483 and 370 nm.

The effect of pH on growth inhibition by the drug was investigated, although at pH 7 and above, yeast growth is adversely affected and also it is not possible to maintain growth media below pH values of 5·4 as suitable buffers are not available. Nevertheless, it was clear that within this range the drug was most potent in the more alkaline media. The most probable interpretation of these findings is that the free base is the species responsible for inhibition. Since the drug is lipid soluble, it will accumulate in cell lipids, for example membranes. Increasing the pH therefore effectively changes the partition of the drug between medium and cells, causing an increase in drug accumulation in cells. All tests of drug action including those described above, were carried out in media containing 0·1 M sodium phosphate buffer at pH 6·2. These conditions represented a compromise between maximizing drug potency and optimizing yeast growth conditions.

The amount of growth achieved in the glucose medium in the presence of various amounts of Lampren was accurately determined and the results are shown in Fig. 3.

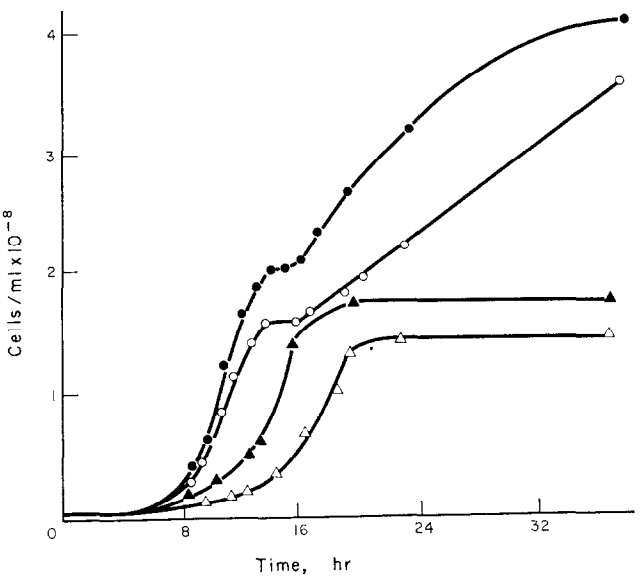

FIG. 3. Effects of Lampren on growth of strain D6 in glucose (2%) medium. ●——●, Control ○——○, 2·5 μg/ml Lampren; ▲——▲ 5 μg/ml Lampren; △——△ 10 μg/ml Lampren.

Cells of *S. cerevisiae* under conditions of anoxia or in the presence of glucose, do not synthesize a respiratory chain. When glucose concentration falls to about 0·2% in the medium, this repression is removed. Synthesis of functional mitochondria then proceeds followed by respiration of the bi-product of glycolysis. The diauxic nature of the growth curve obtained can be clearly seen. It would appear that Lampren at a concentration of 5 μg/ml completely inhibits the derepression of the respiratory system, but an assessment of this aspect of drug activity was made by comparing the

TABLE 1. EFFECT OF LAMPREN ON THE DEVELOPMENT OF RESPIRATION IN GLUCOSE CULTURES OF YEAST

Lampren (μg/ml)	Rate of oxygen uptake (% control)*
0	100
2·5	57
5·0	11
10·0	9

* 10^6 Cells sampled from each culture at stationary phase, strain D6.

TABLE 2. LAMPREN TOLERANCE OF YEAST STRAINS AND SPONTANEOUS LAMPREN-RESISTANT MUTANTS: COMPARISON WITH OLIGOMYCIN (OL), TRIETHYL TIN (TT) AND CHLORIMIPRAMINE (CI)

Lampren-resistant		Tolerance levels (μg/ml) on glycerol medium*			
Strain	Mutant	Lampren	CI	TT	OL
42		0·25	10	0·5	<0·5
	42-1001	2·0	25	2·5	2·0
	42-1002	2·0	50	2·5	2·0
	42-251	2·0	50	2·5	2·0
D26		0·25	10	1·0	0·5
	D26-1001	1·0	50	2·5	1·0
	D26-1002	3·0	75	5·0	1·0
	D26-1003	3·0	75	5·0	1·0
D24		0·25	25	1·0	1·0
	D24-251	1·5	75	2·5	2·0
	D24-501	3·0	75	5·0	2·0
	D24-1001	3·0	75	5·0	2·0
D74		0·25	10	1·0	0·5
	D74-101	2·0	50	0·5	0·5
	D74-102	1·5	25	5·0	2·0
	D74-103	1·0	25	5·0	1·0
	D74-51	0·75	25	1·0	0·5
	D74-52	0·75	25	1·0	0·5
	D74-53	0·5	25	1·0	0·5
D6		<0·25	25	1·0	0·5
	D6-51	0·75	25	1·0	0·5
	D6-52	0·25	25	1·0	0·5
	D6-53	0·75	25	2·5	0·5
	D6-54	0·5	25	2·5	0·5
	D6-101	1·5	25	5·0	0·5
	D6-102	2·0	50	5·0	1·0
	D6-103	0·25	25	1·0	0·5

* Drug concentrations used: Lampren, 0·25, 0·5, 0·75, 1·0, 1·5, 2·0, 3·0, 4·0; CI, 10, 25, 50, 75, 100; TT, 0·5, 1·0, 2·5, 5·0, 10; OL, 0·5, 1·0, 2·0 μg/ml. Strains are completely inhibited in growth at the next higher concentration above the tolerance level.

oxygen uptake of treated and untreated cells. Cells were withdrawn from each of the four cultures at stationary phase, washed and similar amounts inoculated into the oxygen electrode. The results are recorded in Table 1 from which it can be seen that the drug inhibits the development of respiratory activity, but not totally at the higher concentrations. Since mitochondrial function (of fully adapted cells) of this strain in metabolizing glycerol, is sufficiently inhibited to prevent growth at 0·5 μg/ml of the drug on agar, it is surprising that growth, based on respiration and, however slow, can proceed at 2·5 μg/ml. A factor that must be taken into account is the high cell density (about $10^8 \times 2$) at the time of adaptation to respiration in the glucose cultures. In testing the effect of drug–cell ratio, it was found that 0·5 μg/ml Lampren arrested growth in liquid glycerol medium if the inoculum was 10^6 cells/ml of strain D6, but 5 μg/ml was required when the initial inoculum was 10^8 cells/ml to prevent growth.

The effect of Lampren on mitochondrial development was further investigated by an examination of absorption spectra of treated cells. In respiratory adapted cells, cytochromes $a + a_3$, b and c show characteristic absorption peaks in the range 500–650 nm (Fig. 4). Accumulation of the red-coloured drug in treated cells presented a problem due to absorption by the drug itself. The drug could not be washed out of these cells either with water or organic solvents, itself testifying to the binding capacity of Lampren. The problem was largely overcome by adding sodium dithionite which caused bleaching of the drug in the cells. Spectra were then obtained of several strains grown in glucose medium containing the drug in amounts varying from 3 to 10 μg/ml. Typical spectra are shown in Fig. 4. The results indicated that Lampren depressed significantly the synthesis of all cytochromes.

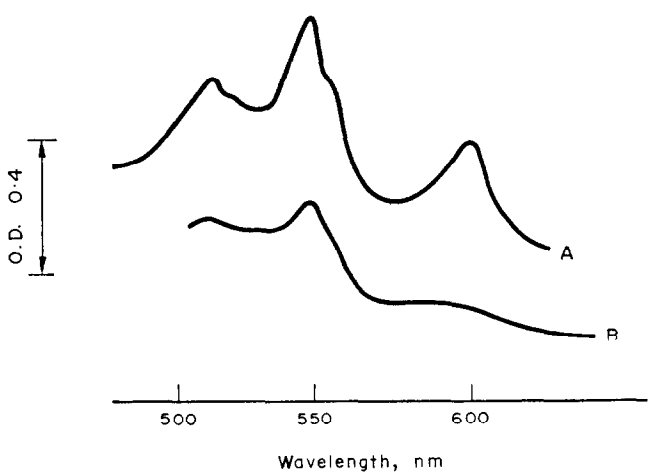

FIG. 4. Absorption spectra of cells of strain D6 in the absence (A) and in the presence (B) of 5 μg/m Lampren grown to stationary phase. Peaks at 605, 562 and 550 nm are the α-peaks of cytochromes a, a_3 b and c respectively. β-Peaks occur at 530 and 520 nm of b and c respectively.

RNA synthesis. The effect of Lampren on cellular biosynthesis was then investigated with respect to RNA synthesis. The uptake of the RNA precursor uracil was measured into cells in the presence and absence of the drug. It was found (Fig. 5) that in glycerol medium, concentrations of the drug from 1 to 10 μg/ml significantly inhibited the

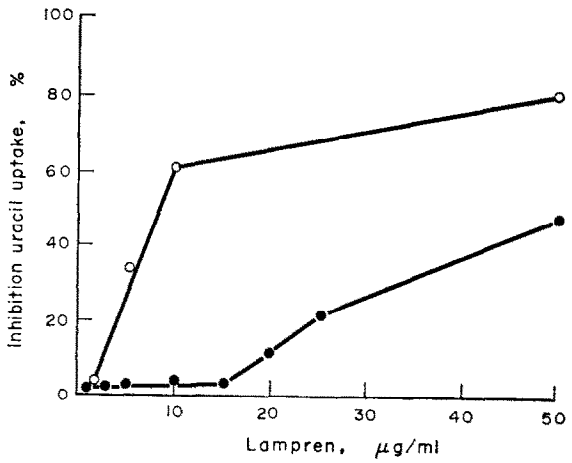

FIG. 5. Inhibition of RNA synthesis in cells of strain D6 in the presence of various concentrations of Lampren. ○——○ Glycerol medium; ●——● glucose medium.

uptake of uracil over the time of pulse labelling (30 min), but in glucose medium concentrations of more than 15 μg/ml were required to show any effect on RNA synthesis. These results further testified to the selective action of Lampren on the mitochondrial system, in which the function of the organelle in providing ATP appears to be the primary target. Restriction in this respect would have an immediate effect on cellular processes generally of which protein synthesis and RNA synthesis are examples.

Mode of action. Drugs which are known to have primary antimitochondrial activity include oligomycin, triethyl tin[5] and chlorimipramine.[6] Cross-resistance relationships were tested between these drugs and Lampren and the results obtained with some representative Lampren-resistant mutants are given in Table 2. All of these mutants except one, were found to have acquired simultaneously, resistance to these other drugs. In general, cross-resistance to chlorimipramine and triethyl tin was more pronounced than resistance to oligomycin. Also, the cross-relationship is quantitative in that mutants resistant to high levels of Lampren are usually resistant to high concentrations of the other drugs. Similarly it was found that mutants isolated as resistant to each of the other drugs were frequently cross-resistant to Lampren and to each other. Cross-relationship between oligomycin, triethyl tin and chlorimipramine has been reported previously.[7]

A high correlation of this type between drugs is indicative of a close relationship in site of action. Although all four compounds are very different regarding chemical structure, they have a common property of being strongly lipophyllic, leading to reactivity with membranes. The tentative conclusion may be drawn that Lampren reacts preferentially with mitochondrial membranes in bringing about its effect in yeast cells. It is worth noting that imipramine has been found by us to bind preferentially to yeast mitochondrial membranes compared with cell membranes,* and that resistant mitochondria bind the drug to a significantly less extent than sensitive ones.

* D. Linstead and D. Wilkie, unpublished observations.

Effect on oxygen uptake in respiring cells. A possible direct effect of Lampren on the respiratory chain was investigated by recording alterations in oxygen uptake of respiring cells following addition of the drug.

Yeast cells grown in glucose medium to early stationary phase were introduced into the oxygen electrode at densities between 10^7 and 10^8 cells/ml, and the rate of consumption of oxygen measured over a period of 5 min. After this time the drug was added as a stock solution in ethanol. In a control experiment, addition of ethanol itself had no detectable effect on rate of oxygen uptake. Low concentrations of Lampren of 0·5–1·0 μg/ml had no detectable effect but addition of 5 μg/ml had the surprising effect of stimulating oxygen consumption to a marked degree, the rate being about 160 per cent of the control rate. Increased oxygen uptake in these circumstances is characteristic of uncouplers of oxidative phosphorylation due to the release of respiration from the limitation imposed by phosphorylation. The degree of stimulation seen by Lampren seemed too great to be explained in this way. In some experiments when high concentrations were used of up to 25 μg/ml, a stimulation of almost 400 per cent was seen in contrast to uncouplers added to yeast suspensions which show a maximum stimulation of about 30 per cent generally.[8] An alternative explanation was suggested by an observation made during these experiments. If sufficient Lampren was added to make the colour visible (about 25 μg/ml) and incubation carried out until all oxygen was removed from the electrode chamber, the drug colour disappeared at the moment of establishing anaerobiosis. When air was re-admitted, the colour rapidly returned. It was concluded that yeast cells are capable of reducing Lampren, but that the drug is rapidly autoxidized in the presence of oxygen. The mechanism of growth inhibition, if this is true, would be that Lampren provides an alternative pathway to the respiratory chain for the oxidation of substrates such as NADH. On this model a part at least, of the terminal portion of the respiratory chain would be by-passed through this pathway which, of course, is not ATP-producing.

Further evidence to support this hypothesis was obtained by the finding that oxygen uptake by a yeast suspension in the presence of Lampren is partially insensitive to cyanide. Potassium cyanide added to respiring yeast cells to a final concentration of 1 mM, inhibited respiration by more than 90 per cent. When Lampren was then added (15 μg/ml), oxygen consumption was reinitiated at a rate of 32 per cent of the cyanide sensitive respiration of the control. This was taken to mean that oxidation was taking place in the yeast cells without the involvement of cytochrome oxidase and possibly other portions of the terminal respiratory chain as well.

The interaction of redox dyes with the mitochondrial respiratory chain is well known, but most of the experiments demonstrating these effects employ isolated mitochondria or submitochondrial particles. Substances such as potassium ferricyanide, methylene blue and phenazine methosulphate have been extensively used as artificial electron acceptors in studying portions of the respiratory chain in isolated mitochondria. The close similarity between the molecular structures of Lampren and phenazine methosulphate led us to suspect that the mechanism of action of Lampren *in vivo* is similar to the well-established mechanism of action of phenazine methosulphate *in vitro*. It was therefore of interest to test this compound for selective inhibition of yeast growth on glycerol medium.

Six strains each sensitive to Lampren and seventeen spontaneous Lampren-resistant mutants were tested for growth inhibition in the usual way on glucose and glycerol

media respectively. Phenazine methosulphate was added to give concentrations of 1, 5, 10 and 50 μg/ml in the various Petri dishes. No growth inhibition was seen on the glucose medium series and there was little or no effect in glycerol medium at the 5 and 10 μg/ml concentrations. At 50 μg/ml, growth on glycerol plates was completely arrested in the case of the sensitive strains but all Lampren-resistant mutants except one, grew normally. A very high correlation between Lampren activity and that of phenazine methosulphate was evident from these results and it was concluded that the two compounds have a similar mode of action. In this respect, Lampren is much more potent than the methosulphate. Because of the light sensitivity of the methosulphate, these experiments were carried out in the dark as far as possible when this compound was being used.

Recovery of cells. The recovery of cells after treatment was examined. Cells of strain D6 were inoculated into liquid, glycerol medium containing 5 μg/ml Lampren. Cells were removed from shaking culture at intervals of 24, 48 and 72 hr in aliquots, washed and plated on glucose medium. Little or no effect was seen on cell viability compared with untreated controls. Also, the colonies that developed from treated cells were mainly respiratory normal and similar to the controls regarding the frequency of respiratory deficient (petite) mutants, namely about 2 per cent. These results showed that cells recovered completely from the inhibitory effects of the drug when plated on glucose medium. However, if cells were plated directly after washing onto glycerol medium, no colonies would develop. Apparently, the drug is still bound to the mitochondria or otherwise continues to manifest its inhibitory effect on respiratory activity in these cells and that a period of organelle resynthesis is necessary on glucose to dilute out the drug.

Studies with mammalian systems. In cultures of human skin fibroblasts, the mitotic index is markedly depressed when the culture medium contains 5 μg/ml Lampren; growth of the cultures is arrested. The mitotic process seems to be totally inhibited at a concentration of 10 μg/ml of the drug. In these experiments, the details of which will be reported later as they are still underway, the drug was added from a stock solution in DMF. DMF when added to cultures alone had no detectable effect. Mitochondria isolated from the livers of young rats, showed endogenous respiratory activity when placed in the oxygen electrode. The effect of adding Lampren at intervals to the preparation on the rate of oxygen uptake was recorded. The results are given in Table 3 from which it is apparent that the drug is affecting the mitochondria in essentially the same way as yeast cells. Stimulation of oxygen uptake in mitochondria is seen at lower

TABLE 3. EFFECT OF LAMPREN ON OXYGEN UPTAKE BY RAT LIVER MITOCHONDRIA

Lampren concen* (μg/ml)	Rate of O_2 uptake (mm of chart/min)	Control (%)
0	4	100
0·5	5	125
1·0	7·6	190
1·5	11	275

* 0·5 μg added at 3-min intervals to 1 ml suspension of mitochondria.

drug concentrations than are required to detect these effects on intact yeast cells. This may be due to the immediate accessibility of reaction sites in the case of the isolated mitochondria. As in the experiments with fibroblasts, Lampren was added from a DMF solution. DMF added alone to the mitochondria had no effect on oxygen uptake.

DISCUSSION

Results of these investigations clearly demonstrate that Lampren selectively inhibits mitochondria in yeast cells. This is the first demonstration of an effect of this kind by the drug and marks the discovery of a new class of such inhibitors, namely, the rimino-phenazines. It would be of considerable interest to make a comparative study of the antimitochondrial activities of the large number of compounds available in this group to see whether there is a correlation with their antibacterial activities.

Because of the liphophillic nature of Lampren and cross-relationship with drugs known to react with mitochondrial membranes, we favour the hypothesis that the drug achieves its effect by attaching likewise to the mitochondrial membrane more readily than to cellular membranes. In doing so, it competes with cytochrome oxidase as an electron acceptor. Withdrawal of hydrogen from cultures of *Mycobacterium smegmatis* has been reported[1] and antimitochondrial activity would seem to parallel closely the antibacterial effect. Similarities between bacterial and mitochondrial systems have been described in recent years and are discussed in the literature.[9]

From the results reported here, administration of Lampren to humans and experimental animals should lead to serious side effects, but only a possible teratological effect in mice is reported so far. This may be due to the fact that there is low absorption of the drug and to its rapid uptake into fat cells and macrophages in which the drug soon appears as crystals.[10] This withdrawal of the drug leads to low serum levels (about 2 μg/ml) soon after administration but the high concentrations in the reticulo-endothelial system has prompted the suggestion that Lampren may be clinically useful as a carrier of antitumour agents in the treatment of reticulosarcomata.[10] Our findings of antimitotic activity suggests that the drug may have a direct use in this connection.

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REFERENCES

1. C. V. BARRY, J. G. BELTON, J. F. O'SULLIVAN and D. TWOMEY, *Nature, Lond.* **162,** 622 (1948).
2. D. WILKIE, *Med. Biol. Illus.* **22,** 119 (1972).
3. B. EPHRUSSI, *Nucleocytoplasmic Relations in Microorganisms*, Oxford University Press, Oxford (1953).
4. C. WIDNELL and J. TATA, *Biochem. J.* **92,** 313 (1964).
5. J. R. MANGER, *FEBS Letts* **4,** 61 (1969).
6. A. R. HUGHES and D. WILKIE, *Biochem. Pharmac.* **19,** 2555 (1970).
7. D. LINSTEAD and D. WILKIE, *Biochem. Pnarmac.* **20,** 839 (1971).
8. E. RACKER, *Membranes of Mitochondria and Chloroplasts*, Van Nostrand Reinholt, New York (1970).
9. D. B. ROODYN and D. WILKIE, *The Biogenesis of Mitochondria*, Methuen, London (1968).
10. M. L. CONALTY, *Ir. J. Med. Sc.*, No. 491 497 (1966).