Cellular Oxidative Phosphorylation. III. Measurement in Chemically Modified Cells*

Bruce E. Morton† and Henry A. Lardy

ABSTRACT: The specificity of action of surfactants on the membranes of bovine spermatozoa was studied by observing both oxidative phosphorylation and the ability of the cell to exclude the stain, erythrosin B. Surfactants were found that specifically altered the function of either the cell membrane, the mitochondrial membrane, neither, or both. Filipin, a polyene antibiotic found to be specific for the cell membrane, markedly stimulated net P_i uptake, respiration, and the observed P:O ratio of bovine epididymal spermatozoa. The measurement of oxidative phosphorylation in ejaculated sperm and ascites tumor cells was also influenced by filipin. Net P_i uptake was not observed in *Escherichia coli* even after the cell structure was modified in several ways. The action of the surfactants upon mammalian cells and possible applications are discussed.

It was demonstrated in paper II of this series (Morton and Lardy, 1967b) that the readily measurable net P_i uptake associated with the oxidative phosphorylation of intact bovine epididymal sperm cells (Morton and Lardy, 1967a) could be increased to near "theoretical" P:O ratios if the membranes of the cell were physically modified. To extend this work, chemical modification of cellular membranes was attempted. For several reasons the polyene antibiotics seemed likely to be useful agents for this purpose. Kinsky (1961) had shown them to cause a loss of cytoplasmic components of *Neurospora* to the external medium. Lampen *et al.* (1962) had demonstrated a correlation between sterols in cellular membranes and susceptibility to the antibiotic activity of polyenes. Since most mammalian cell membranes do contain sterols and are susceptible to polyenes, whereas mitochondria are much less susceptible to the cholesterol-specific surfactant, saponin, than to other surfactant types (Pressman and Lardy, 1956), it appeared that conditions might be established for altering the cellular membrane without damaging the mitochondrial membranes.

Disruption of the mitochondria of spermatozoa eliminates net P_i uptake (Morton, 1962). This, together with the fact that the normal cell membrane is impermeable to certain dyes (Mayer *et al.*, 1951), made it possible in the present work to determine whether chemical agents attacking biological membranes alter the permeability of the cellular membrane, the mitochondrial membrane, or both. Filipin and certain other compounds with affinity for cholesterol have been found to have the desired selectivity (Demel *et al.*, 1965). They are useful in the study of cellular oxidative phosphorylation in sperm and other cells when it is desirable to increase the permeability of the cell membrane without lysis and with minimum alteration of the physical organization of the insoluble components of the cytoplasm. It is hoped that the techniques developed in this paper as well as the two preceding it, may aid in relating the discoveries made at the mitochondrial and submitochondrial levels with knowledge of the energy metabolism of the cell as a whole.

Materials and Methods

The materials and methods of this report are similar to those described in the two preceding papers (Morton and Lardy, 1967a,b). Filipin was obtained through the courtesy of Dr. Kingsley Mann, Upjohn Laboratories; other polyenes were gifts of Dr. J. O. Lampen when he was at the Squibb Institute for Medical Research.

Vitamin A (80% all *trans*) was the gift of Professor Hector DeLuca and Dr. Maija Zile of the Department of Biochemistry, University of Wisconsin. The other surfactants and reagents were of the highest quality available commercially. The *Escherichia coli* cells and penicillin and lysozyme spheroplasts were kindly prepared by Mr. Mel DePamphilis. The Sarcoma 180 was a strain obtained from Dr. Anna Marie Williams, Department of Medicine, University of Wisconsin.

Results

Epididymal Spermatozoa. Ethanol and 2,3-propylene glycol, solvents commonly used to dissolve antibiotics for studies with mitochondria, are somewhat toxic to spermatozoa and require the use of extensive controls. Better solvents were therefore sought. Dimethyl sulfoxide (DMSO), an agent that increases the passage of other molecules through cellular membranes (Jacob

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† National Institutes of Health Predoctoral Fellow, 1963–1965; present address: Department of Biology, Massachusetts Institute of Technology, Cambridge, Mass.

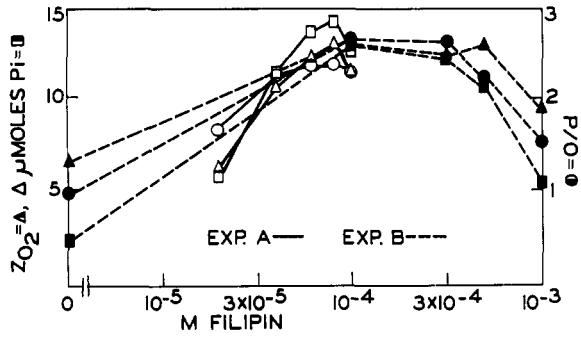

FIGURE 1: The response of bovine epididymal spermatozoa to filipin; methods of incubation as in Morton and Lardy (1967a). Conditions: 37° pH 7.0, 15 mM H_3PO_4 (Tris salt), 40 mM KF, 0.5 mM ATP,[1] 10 mM $MgSO_4$, 37.5 mM 2-deoxyglucose, 0.1 mg/ml of Sigma type IV yeast hexokinase, 10 mM α-ketoglutarate, and 9.2 × 10^8 cells/ml.

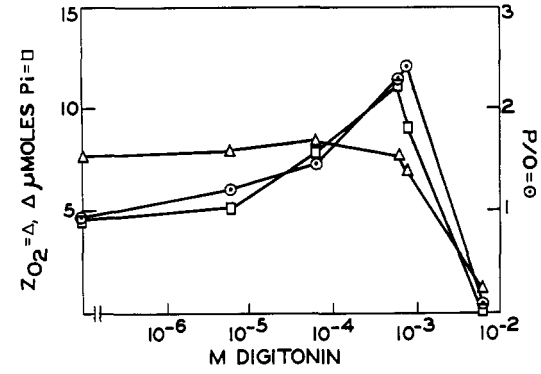

FIGURE 2: The response of bovine epididymal spermatozoa to digitonin. Incubation conditions the same as in Figure 1, 8.5 × 10^8 cells/ml.

et al., 1964), was found to have no effect upon respiration or net P_i uptake of epididymal sperm cells at a 1% final concentration; 10% DMSO inhibited net phosphate uptake 30% but did not alter oxygen consumption. Cells treated with DMSO did not become permeable to erythrosin B (Mayer *et al.*, 1951), indicating that their cellular membrane retained its ability to exclude large molecules. The fact that this powerful solvent was nontoxic in this system at 1% final concentration made it a very useful vehicle for all of the agents used in the present study to modify membrane permeability. Dimethylformamide was found to be as nontoxic as DMSO to spermatozoan oxidative phosphorylation.

Filipin, a polyene antibiotic of the pentaene group (Berkoz and Djerassi, 1959), was found to enhance oxygen consumption, phosphate esterification, and the P:O ratio of bovine epididymal spermatozoa (Figure 1). The optimum concentration range was from 3 × 10^{-5} to 3 × 10^{-4} M. Other experiments showed that the lowest concentration of filipin that would elicit a detectable increase in ΔP_i was about 10^{-6} M. In the presence of 10^{-4} M filipin the epididymal sperm were immotile (even in the absence of fluoride) and permeable to erythrosin B as indicated by their red coloration in the presence of this dye. The usual approximately 10% of the control cells were stained red.

Digitonin, another agent known to bind cholesterol, was found not to enhance respiration but it did increase phosphate uptake (Figure 2) and caused the cellular membrane to become permeable to erythrosin B. The highest concentration tested (10^{-2} M) is that used by Cooper and Lehninger (1956) to disrupt rat liver mitochondria into "digitonin particles." Sperm mitochondria are evidently susceptible to damage by this concentration of digitonin as well. An important difference between filipin and digitonin is that while both appear to damage mitochondria at about the same concentration, filipin altered cellular membrane permeability at about one-tenth the concentration required for digitonin. This gave some separation in the response of the two membranes to filipin while in the case of digitonin the maximal effect upon the cellular membrane probably did not occur before the mitochondrial membrane began to be altered. Further support of this contention is the failure of digitonin to induce the striking increase in oxygen consumption noted in the presence of filipin.

Table I shows the response of rat liver mitochondria incubated in the system used in these reports to measure cellular oxidative phosphorylation. The concentrations of surfactants employed here were those which increased the observed P:O ratio maximally in spermatozoa (Figures 1 and 2). Filipin at 10^{-4} M only very slightly reduced the P:O ratio while 8.5 × 10^{-4} M digitonin

TABLE I: Response of Rat Liver Mitochondria to Filipin, Digitonin, and DMSO in the System used to Measure Cellular Oxidative Phosphorylation.[a]

Agent Added	Concn	P:O	Q_{O_2} (N)
None	...	2.9	69
DMSO	1%	3.4	60
DMSO + filipin	1%, 10^{-4} M	3.2	64
Digitonin	6.5 × 10^{-4} M	2.6	74
Digitonin	8.5 × 10^{-4} M	2.0	85
85-μ glass beads	1.25 g/ml	2.3	62

[a] Conditions were the same as in Figure 1 except the temperature was 30°. There was 1.20 mg of mitochondrial N/ml of incubation mixture.

[1] Abbreviations used in this report: ATP, adenosine triphosphate.

TABLE II: P:O Ratios and Z_{O_2} Values Observed with Chemically Modified Bovine Epididymal Spermatozoa.[a]

Expt	Substrate	Addition	P:O	Z_{O_2}
A	α-Ketoglutarate	No filipin	1.0	6.5
	α-Ketoglutarate	10^{-4} M filipin	2.6	13.0
B	α-Ketoglutarate	33 v/v glass beads	2.2	11.1
	α-Ketoglutarate	10^{-4} M filipin	2.4	15.1
	Malate	10^{-4} M filipin	2.5	13.0
	α-Glycerophosphate	10^{-4} M filipin	1.6	22.3
	Endogenous	10^{-4} M filipin	2.1	6.6

[a] Incubation conditions were the same as in Figure 1 except for substrates, all of which were 10 mM; expt A, 9.1 × 10^8 cells/ml, and expt B 5.0 × 10^8 cells/ml.

clearly bordered on toxicity as in the case with spermatozoa. The relatively low rate of α-ketoglutarate oxidation results from the presence of 40 mM fluoride. Rat liver mitochondria were not disrupted into submitochondrial particles by treatment with 10^{-2} M filipin at 2° for 20 min, but such treatment diminished the phosphorylation efficiency by about one-third.

Table II shows the results obtained thus far for bovine epididymal spermatozoa in the presence of 10^{-4} M filipin. Comparison with data of Table I in the first paper of this series (Morton and Lardy, 1967a) emphasizes the marked stimulation of both respiration and net P_i uptake caused by the presence of filipin. Filipin was found to be unstable. It was stored only as long as 10^{-4} M caused the sperm to become permeable to erythrosin B.

The polyene antibiotics amphotericin B, candicidin, candidin, and nystatin were found to vary in their effects on this system. The tetraene, nystatin, resembled filipin but required the much higher concentration of about 0.3 mg/ml for full activity as would be predicted from the data of Egorenkova (1964) who showed antifungal activity of these antibiotics to increase with increasing unsaturation. However, the heptaene, amphotericin B, was inactive at 3 mg/ml which indicates that either the antifungal activity of that compound is based upon a different mechanism than that of filipin and nystatin or that the cell membranes of spermatozoa are more selective than fungi in their susceptibility to polyene antibiotics of differing structures. The candicidin preparation caused uncoupling of oxidative phosphorylation at 10 μg/ml. However, the water-soluble fraction of this mixture which was made equivalent to 10 μg/ml of the original mixture was inactive. Candidin uncoupled at 5 μg/ml. Lardy *et al.* (1958), using the same antibiotic samples, found both candicidin and candidin to have no effect upon the oxidative phosphorylation of rat liver mitochondria. That bull sperm oxidative phosphorylation is sensitive to these polyenes suggests an interesting difference in mammalian mitochondria of different sources.

Saponin, a cholesterol-specific natural product (Schulman and Rideal, 1937), was found to increase the P:O ratio of epididymal sperm maximally at 0.3 per cent but did not enhance the Z_{O_2}. At a concentration of 1%, saponin increased the respiration 10% but the net P_i uptake remained constant. Although this agent was earlier believed, on the basis of electron microscopic observations, to dissolve hexagonal holes in the membranes of certain cells and viruses (Dourmashkin *et al.*, 1962), it has been suggested that the hexagonal patterns are due to saponin–cholesterol complexes formed on the surface of the membranes (Bangham and Horne, 1962; Glauert *et al.*, 1962). Saponin caused the sperm to lose their ability to exclude erythrosin B (60% stained at 0.3% saponin and 100% stained at 1% saponin).

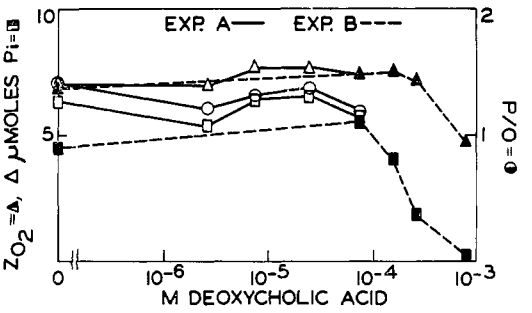

FIGURE 3: The response of bovine epididymal spermatozoa to deoxycholic acid. Incubation conditions the same as in Figure 1. Experiment A, 8.2 × 10^8 cells/ml; expt B, 7.6 × 10^8 cells/ml.

Deoxycholic acid is a surfactant that does not specifically bind steroids. Although it activates the ATPase of liver mitochondria (Pressman and Lardy, 1956) it did not enhance respiration of epididymal spermatozoa (Figure 3); at higher concentrations it inhibited respiration and abolished P_i uptake. This agent is obviously able to penetrate the sperm cell, but did not alter the ability of the membrane to exclude erythrocin B.

High concentrations of vitamin A cause hemolysis of mammalian erythrocytes (Dingle and Lucy, 1962) while

TABLE III: The Response of Ascites Tumor Cells to Chemical Modification of the Cell Membrane.

Cell Type and Substrate	Additions	Z_{O_2}	($\Delta\mu$moles of P_i/ml)	P:O
Ehrlich ascites β-hydroxy-butyrate	None	174	2.8	0.6
	1% DMSO	181	2.3	0.5
	3×10^{-3} M filipin + 0.3% DMSO	190	2.0	0.5
	10^{-4} M filipin + 1% DMSO	142	2.7	0.8
	3×10^{-4} M filipin + 3% DMSO	69	1.7	1.0
Sarcoma 180, Tris–lactate	None	128	4.4	0.6
	10^{-4} M filipin + 1% DMSO	116	4.7	0.7
	10^{-3} M filipin + 10% DMSO	70	6.3	1.6

[a] Ehrlich ascites tumor, 3.8×10^7 cells/ml. Incubation conditions were as in Figure 1 except KF was 10 mM and 10 mM β-hydroxybutyrate was substituted for α-ketoglutarate. Sarcoma 180, 12.6×10^7 cells/ml. Conditions were the same as above except the substrate was 10 mM L-(+)-lactic acid (Tris salt).

physiological levels protect against hemolysis (Dingle, 1964). The effect was found to be correlated with the nutritionally active isomers and these were found to complex with a lecithin–cholesterol monolayer (Dingle, 1964). The addition of concentrations of vitamin A alcohol up to 3×10^{-4} M caused only slight depression in net P_i uptake and no staining of the epididymal sperm by erythrosin B; at 10^{-3} M the cells did stain and also lost their ability to fix P_i from the medium. The cells at this concentration also appeared to be covered with small amorphous particles. Possibly the vitamin precipitated upon their surface.

While filipin and related compounds at the proper concentrations apparently modify only the cell membrane, and deoxycholic acid modified only the mitochondrial membrane, Triton x-100, and sodium laurylsulfate were found to represent a third category of surfactants which attack both mitochondrial and cellular membranes. These surfactants at 0.01% caused a depression of both Z_{O_2} and P:O and permitted staining by erythrosin B. At 0.1%, respiration and phosphate uptake were negligible. Koefoed-Johnson and Mann (1954) found sodium laurylsulfate and other detergents to cause a diminution of glycolysis, motility, and normal respiration.

Ejaculated Spermatozoa. Filipin at 10^{-4} M did not increase oxygen uptake of ejaculated bovine spermatozoa but increased the measured P:O slightly. The effect on respiration appears to be a compromise between the striking stimulation obtained with filipin in epididymal spermatozoa and the inhibition that follows bead treatment of ejaculated spermatozoa.

Ascites Tumor Cells. Filipin enhanced net phosphate uptake and P:O ratios of Sarcoma 180 ascites cells but did not enhance oxygen consumption (Table III). Higher concentrations of filipin were required to obtain an effect on phosphate uptake by tumor cells than were necessary with spermatozoa. It is assumed that 3 and 10% DMSO exerted an inhibitory effect on net P_i uptake with these cells as was the case with bull sperm.

E. coli, like most bacteria, do not contain cholesterol in their membranes and thus are not susceptible to polyenes. Therefore, other means of altering their cell membranes were sought in an effort to obtain net phosphate uptake. Leive (1965) treated these cells with EDTA to make them permeable to actinomycin; however, the use of this method did not increase the net P_i observed here. The more drastic osmotic shock method, used by Neu and Heppel (1965) to cause the loss of certain enzymes and 260-mμ-absorbing compounds, was also employed. Although they report 60–90% of *E. coli* cells viable after this procedure, we found the cells osmotically shocked from 11% w/w sucrose respired at only 5% the rate of the controls and did not remove P_i from the incubation medium described in the first paper of this series (Morton and Lardy, 1967a) with lactate as the substrate. Penicillin spheroplasts and lysozyme spheroplasts respired at a rate only slightly lower than the normal control cells but there was no net P_i uptake. It was concluded that methods suitable for studying sperm and tumor cell phosphorylation are not yet applicable to bacteria.

Discussion

It is at present unclear why filipin causes such a marked stimulation of respiration in epididymal sperm while digitonin which is also cholesterol specific and glass bead treatment which also increases sperm cell membrane permeability do not. The possibility that filipin might remove the acrosome as glass beads do (Morton and Lardy, 1967b) was eliminated by examination with Giemsa stain (Iype *et al.*, 1963). This, coupled with the response of Sarcoma 180 to filipin suggests that filipin leaves the cell membrane in position but modifies its permeability.

The method for observing cellular oxidative phosphorylation, developed earlier in this series, can be used to clarify the membrane specificity of the various surfactants. Deoxycholate can pass through the sperm

cell membrane, leaving it apparently functional, to attack the mitochondrial membrane. The finding that the opposite is true for certain polyene antibiotics is of interest in the measurement of cellular oxidative phosphorylation and the study of the influence of the cytosol and cell membrane upon this process.

With the aid of compounds such as filipin it may be possible to bypass cell membrane pumps as well as barriers and introduce compounds into the energetically functional cell at concentrations before impossible. It may even be possible to introduce large molecules such as alien nucleic acids, enzymes, or other substances into the cytoplasm and specifically alter certain processes. Thus one could approach some problems related to correction of genetic defects, enzyme biosynthesis, and the mechanism of action of hormones.

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