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Synthesis of ATP coupled with action of membrane protonic pumps at the octane–water interface

ATP SYNTHESIS in mitochondria is one of the most important energy-producing processes in the cell. We have studied the main stage of this process, the coupling of ATP synthetase to the action of membrane protonic pumps at an octane–water interface.

Two hypotheses exist concerning this coupling of respiration to phosphorylation. That of Williams[1] suggests direct proton transfer in lipid phase from respiration enzymes to ATP synthetase, whereas that of Mitchell[2] postulates an essential additional stage of transmembrane proton transfer, resulting in the formation of a membrane potential. We have carried out ATP synthesis in non-equilibrium conditions (acidic concentration in the hydrophobic phase), thus supporting William's hypothesis.

We had previously shown that the mitochondrial ATPase can transfer protons from water to octane by ATP hydrolysis[3]. We therefore carried out the reverse reaction, and observed ATP synthesis coupled to the action of purified mitochondrial ATPase adsorbed at an octane–water interface. The proton flow through the ATPase complex from octane to water was caused by an excess concentration (with respect to that of the equilibrium) of the undissociated acid or Lewis form in the octane phase.

The excess concentration of acid was produced by oxidation of the reduced form of nicotinamide adenine dinucleotide (NADH) by ferricyanide with the participation of the enzymes comprising the initial step in the respiratory chain of mitochondria. For this purpose, submitochondrial particles (SMP) (for isolation techniques, see refs 4 and 6), were introduced into the octane–water system, to which was added one of the ATPases to be examined and rotenone, an inhibitor of NADH oxidase which prevents respiration and thus the synthesis of ATP in SMP. If NADH is added to the octane–water system containing a proton acceptor 2,4-dinitrophenol (DNP), $K_3Fe(CN)_6$, SMP and rotenone, the maximum shift in the positive direction of the potential difference measured in the chain, Au–air–octane, proton acceptor (RO^-, ROH)–water, enzyme, substrate–water, saturated KCl–Hg_2Cl_2,Hg, was 0.52 V. ATP synthesis at the interface was detected by a decrease in potential difference measured in this chain on addition of ADP and inorganic phosphate to the system. Equilibrium octane–water systems containing 50 mM Tris-HCl (1 mM) and DNP were used. Enzyme and substrates were added just before measuring the potential differences by the dynamic capacitance method[3,8–10]. Voltages plotted in the figures are referred to the initial value.

When 0.2 mM NADH was added to the system containing SMP, $K_3Fe(CN)_6$ and oligomycin-sensitive ATPase, a potential shift in the positive direction was observed at the octane–water interface. Subsequent addition of 1 mM ADP and 1 mM inorganic phosphate caused the potential difference to shift in the negative direction. At sufficiently high ADP concentrations this negative shift was below the initial value (Fig. 1). Addition of oligomycin (points C and C′, Fig. 1) eliminated almost completely the action of ADP and phosphate on the potential difference in the oligomycin-sensitive ATPase system.

Instead of SMP, another proton pump, such as bacteriorhodopsin sheets (obtained from *Halobacterium halobium*), was used. As we have shown previously[8], bacteriorhodopsin sheets are also capable of transferring protons from water to octane by the action of light. Figure 2 shows that when light is switched on, bacteriorhodopsin sheets cause a potential change (0.5 V) at the octane–water interface. When the ATPase reaction starts, however, the potential returns to its initial value. When oligomycin is added in the presense of oligomycin-sensitive ATPase of *p-N,N*-di(2-chloroethyl)aminophenylacetic acid, an alkylating agent and inhibitor of soluble mitochondrial ATPase, bacteriorhodopsin sheets again generate the photopotential. Figure 2 shows the change in potential difference in the above chain in the presence of bacteriorhodopsin sheets on illumination and in the dark, as affected by the concentration of

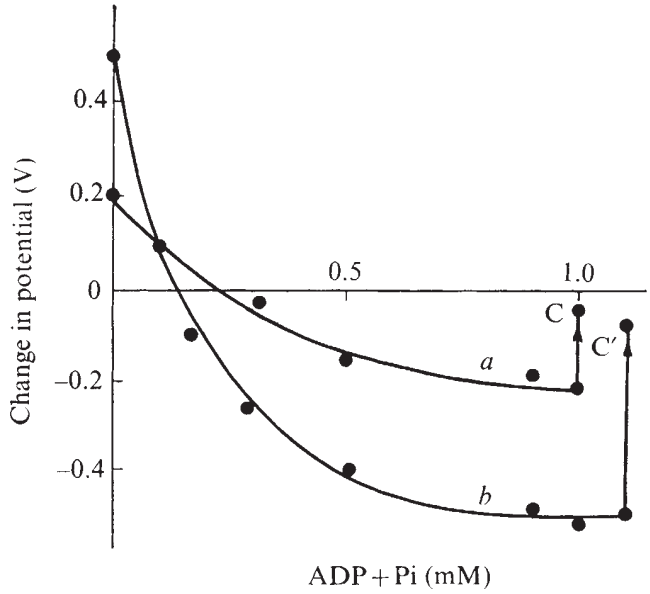

Fig. 1 Shift in negative direction of potential difference in the chain (see text) as affected by the concentration of substrate ADP+Pi. Composition of incubation medium: *a* 0.1 mg ml^{-1} oligomycin-sensitive ATPase, 0.1 mg ml^{-1} SMP (isolated by the technique used in ref. 4), 2.5 μg ml^{-1} rotenone, 50 mM Tris-HCl, 1 mM DNP, 1 mM $MgSO_4$, 1 mM $K_3Fe(CN)_6$ and 0.2 mM NADH. *b*, As *a* except SMP was isolated by the technique used in ref. 2. At points C and C′ 2 μg ml^{-1} oligomycin was added. The following experiments were run as controls: (1) Oligomycin-sensitive ATPase was excluded from the complete system. In these conditions, addition of ADP and Pi does not affect the potential difference. (2) SMP was excluded from the complete system. Addition of ADP and inorganic phosphate does not affect the potential difference. (3) NADH is excluded from the complete system. A very small potential change (no more than 25 mV) was observed on addition of 1 mM ADP and 1 mM Pi. Inorganic phosphate was excluded from the complete system. A very small potential change (no more than 25 mV) was observed on addition of 1 mM ADP.

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Table 1 ATP synthesis at the octane–water interface due to the excess concentration of PCP in octane

ATPase	Experimental conditions	Amount of ATP in sample ($\times 10^{-6}$ M)	Mean amount of ATP synthesised ($\times 10^{-6}$ M)
Oligomycin-sensitive ATPase (100 μg ml^{-1}) +ADP (10^{-4} M)	−PCB	3.1	—
	+PCB	4.2	1.1
	+PCB+oligomycin (1 g ml^{-1})	2.9	—
Soluble mitochondrial ATPase before heat treatment (130 μg ml^{-1}) +ADP (3.10^{-5} M)	−PCB	2.5	—
	+PCB	3.7	1.1

the substrate ADP and inorganic phosphate. When ADP and inorganic phosphate are added to a system with an active proton pump, the potential rapidly decreases.

ATP synthesis by oligomycin-sensitive ATPase, adsorbed on the octane–water interface, also takes place after addition of pentachlorphenol–octane solution to the octane phase. The system contained 1 ml octane and 2 ml 20 mM phosphate buffer (*p*H=7.5) to which were added 40 g hexokinase protein, 10^{-4} M ADP, 20 mM glucose, 2 mM $MgSO_4$ and 100 μg protein of oligomycin-sensitive ATPase isolated from the mitochondria of ox heart by Racker's method[2]. After introduction of 0.02 ml PCP into octane, its concentration in the complete system was 5×10^{-5} M. After 1 min the reaction was stopped by perchloric acid and neutralised with NaOH. The amount of ATP formed was determined fluorimetrically by the glucose-6-phosphate dehydrogenase method. In the control tests PCP and octane were excluded from the system. The results of these tests (Table 1) show that in the control tests ATP is present as an impurity in the reactants used and can be formed according to the equation 2ADP→ATP+AMP, because of the presence of myokinases in the enzymes used. In the complete system the amount of ATP formed was 30–50% higher than in control tests in which PCP was not added. The experimental error was no greater than 10%. In the experiments with oligomycin-sensitive ATPase, oligomycin lowered the ATP level to the control value.

Figure 3 shows a scheme for the action of ATPase at the interface, based on the experimental data described above. It is clear from this scheme that under the action of light, in the presence in the non-aqueous phase of a proton acceptor (such as a weak acid, ROH), bacteriorhodopsin transfers protons to this phase. Of course, protons do not exist in the free form in the non-aqueous phase, but are captured by RO^- or ROH to form ROH or ROH_2^+ (Lewis acid form[10]).

If the charged Lewis acid form alone acted in this process as proton donor, the potential would always be positive and equal to, or less than, the initial positive charge of octane obtained in the case of SMP (Fig. 1). The change in the potential sign in the negative direction observed in the experiment with SMP must mean that ATP synthesis is also brought about by the elimination of protons from the neutral ROH form of DNP. In this case the anion remains in the non-aqueous phase and the proton goes into water. To verify this assumption, we used dimethoxybenzene instead of DNP as Lewis-type proton acceptor in the non-aqueous phase. In this case, when NADH is added to the system containing soluble mitochondrial ATPase and SMP, the potential shifts in the positive direction by 0.2 V. At high ADP and inorganic phosphate concentrations it does not shift in the negative direction.

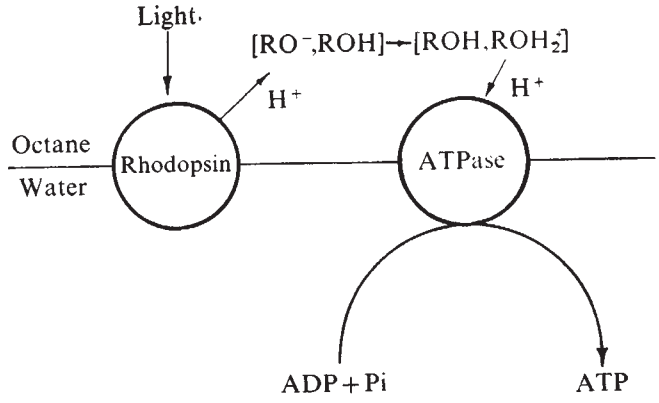
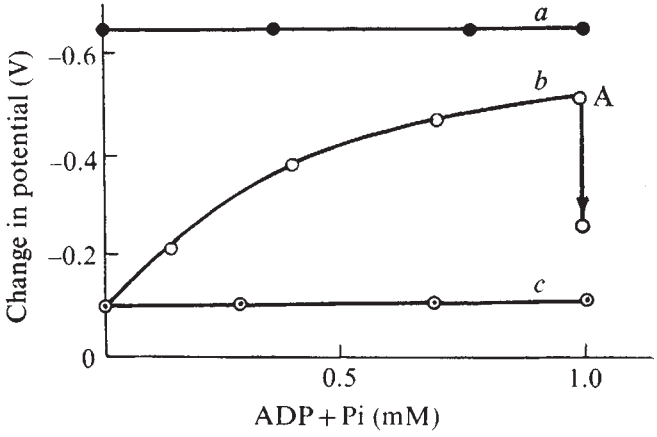


Fig. 3 Scheme for ATP synthesis at octane–water interface brought about by action of bacteriorhodopsin sheets.

Fig. 2 Dependence of potential difference measured in the chain (see text) on the concentration of substrate ADP + Pi. Composition of incubation medium: *a*, 30 μg ml^{-1} bacteriorhodopsin sheets, 1 mM DNP, 50 mM Tris-HCl and 1 mM $MgSO_4$ in the dark; *b*, as *c* plus 10^{-8} M soluble mitochondrial ATPase; *c*, as *a*, *b* and *c* under illumination. *p*H = 7.9. At A, 1 mM *p*-*N*,*N*-di-(2-chloroethyl)aminophenylacetic acid has been introduced into the cell



The interpretation of our experimental data obtained from measurements of the potential difference in the above chain is being verified by direct determinations of the amount of ATP synthesised. Our data show that the presence of a transmembrane potential is not a necessary condition for ATP synthesis by ATPase. The energy of proton (acid) solvation can be used for ATP synthesis.

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Antiviral, immunosuppressive and antitumour effects of Ribavirin

THE compound 1-β-D-ribofuranosyl-2,2,4-triazole-3-carboxamide (Ribavirin) has been reported to inhibit the replication of both RNA and DNA viruses *in vitro*, and to inhibit influenza virus infection of tissue cultures and mice[1–3]. The compound acts by interfering with guanidine monophosphate formation and subsequent nucleic acid synthesis; at a concentration which completely inhibited influenza viral polypeptide production in tissue culture, however, there was no demonstrable effect on cellular protein synthesis[4]. To test the activity of Ribavirin further, we examined the effect of this compound on influenza virus infection of ferrets. Four adult ferrets were inoculated intraperitoneally with 100 mg kg^{-1} d^{-1} of Ribavirin each for 7 d; this dose although it did not cause deaths or other signs of toxicity, was probably close to the toxic level as judged by standard tests in mice. Two hours after the second inoculation of drug, these animals and four untreated ferrets were lightly anaesthetised and inoculated intranasally with $10^{3.0}$ ferret-infective doses of egg-grown influenza virus A/Port Chalmers/73 (H3N2). Following virus infection, the drug-treated ferrets were given 5 further doses of Ribavirin. The response of the animals to virus infection was measured, as described previously[5,6]. The results are shown in Table 1. Ribavirin had a marked effect on the response of ferrets to influenza virus infection; compared with control ferrets, influenza virus-infected animals treated with Ribavirin did not exhibit a febrile reaction to infection, did not develop a significant increase in nasal wash protein and did not develop either local or serum antibody. In addition, the titre of virus in nasal washings collected 3 d after virus infection from drug-treated animals was 100-fold less than that found in control ferrets.

Table 1 Response of ferrets to infection with influenza virus A/Port Chalmers/73

Response to infection*	Control ferrets	Ferrets treated with Ribavirin (100 mg kg^{-1} d^{-1})
Rise in temperature† (°C)	39.3–40.9	39.6–39.6
Virus titre in nasal wash at 48 h post-infection (EID_{50} ml^{-1})	$10^{6.30}$	$10^{4.35}$
Nasal wash protein concentration; pre- and 7 d post-infection (mg per 100 ml)	0.28–1.22	0.26–0.43
Nasal wash neutralising antibody titre; pre- and 7 d post-infection	<2–1:14	<2–<2
Serum HI antibody response (g.m.t.)	<5–463	<5–<5

*Mean values for four ferrets.
†Increase in temperature from mean, pre-inoculation temperature to temperature at 48 h post-infection.

The absence of both a serum and a local antibody response in Ribavirin-treated ferrets infected with influenza virus may have been due to the antiviral activity of the compound which reduced virus replication to a level that did not induce antibody production (Table 1). Alternatively, Ribavirin may also have an immunosuppressive effect. To test this latter possibility, a group of guinea pigs was inoculated intraperitoneally with 100 mg kg^{-1} d^{-1} of Ribavirin for 10 d. On the second day, the drug-treated animals and a group of untreated guinea pigs were inoculated intramuscularly with 400 IU of inactivated influenza virus A/England/42/72 (H3N2) vaccine in an equal volume of Freund's complete adjuvant (FCA). Serum samples were collected before and 10 d after immunisation, and tested for serum haemagglutination inhibiting (HI) antibody. Some guinea pigs in each group were inoculated intraperitoneally with 10 ml of sterile paraffin 4 d after immunisation and were killed 6 d later; the peritoneal macrophages were collected from these animals and tested for cell-mediated immunity to influenza virus by the macrophage migration inhibition test. Ten days after immunisation, peripheral blood specimens were collected from the guinea pigs, and the buffy coat cells incubated with 2.0 μg ml^{-1} of phytohaemagglutinin and tested for lymphocyte transformation. The results are shown in Table 2. Guinea pigs immunised with influenza virus vaccine in FCA produced serum HI antibody by 10 d, post-inoculation (geometric mean titre g.m.t. = 1 : 124); at this time however, no antibody was detectable in serum from Ribavirin-treated animals. The migration of peritoneal macrophages from both control and drug-treated guinea pigs was significantly inhibited in the presence of 40 IU of influenza virus A/England/42/72 vaccine. Furthermore, lymphocytes from drug-treated and control animals were both transformed by PHA to approximately the same degree (Table 2). The results indicate that Ribavirin, at

Table 2 Immune response of guinea pigs to inoculation with 400 IU of inactivated influenza virus A/England/42/72 vaccine in FCA

Ribavirin treatment	Animal no.	Serum HI antibody response (g.m.t.) day −1	Serum HI antibody response (g.m.t.) day 10	Delayed hypersensitivity response (MMI test) to 40 IU influenza vaccine at day 10, post-infection: Test ± s.d.*	Control ± s.d.	Inhibition %	Response to PHA (2.0 μg ml^{-1}) at day 10, post-inoculation: Test (c.p.m. × 10^{-4})	Control (c.p.m. × 10^{-4})	Fold stimulation
100 mg kg^{-1} d^{-1} from day −1 to 10	1	<5	<5	NT	NT	NT	22.2	2.1	10.7
	2	<5	<5	0.25 ± 0.01	0.48 ± 0.04	54†	7.9	1.3	6.1
	3	<5	<5	0.66 ± 0.02	1.25 ± 0.11	47†	NT	NT	NT
	4	<5	<5	NT	NT	NT	NT	NT	NT
		(<5)	(<5)						
Nil	5	<5	512	0.75 ± 0.05	1.79 ± 0.22	58†	NT	NT	NT
	6	<5	128	1.33 ± 0.68	2.91 ± 0.57	54†	NT	NT	NT
	7	<5	32	0.47 ± 0.06	0.73 ± 0.02	36†	13.2	1.7	7.8
	8	<5	128	0.31 ± 0.01	0.40 ± 0.01	22†	6.3	1.7	3.7
		(<5)	(124)						

*Mean area of macrophage migration ± standard deviation.
†Significant inhibition of macrophage migration ($P = < 0.05$).
NT, not tested.

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