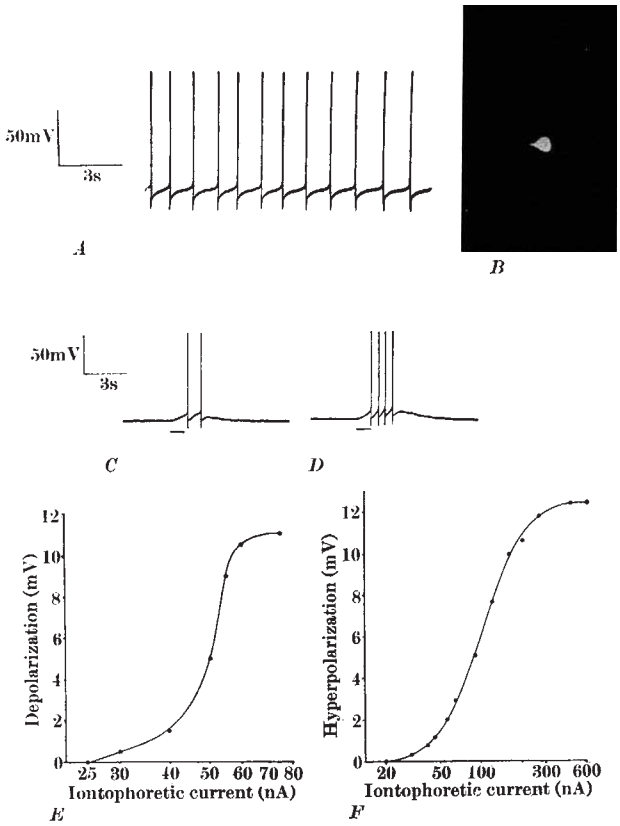

Fig. 1. *A*, Microelectrode recording of spontaneous action potentials from a neurone in the sixth abdominal ganglion of the cockroach. *B*, Photograph in ultraviolet light of a neurone that had been penetrated with a microelectrode filled with Procion yellow. The location of the microelectrode can be thus determined. The electrode was in the cell (shown white in the photograph) and not in an axon. *C*, Iontophoretic injection of acetylcholine (50 nA). *D*, Iontophoretic injection of acetylcholine (120 nA). *E*, Dose response relationship between amount of acetylcholine injected onto neurone (iontophoretic current) and the response of the neurone (mV depolarization). *F*, Dose response relationsbip between amount of GABA injected onto neurone (iontophoretic current) and the response of the neurone (mV hyperpolarization).

photograph of such a preparation. The marked cell fluoresces a bright yellow orange colour (white in the photograph). Such histological studies show that the electrode is actually in the cell body and not in a branch of the axon.

Iontophoretic application of acetylcholine from a second electrode close to the neurone surface led to a depolarization of the membrane. When the amount of acetylcholine injected onto the neurone was increased, the depolarization increased and the cell fired off a series of action potentials. Fig. 1*C* shows the effect of passing 50 nA of current through the electrode. There was a depolarization of the cell and two action potentials followed. When a larger current was passed through the iontophoretic electrode (Fig. 1*D*, 120 nA) there was a larger depolarization and four action potentials. Experiments where the solution in the iontophoretic electrode was NaCl showed that the depolarization was not caused by the iontophoretic current but by the acetylcholine. The response of the cell was proportional to the amount of acetylcholine applied. Fig. 1*E* shows the relationship between the cell's response and the amount of acetylcholine applied. The threshold to acetylcholine was $1{\cdot}31 \times 10^{-13}$ M.

A similar series of experiments was carried out using gamma amino butyric acid (GABA) in the iontophoretic electrode. GABA hyperpolarized the nerve cell, the hyperpolarization being proportional to the amount of GABA injected onto the neurone (Fig. 1*F*). GABA also stopped any spontaneously active potentials in the nerve cell. The threshold to GABA was $1{\cdot}05 \times 10^{-13}$ M.

The effect of acetylcholine is to increase the membrane permeability to sodium ions while the effect of GABA is to increase the membrane permeability to chloride ions. This was shown by changes in the ionic composition of the saline around the neurone and the injection of ions into the cell body.

The sensitivity of the insect neurone to acetylcholine or GABA compares favourably with that of the crustacean nerve muscle junction[11] or the mollusc neurones[12]. We have carried out a series of experiments using the same iontophoretic electrode on insect neurones and snail neurones and have found that both have a similar sensitivity to acetylcholine. Measurements of reversal potentials in insect neurones indicate that the EPSP and the IPSP are the same as those for acetylcholine and GABA respectively and that there is a similar change in ionic permeability between the natural transmitter and the chemical applied on the post synaptic membrane.

Our work strengthens the evidence that acetylcholine is the excitatory transmitter in the insect central nervous system and that GABA is the inhibitory transmitter. The breakdown of the acetylcholine between the site of application and the site of action probably explains why relatively high concentrations of acetylcholine have had to be used in the past to elicit excitation of insect neurones. The research has been supported by a grant from the Agricultural Research Council.

G. A. Kerkut
R. M. Pitman
R. J. Walker

Department of Physiology and Biochemistry,
University of Southampton.

Received April 4, 1969.

[1] Roeder, K. D., *Bull. Johns Hopkins Hosp.*, **83**, 587 (1948).
[2] Twarog, B., and Roeder, K. D., *Ann. Ent. Soc. Amer.*, **50**, 231 (1957).
[3] Yamasaki, T., and Narahashi, T., *Nature*, **182**, 1805 (1958).
[4] Colhoun, E. H., *Adv. Insect. Physiol.*, **1**, 1 (1963).
[5] Callec, J., and Boistel, J., *CR Soc. Biol.*, **161**, 442 (1967).
[6] Treherne, J. E., and Smith, D. S., *J. Exp. Biol.*, **43**, 13 (1965).
[7] Hagiwara, S., and Watanabe, A., *J. Cell. Comp. Physiol.*, **47**, 514 (1956).
[8] Callec, J., and Boistel, J., *CR Soc. Biol.*, **160**, 1943 (1966).
[9] Rowe, E. C., thesis, Univ. Michigan (1964).
[10] Kerkut, G. A., Pitman, R. M., and Walker, R. J., *Life Sci.*, **7** (1), 605 (1968).
[11] Takeuchi, A., and Takeuchi, N., *J. Physiol.*, **170**, 296 (1965).
[12] Gerschenfeld, A., and Stefani, E., *J. Physiol.*, **185**, 668 (1966).

Mechanism of Coupling of Oxidative Phosphorylation and the Membrane Potential of Mitochondria

The existence of an electrical potential difference at the mitochondrial membrane is one of the postulates of Mitchell's chemiosmotic theory of oxidative phosphorylation[1,2]. A shunting of the mitochondrial membrane leads to the uncoupling of oxidative phosphorylation. It has been demonstrated that uncoupling agents increase the proton permeability of phospholipid micelle membranes[3] and artificial bimolecular membranes[4–7]. Mitchell and Moyle[8] showed that uncouplers can operate as proton carriers in mitochondrial membranes.

This communication is an attempt to prove directly the existence of a potential difference at the mitochondrial membrane. If such a potential exists, compounds inducing equal permeability for H^+ ions should produce equal uncoupling effects. The penetrating cations should be actively transported through the membrane into mitochondria while penetrating anions should go out of mitochondria but into sonic mitochondrial particles (SMP) which have the opposite membrane polarity. Cation transport in mitochondria should decrease the *p*H of extramitochondrial space while anion transport in SMP should increase the *p*H of extra-SMP space.

Our methods have been described in refs. 4, 9–12. Fig. 1*A* is a plot of uncoupling efficiency in mitochondria against that in bimolecular membranes prepared from mitochondrial phospholipids. The concentrations of uncouplers inducing two-fold stimulation of succinate oxidation in state 4 are plotted against the concentrations of the same agents that increase the membrane proton conductance by 5×10^{-9} mho/cm^2. There is a good correlation between these two parameters for most of the uncouplers.

A number of synthetic ions have been found which possess hydrophobic groups and penetrate the membrane without changing the membrane permeability for H^+, OH^- or other ions present in the incubation mixture (Fig. 1*B*).

Fig. 1*C* shows the *p*H responses on the addition of such ions to mitochondria and SMP in state 4. These results and other tests show that synthetic penetrating cations brought about the same alterations in mitochondrial functions as did Ca^{++} ions or K^+ ions in the presence of valinomycin. The active transport of K^+ into mitochondria in the presence of carriers has been described by Pressman and coworkers[13]. Our experiments suggest that such an effect takes place with all penetrating cations.

To prove that ions are translocated through the mitochondrial membrane under the influence of the electrical field it was necessary to demonstrate the active transport of penetrating anions in the opposite direction. The result of a typical experiment is shown in Fig. 1*D*. It is seen that anion concentration in the incubation medium decreases when SMP hydrolyse ATP or oxidize succinate. Approximate calculation of the concentration gradient across the SMP membrane after energy dependent accumulation of the anion gave the value of > 1,000 which corresponds to a membrane potential of > 180 mV. The total electrochemical potential difference across the membrane (taking into account *p*H changes) is more than 200 mV.

Assuming the membrane potential as proved we must consider the following three possibilities. (1) According to Mitchell[2] both the respiratory chain and ATPase are H^+-pumps. (2) According to Chappell and Crofts[14] the membrane potential is produced by a special H^+-pump driven by hydrolysis of a high energy intermediate. (Mitchell and Moyle's experiments[15] call for a more precise definition of this hypothesis: if O_2 oxidizes, a hydrogen donor H^+ and OH^- will appear on the outside of the mitochondria; when $Fe(CN)_6^{4-}$ is used instead of a hydrogen donor, $Fe(CN)_6^{3-}$ and OH^- will appear outside.) (3) Only the respiratory chain can directly produce the membrane potential, whereas ATP energy is to be utilized for this purpose through the reverse electron transfer.

We thank Mr L. L. Grinius, Mr U. P. Kadziauskas and Mrs V. V. Kuliene for help with the experiments,

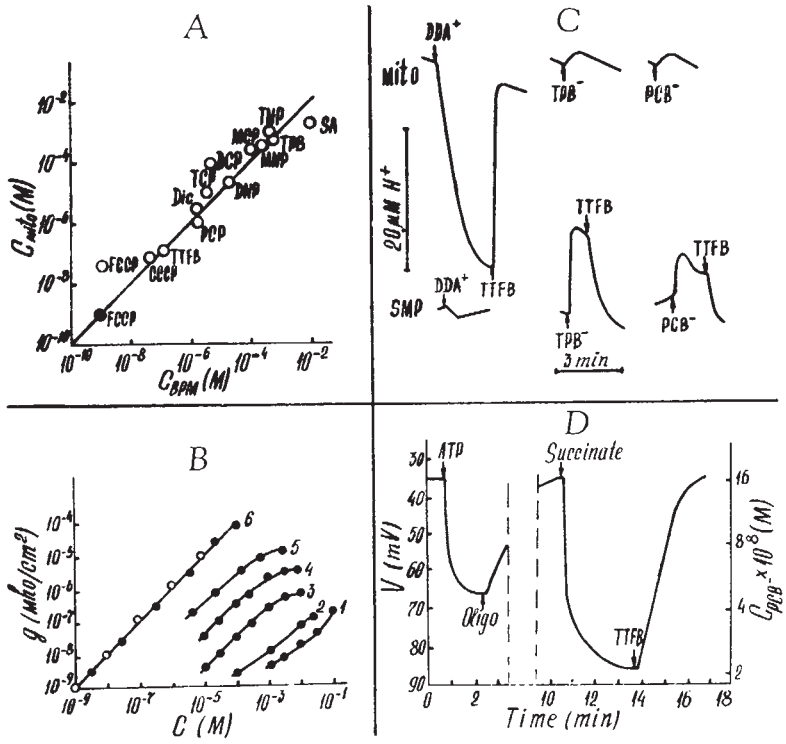

Fig. 1. Effects of uncouplers and penetrating ions on bimolecular phospholipid membranes, mitochondria and sonic mitochondrial particles. *A*, Correlation of effects of uncouplers in mitochondria (C_{mito}) and in membranes (C_{BPM}). CCCP *m*-chlorocarbonylcyanidephenylhydrazone; DCP, 2,4-dichlorophenol; Dic, dicoumarol; DNP, 2,4-dinitrophenol; FCCP, *p*-trifluoromethoxycarbonylcyanidephenylhydrazone; MCP, *p*-methyl-*m*-chlorophenol; MNP, *m*-nitrophenol; PCP, pentachlorophenol; SA, salicylic acid; TCP, 2,4,5-trichlorophenol; TNP, 2,4,6-trinitrophenol; TTFB, tetrachlorotrifluoromethylbenzimidazole. Filled circle indicates C_{mito} for FCCP in water phase of incubation mixture. *B*, Dependence of membrane conductance on concentration of penetrating ions: (1) *N*,*N*-dimethyl-*N*,*N*-dibenzylammonium cation (DDA^+); (2) DDA^+ in the presence of 10^{-9} M tetraphenylboron anion (TPB^-); (3) DDA^+ and 10^{-8} M TPB^-; (4) DDA^+ and 10^{-7} M TPB^-; (5) DDA^+ and 3×10^{-7} M phenyldicarbaundecaboran anion (PCB^-); (6) TPB^- (●) and PCB^- (○). *C*, *p*H responses of beef heart mitochondria (mito) and sonic particles (SMP) on addition of penetrating ions. Incubation mixture contained 0·26 M sucrose, 0·015 M sodium succinate, 0·9 mM EDTA, 4·7 mM $MgSO_4$, 2 mg mitochondrial or particle protein per ml. Concentrations of additions: 0·9 mM DDA^+, 60 μM PCB^-, 90 μM TPB^-, 9 μM TTFB. Samples with DDA^+ contained 0·3 μM TPB^-. *D*, Energy-dependent transport of penetrating anions into sonic mitochondrial particles. Ordinate: "inner" potential of selective "PCB-electrode" and corresponding PCB^- concentration in the water phase of incubation mixture. Particles (1 mg protein/ml.) were incubated in 0·25 M sucrose, 0·005 M $MgCl_2$, 0·03 M *tris* HCl (*p*H 7·5). Concentrations of additions: 5×10^{-4} M ATP, 15 mM succinate, 3×10^{-6} M TTFB, 2·5 μg/ml. oligomycin. PCB^- concentration in the inner compartment of the selective electrode is 7×10^{-7} M.

Dr U. V. Zeifman and Dr O. Yu. Okhlobistin for providing some of the substances and Miss T. Kheifets for translating the manuscript into English.

E. A. Liberman
V. P. Topaly
L. M. Tsofina

Institute of the Problems of
Information Transmission,
USSR Academy of Sciences,
Moscow.

A. A. Jasaitis
V. P. Skulachev

Department of Bioenergetics,
Laboratory of Bioorganic Chemistry,
Moscow State University, USSR.

Received March 3; revised April 18, 1969.

[1] Mitchell, P., *Nature*, **191**, 144 (1961).
[2] Mitchell, P., *Chemiosmotic Coupling in Oxidative and Photosynthetic Phosphorylation* (Glynn Research Laboratories, Bodmin, 1966).
[3] Chappell, J. B., and Haarhoff, K. N., in *Biochemistry of Mitochondria* (edit. by Slater, E. C., Kaniuga, Z., and Wojtczak, L.), 75 (Academic Press, London, 1967).
[4] Liberman, E. A., Mokhova, E. N., Skulachev, V. P., and Topaly, V. P., *Biofizika* (USSR), **13**, 188 (1968).
[5] Skulachev, V. P., Sharaf, A. A., Yaguzhinsky, L. S., Jasaitis, A. A., Liberman, E. A., and Topaly, V. P., *Currents in Modern Biology*, **2**, 98 (1968).
[6] Liberman, E. A., and Topaly, V. P., *Biofizika* (USSR), **13**, 1025 (1968).
[7] Hopfer, U., Lehninger, A. L., and Thompson, T. E., *Proc. US Nat. Acad. Sci.*, **59**, 484 (1968).
[8] Mitchell, P., and Moyle, J., *Biochem. J.*, **105**, 1147 (1967).
[9] Hansen, M., and Smith, A. L., *Biochim. Biophys. Acta*, **81**, 214 (1964).
[10] Crane, F. L., Gleen, J. L., and Green, D. E., *Biochim. Biophys. Acta*, **28**, 475 (1956).
[11] Mueller, P., Rudin, D. O., Tien, H. T., and Wescott, W. C., *J. Phys. Chem.*, **67**, 534 (1963).
[12] Liberman, E. A., and Topaly, V. P., *Biofizika* (USSR), **14**, 562 (1969).
[13] Moore, C., and Pressman, B. C., *Biochem. Biophys. Res. Commun.*, **15**, 562 (1964).
[14] Chappell, J. B., and Crofts, A. R., in *Regulation of Metabolic Processes in Mitochondria* (edit. by Tager, J. M., Papa, S., Quagliarello, E., and Slater, E. C.), 293 (Elsevier, Amsterdam, 1966).
[15] Mitchell, P., and Moyle, J., in *Biochemistry of Mitochondria* (edit. by Slater, E. C., Kaniuga, Z., and Wojtczak, Z.), 53 (Academic Press, London, 1967).

Triallelic Complementation and the Subunit Structure of Enzymes

Interallelic complementation makes a genetical approach to the problem of enzyme structure possible. It is expressed as the partial to complete restoration of the normal phenotype in heterocaryons or heterozygotes derived from different allelic mutants. Detection of complementation between alleles of any specific gene suggests that such a gene controls an enzyme with quaternary structure. Moreover, this effect shows that the enzyme contains identical subunits.

Complementation maps are helpful in estimating the degree of structural damage to the protein subunits synthesized under the control of the complementing alleles. Modern theory of interallelic complementation[1] arises from data obtained chiefly from investigations using microorganisms. All complementation maps so far constructed have been based on the results of interactions of pairs of alleles.

Although it is known that most enzymes with a quaternary structure consist of more than two identical subunits, there are no complementation data which reflect the degree of complexity found in the quaternary structure of enzymes.

The presence of more than two identical subunits in the structure of an enzyme can be recognized provided some modifications are made in the usual complementation test. It may be possible to study the interaction of more than two types of identical subunits differing in their mutational damage, that is, to study complementation between more than two heteroalleles simultaneously. It might be possible to obtain positive complementation between three alleles, for example, which fail to complement each other in pairs, or, vice versa, to obtain negative complementation between three heteroalleles which are capable of complementation in pairs, provided the enzyme under consideration is composed of more than two identical subunits.

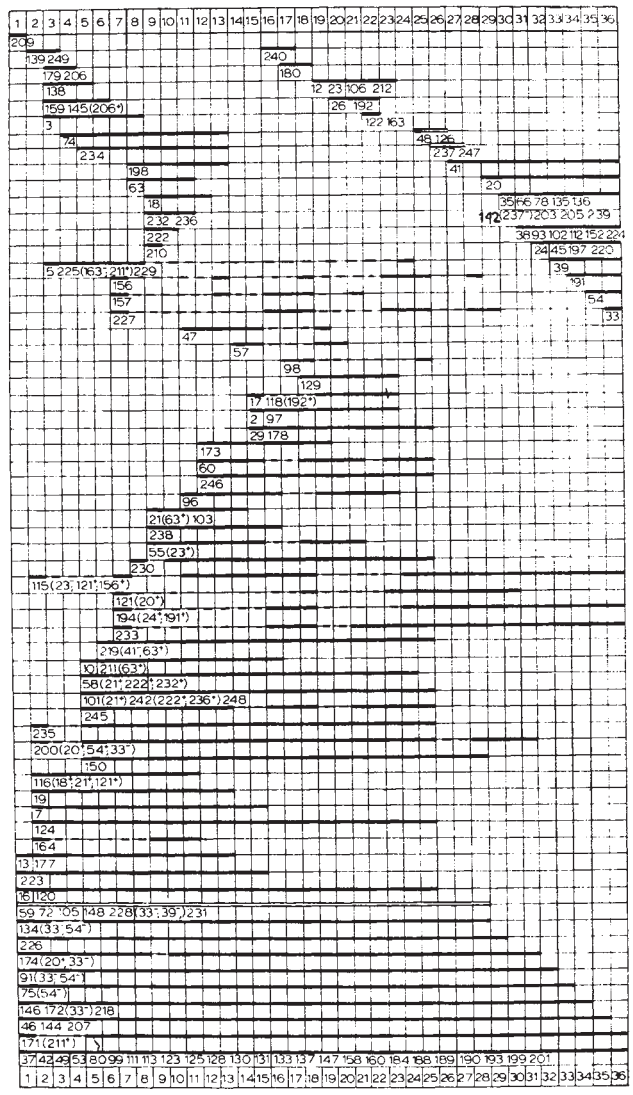

Fig. 1. Complementation map of the ad_2 locus in *S. cerevisiae*[8] constructed from data on interactions between pairs of alleles. Overlapping and non-overlapping lines correspond to non-complementation and complementation, respectively. Dotted lines represent deviations from linearity on this map. Figures at the top and bottom of the complementation map show the numbers of complons. Some of the results of the complementation test could not be represented on this map as overlapping or non-overlapping lines. Such cases are listed in brackets near the corresponding allele number. For example, mutant 219 [complons: 6–25] cannot complement with mutant 41, although their functional defects are represented as non-overlapping ones. At the same time mutant 219 does complement with mutant 63, although the functional defect of the latter overlaps the functional defect of 219 on this map. This contradiction is shown as follows: 219 (41-63+). Mutations 48, 237 and 105 which were used in the triploid complementation experiments are shown by an open line, =.

Different genetical systems can be used to estimate the number of identical subunits in the protein *in vivo* through the study of multiallelic complementation; we shall mention three. These are multiple heterokaryons in fungi[2], phage-bacterium systems with multiple infection (A. Maysurian, personal communication) and the system of polyploid yeast which appears to have some advantages. A polyploid series in yeast can be easily developed[3,4].