BOT may interact with some membrane component involved in the mechanism of exocytosis in cholinergic nerve terminals.

Note added in proof: Pumplin and Reese (*18*), on the basis of freeze-fracture studies of frog neuromuscular junction treated with BOT, brown widow spider venom, and calcium ionophores, suggest that BOT may block a step in the release of ACh after the stage of entry of cations into nerve terminal.

ING KAO, DANIEL B. DRACHMAN
Department of Neurology, Johns Hopkins Medical School, Baltimore, Maryland 21205

DONALD L. PRICE
Departments of Neurology and Pathology, Johns Hopkins Medical School, Baltimore, Maryland 21205

References and Notes

1. C. Lamanna, *Science* **130**, 763 (1969).
2. A. C. Guyton and M. A. MacDonald, *Arch. Neurol. Psychiat.* **57**, 578 (1947); V. B. Brooks, *J. Physiol. (London)* **123**, 501 (1954); L. L. Simpson, in *Neuropoisons: Their Pathophysiological Actions*, L. L. Simpson, Ed. (Plenum, New York, 1971), p. 303.
3. A. S. V. Burgen, F. Dickens, L. J. Zatman, *J. Physiol. (London)* **109**, 10 (1949).
4. C. O. Hebb, K. Krnjević, A. Silver, *ibid.* **171**, 504 (1964); D. Elmqvist and D. M. J. Quastel, *ibid.* **179**, 389 (1965); L. T. Potter, *ibid.* **206**, 145 (1970).
5. P. Fatt and B. Katz, *ibid.* **117**, 109 (1952); J. del Castillo and B. Katz, *ibid.* **124**, 560 (1954); B. Katz and R. Miledi, *Proc. R. Soc. London Ser. B* **161**, 453 (1965); *J. Physiol. (London)* **224**, 665 (1972).
6. B. Katz, *The Release of Neural Transmitter Substances* (Liverpool Univ. Press, Liverpool, 1969), p. 601; D. Elmqvist and D. S. Feldman, *J. Physiol. (London)* **181**, 487 (1965).
7. R. Birks, H. E. Huxley, B. Katz, *J. Physiol. (London)* **150**, 134 (1960); R. Couteaux and M. Pecot-Dechavassine, *C. R. Acad. Sci. Ser. D* **271**, 2346 (1970); J. E. Heuser and T. S. Reese, *Anat. Rec.* **172**, 329 (1972); B. Ceccarelli, W. P. Hurlbut, A. Mauro, *J. Cell Biol.* **54**, 30 (1972); F. Dreyer, K. Peper, K. Akert, C. Sandri, H. Moore, *Brain Res.* **62**, 373 (1973); J. E. Heuser, T. S. Reese, D. M. D. Landis, *J. Neurocytol.* **3**, 109 (1974); M. H. Ellisman, J. E. Rash, L. A. Staehelin, K. R. Porter, *J. Cell Biol.* **68**, 752 (1976).
8. A. J. Harris and R. Miledi, *J. Physiol. (London)* **217**, 497 (1971).
9. O. A. Trowell, *Exp. Cell Res.* **16**, 118 (1959).
10. M. J. Karnovsky, *J. Cell Biol.* **35**, 213 (1967).
11. Botulinum toxin, type A, is a gift from Dr. E. J. Schantz, University of Wisconsin; E. J. Schantz, in *Botulinum*, K. H. Lewis and K. Cassel, Eds. (U.S. Public Health Service Publ. No. 999-FP-1, Washington, D.C., 1964), pp. 91–104.
12. N. Spitzer, *Nature (London) New Biol.* **237**, 26 (1972); D. A. Boroff, J. del Castillo, J. H. Evoy, R. A. Steinhardt, *J. Physiol. (London)* **240**, 227 (1974).
13. D. B. Foreman, J. L. Mongar, B. D. Gomperts, *Nature (London)* **245**, 249 (1973).
14. H. Kita and W. Van der Kloot, *ibid.* **250**, 658 (1974).
15. Black widow spiders were gifts from Dr. A. W. Clark of University of Wisconsin and Dr. T. Smythe, Jr., of Pennsylvania State University.
16. H. E. Longenecker, Jr., W. P. Hurlbut, A. Mauro, A. W. Clark, *Nature (London)* **225**, 701 (1970); M. Okamoto, H. E. Longenecker, Jr., W. F. Riker, Jr., S. K. Song, *Science* **172**, 733 (1971).
17. D. W. Pumplin and J. del Castillo, *Life Sci.* **17**, 137 (1975).
18. D. W. Pumplin and T. S. Reese, *Neurosci. Abstr.*, in press.
19. We thank Chi Yin Choy for assistance. Supported by NIH research grants 5 P01 NS 10920, 5R01 HD04817, NS 10580, and by an NIH academic career development award to I.K.

4 June 1976

Mitochondria: System for Prolonged Maintenance and Repeated Measurements of Energy-Linked Functions

Abstract. *A combination of long-lived mitochondria and a system equipped with gas and liquid exchange hollow fibers permits the maintenance and repeated assay of mitochondria under controlled conditions for periods in excess of 48 hours at room temperature.*

The quasi-autonomous nature of mitochondria reinforces the need to assess their intrinsic metabolic capabilities as expressed within the living cell. Relatively quick and versatile polarographic and spectrophotometric methods of assay have proved valuable in elucidating specific mitochondrial functions. However, extrapolation of these functions to the whole cell requires that they be examined over extended periods of time at or near equilibrium conditions that simulate the homeostatic milieu of a living cell.

Shown schematically in Fig. 1 is a prototype system that permits control over the mitochondrial milieu while allowing repeated assay for oxygen consumption and related energy-dependent functions of the same mitochondria. The essentials of the system are (i) a monitoring chamber equipped with polarographic O_2 electrode, stirrer, and rubber septum; (ii) peristaltic pump; (iii) gas permeator and liquid exchange hollow fibers; and (iv) inert tubing. The gas and chemical constituents of the circulating mitochondrial suspension can be maintained or moderated by manipulating the external gas and liquid medium with which the suspension is in near equilibrium. To measure O_2 consumption, a suitable O_2 partial pressure is established in the mitochondrial suspension, the flow interrupted, and the disappearance of O_2 from the fixed volume chamber determined by the classic polarographic technique (*1*). Adenosine diphosphate (ADP), cofactors, inhibitors, and so forth, may be added through the septum. In practice the flow pump is turned on again before the O_2 is depleted to avoid anoxia. Alternatively, a bypass can be installed parallel to the chamber so that a portion of the mitochondrial suspension is "trapped" for assay while the remainder continues to circulate (*2*).

A continuous recorder trace from a typical series of polarographic assays is shown in Fig. 2. A first and then a second portion of mitochondria were gently injected through the septum into the circulating reaction medium. Circulation was then stopped and ADP added to produce a state 3 oxidation rate. The pump was then turned on and air introduced into the circulating mitochondrial suspension through the gas permeator hollow fiber. Within a few minutes the flow was again stopped and ADP injected in the cham-

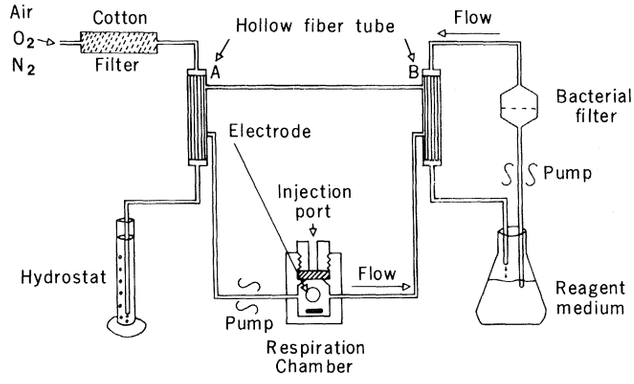


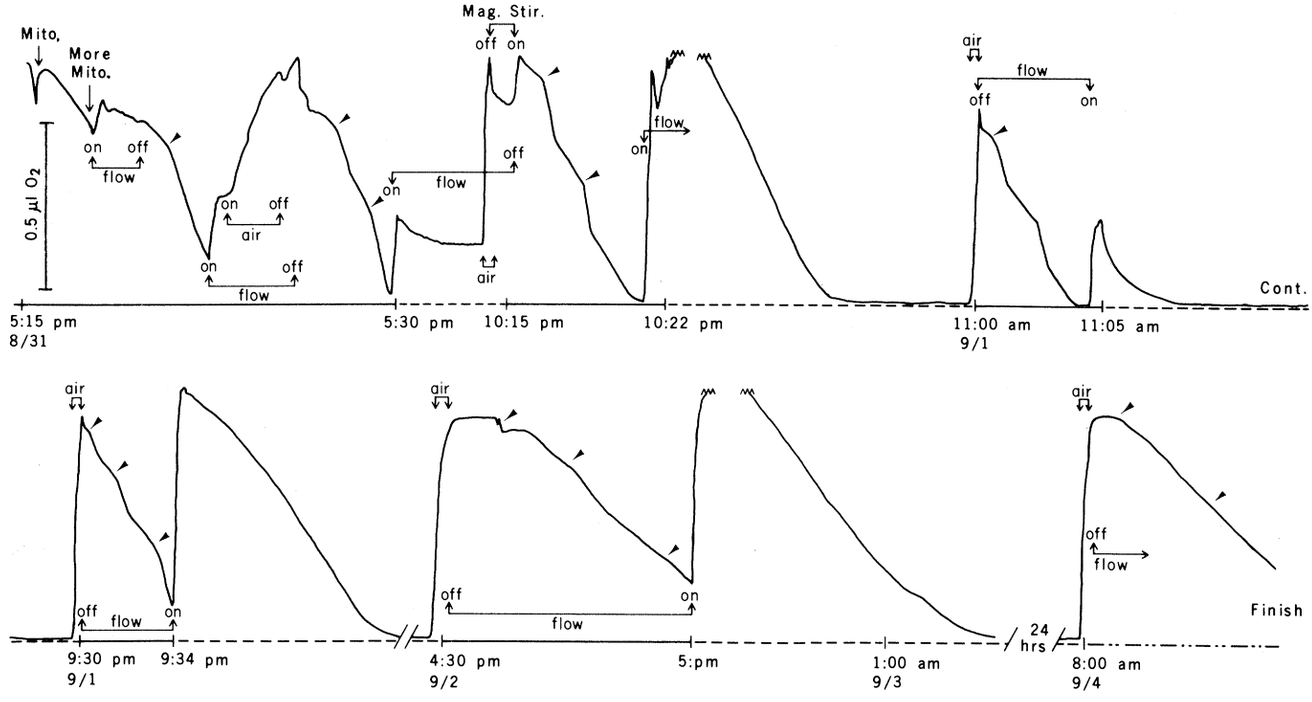
Fig. 1. Prototype mitochondrial maintenance system. Mitochondria suspended in the reagent medium described in the legend to Fig. 3 are circulated (0.5 to 1 ml/min) counterclockwise through the respiration chamber, around the exterior surfaces of liquid exchange (*B*) and gas permeator hollow fibers (*A*), and back to the pump. The mitochondrial suspension is in near equilibrium with a large volume (50 to 100 ml) of complete reaction medium (less mitochondria), which is pumped continuously in counterflow (0.5 to 1 ml/min) through the liquid exchange hollow fibers. A Millipore filter helps prevent occlusion of the liquid exchange hollow fibers (Bio-Rad 80), which have a nominal cutoff for molecules of molecular weight 30,000. The silicone rubber gas permeator membranes (Bio-Rad 5) are essentially nonporous to other than gas molecules. Gas flow through the permeator fibers is maintained at 5 to 10 ml/min against a hydrostatic head of about 20 cm. For more rapid aeration before assay, gas outflow through the permeator can be restricted and the pressure increased up to 8 pounds per square inch. A soft rubber septum at the chamber serves as an injection port. The volume of the closed system, which includes the 1.5-ml chamber, tubing of ⅛-inch inner diameter, and small volume hollow fibers (Bio-Rad π tubes) is approximately 6 ml. A smaller circulating volume but with slower gas exchange rates is achieved by replacing hollow fiber *B* with a dual-function gas and liquid transfer hollow fiber (Bio-Rad 80/5) and incorporating a gas permeator hollow fiber in the external medium flow just before it enters *B*.

ber to obtain additional state 3 and 4 rates. Aeration and polarographic assay were repeated 4½ hours later and at various intervals for the next 3 days. The partial pressure of O_2 was recorded (at a much slower chart speed) between assays.

Mitochondria that are reasonably long-lived are essential to the system. Avocado mitochondria (*3*) and beef heart mitochondria (*4*) have demonstrated this capacity. Results of experiments with both types of organelles are shown in Fig. 3. The avocado mitochondria had been isolated and held at 0°C for 24 hours before injection into the incubation medium. Repeat measurements of respiratory control demonstrated an improvement in this energy-linked function over the first 4 to 5 hours at 25°C. Consistent with evidence that the improvement of respiratory control is dependent on metabolic energy (*3*), the rate and degree of recovery was greatest for the organelles maintained under higher O_2 partial pressure. However, at low O_2 partial pressure some respiratory control was retained for a longer period, which raises the possibility that optimal maintenance of respiratory control may require variable or programmed levels of O_2. Freshly isolated beef heart mitochondria did not show an improvement in respiratory control. Two polarographic assays in close succession (15 and 16 hours after injection) reveal a rather sharp drop in respiratory control which may be attributed to injury as a result of too rapid or too extreme aeration.

In the present system, injury to the mitochondria accrues from shearing forces at the pump and elsewhere, and improvements in circulating device and hollow fiber design are called for. Other modifications could include the addition of monitors for *p*H, specific ions, spectrophotometric data, and so forth, placed in series along the mitochondrial stream.

More complex sensing and feedback loops can also be incorporated to maintain homeostatic conditions or to produce a programmed sequence of changes in ambience. Refinements in design and detailed study of multiple variables will be required to achieve optimal conditions for varying experimental objectives or types of mitochondria. Nonetheless, results obtained with the simple, prototype system illustrate its potential in studying the capacity of mitochondria to maintain energy-linked functions under controlled conditions.



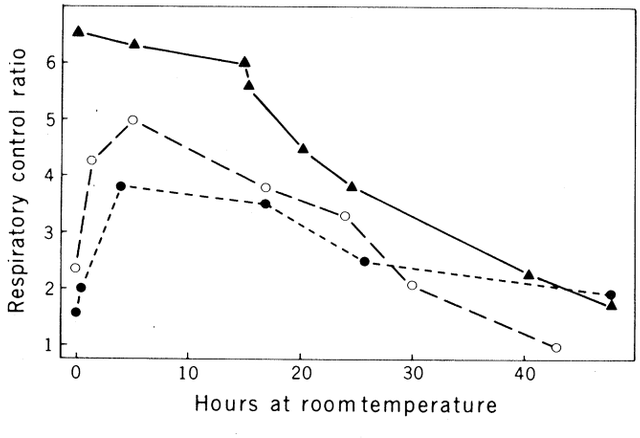


Fig. 2 (top). Several cycles of oxygen consumption and replenishment over a period in excess of 3 days. Chart speeds were 1 inch per 2 minutes (———) or 1 inch per 5 minutes (— — —) during the consecutive assays, and 1 inch per hour (----) in intervals between assays. Flow (*off* and *on*) refers to the flow (1 ml/min) of mitochondrial suspension. Arrows designate the additions of stoichiometric amounts of ADP. The volume of external reagent medium was 100 ml and its flow rate was also 1 ml/min; *Mito.*, mitochondria; *Mag. Stir.*, magnetic stirrer. *Cont.*; continued on next line. Fig. 3 (bottom). Changes in the respiratory control of circulating beef heart mitochondria (triangles) and avocado mitochondria supplied in the intervals between assay with air (open circles) or no gas (closed circles) at the gas permeator. For all three systems small (unmeasured) amounts of O_2 entered the mitochondrial flow via exchange with the external reagent medium, which was not deaerated. The medium for beef heart mitochondria consisted of 0.25*M* sucrose, 10 m*M* phosphate (*p*H 7.2), 5 m*M* (ethylenedinitrilo)tetraacetic acid, 10 m*M* α-ketoglutarate, 2.5 m*M* $MgCl_2$, and bovine serum albumin (0.66 mg/ml). The medium for avocado mitochondria consisted of 0.25*M* sucrose, 66 m*M* phosphate (*p*H 7.2), 1 μm $MgCl_2$, 33 μ*M* thiamine pyrophosphate, 3.3 μ*M* coenzyme A, 0.1 m*M* nicotinamide adenine dinucleotide, 10 m*M* α-ketoglutarate, and chloramphenicol (100 μg/ml) (*5*).

Both beef heart and avocado mitochondria have exhibited some residual respiratory control after 48 hours of continuous circulation at room temperature. A priori there is no reason to suspect that the mitochondria should not maintain energy-linked functions for extended periods of time if presented with a favorable ambience. With improvements in system design and by successive approximations of the milieu that will sustain functional integrity, it should be possible to define the true extent of mitochondrial autonomy or, conversely, to determine the extent of mitochondrial-cytoplasm interdependency in normal, stressed, or diseased cells.

ROGER J. ROMANI
Department of Pomology,
University of California,
Davis 95616

References and Notes

1. B. Chance and G. Williams, *J. Biol. Chem.* **217**, 383 (1955).
2. When placed in circulation, the assay additives quickly disperse through the entire mitochondrial suspension. Subsequent near-equilibrium concentrations depend on the volume and renewal frequency of the external medium. A novel method to achieve rapid changes in mitochondrial milieu has been described [B. Arkles and W. Bringar, *J. Biol. Chem.* **250**, 8856 (1975)] wherein the mitochondria are immobilized on alkyl-silyated glass surfaces.
3. R. Romani, S. E. Tuskes, S. Ozelkok, *Arch. Biochem. Biophys.* **164**, 743 (1974).
4. M. Goldblatt and R. Romani (in preparation) have obtained long-lived beef heart mitochondria by a minor modification of established isolation procedures [G. Hogeboom, *Methods Enzymol.* **1**, 16 (1955)].
5. Chloramphenicol provides control of bacterial growth only for the first 24 hours. Evidence from noncirculating "batch" incubations of mitochondria (*3*) suggests that proliferation of bacteria is not limiting to maintenance of RC. More definitive experiments, including those under way to determine the course of protein synthesis, call for completely sterile systems.
6. The valuable assistance of S. E. Tuskes and M. Goldblatt is gratefully acknowledged.

16 March 1976; revised 26 May 1976

Cytokine Inhibition of DNA Synthesis: Effect on Cyclic Adenosine Monophosphate in Lymphocytes

Abstract. *A greater than twofold increase of intracellular adenosine 3',5'-monophosphate (cyclic AMP) inhibits DNA synthesis in stimulated rat lymphocytes. A two- to fourfold rise of intracellular cyclic AMP, starting at 16 hours, was produced by purified inhibitor of DNA synthesis added to such cells either at 0 or 16 hours, in close association with the initiation of DNA synthesis.*

Helper and suppressor lymphocytes, which play a role in regulating immune responses (*1*), produce various diffusible substances (immunoregulatory lymphokines) which may be the actual mediators of lymphocyte cooperation and suppression (*2*). We have investigated the properties of a mediator of nonspecific suppression designated inhibitor of DNA synthesis (IDS), which is a glycoprotein with a molecular weight of approximately 80,000. This glycoprotein inhibits DNA synthesis of lymphocytes and other cells while sparing other forms of macromolecular synthesis, and is quite distinct from lymphotoxin, a proliferation inhibitory factor (PIF), and a cloning inhibitory factor (*3–5*). It is a product of T cells, being made by thymocytes and peripheral T lymphocytes stimulated with T-cell mitogens; it is produced in large amounts often when nonspecific suppressor cell activity is enhanced (*6*). We now report our studies on how IDS might act by way of a second messenger such as cyclic adenosine monophosphate (AMP).

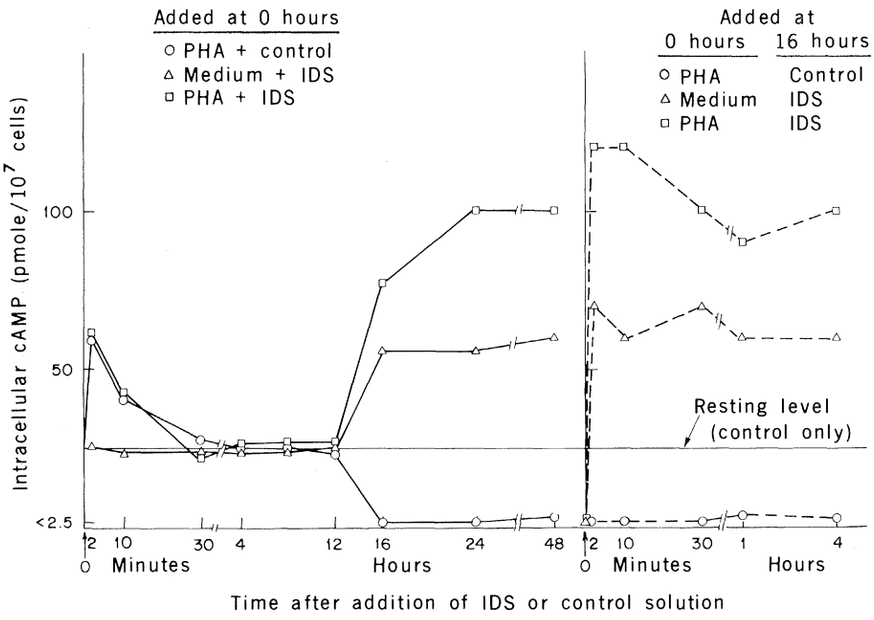


Fig. 1. Changes in cyclic AMP (*cAMP*) in rat lymph node cultures stimulated with phytohemagglutinin (PHA) with or without addition of IDS at 0 or 16 hours. Resting levels (cells incubated in medium alone or with added control solution) varied between 24 and 26 pmole/10^7 cells. Standard deviation of the cyclic AMP assay was always < 2.0 pmole/10^7 cells.

In most types of rapidly dividing cells, intracellular cyclic AMP concentrations are low, while those in many differentiated nondividing cells is high. A number of lines of evidence suggest that intracellular cyclic AMP may actually regulate cell proliferation, with high concentrations acting to inhibit DNA synthesis while lower ones promote it (*7*). It has been proposed that a similar system is operative in lymphocytes stimulated with antigen or lectins (*8*); the relationship in these cells, however, is much less clear-cut. Lymphocytes stimulated with optimum concentrations of concanavalin A show an immediate rise of cyclic AMP, reaching a peak within 2 minutes (*8, 9*). The relation of this early change in cyclic AMP level to DNA synthesis, which occurs many hours later, is not understood. Addition of exogenous cyclic AMP, dibutyryl cyclic AMP, or cholera toxin—all of which raise intracellular cyclic AMP levels—inhibits the DNA synthesis of lectin-treated lymphocytes (*10*). Addition of cyclic GMP (guanosine monophosphate) to lymphocyte cultures promotes DNA synthesis and cell proliferation, and it has been suggested that the critical factor determining whether a cell proceeds to a proliferative or differentiated state may be the intracellular ratio of these two nucleotides (*11*). This suggestion is not universally accepted (*12*).

The IDS was prepared as described (*3*). Briefly, normal DA (Dark August) rat lymph node cells (5×10^6/ml) were cultured with 2.0 μg of concanavalin A (Difco) per milliliter in 10 percent fetal calf serum (Microbiological Associates) in RPMI 1640 medium with Pen-Strep (Gibco) for 48 hours, then washed and recultured without concanavalin A. The 24- and 48-hour supernatants from the cultures were pooled, concentrated 25-fold (Amicon filtration chamber, UM-10