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).

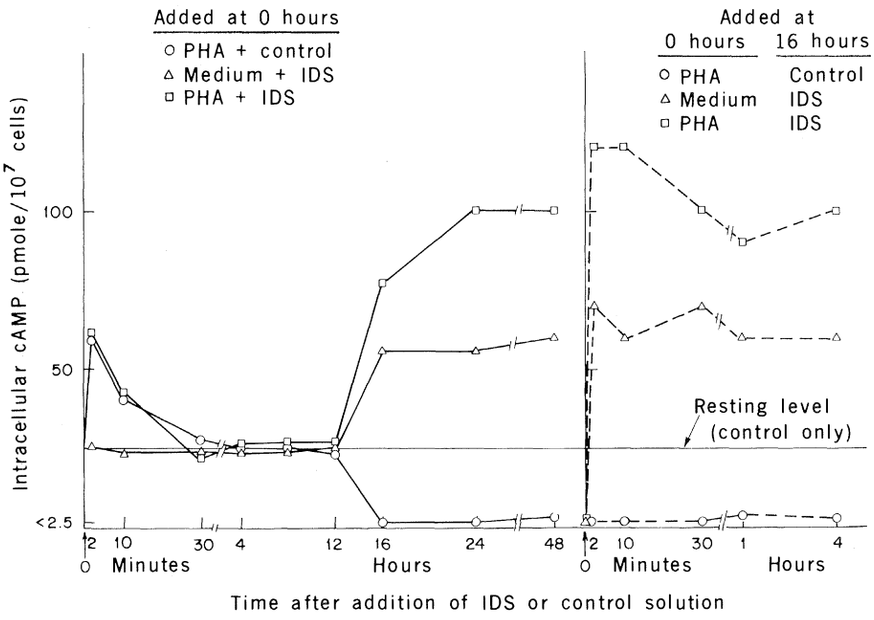

Fig. 1. Changes in cyclic AMP (*cAMP*) in rat lymph node cultures stimulated with phytohemagglutinin (PHA) with or without addition of IDS at 0 or 16 hours. Resting levels (cells incubated in medium alone or with added control solution) varied between 24 and 26 pmole/10^7 cells. Standard deviation of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

membrane), and filtered through Sephadex G-100 (Pharmacia). Pooled fractions containing IDS were chromatographed on DEAE cellulose (Bio-Rad) and eluted with increasing concentrations of sodium chloride; the active fractions were pooled and dialyzed against Fischer's medium. Activities of migration inhibitory factor, lymphotoxin, PIF, or mitogenic factor were not detectable in the purified IDS (*4*). Control supernatants, in which the concanavalin A was added only at the end of culture, were processed in the same way.

In a first series of experiments, cyclic AMP (Sigma) or dibutyryl cyclic AMP (Sigma) was added in various concentrations at 0 time to microplate (Linbro) cultures of normal DA rat lymph node cells (5×10^5 cell/0.2 ml per well) with 1 μl of phytohemagglutinin-P (PHA-P; Difco) in 10 percent fetal calf serum in RPMI 1640 medium with Pen-Strep. The cultures were harvested at 28 hours, after a 4-hour treatment with ^{3}H-labeled thymidine (New England Nuclear; 1.0 μc per well). Both nucleotides inhibited induced DNA synthesis (Table 1), as has been reported (*10*). The inhibition was maximum with added cyclic AMP at $10^{-3}M$ and dibutyryl cyclic AMP at $5 \times 10^{-4}M$. In this same experiment, with both nucleotides in inhibitory concentrations, the percentage of blast cells was decreased from approximately 38 to 9 percent of surviving cells at 28 hours.

Intracellular cyclic AMP was determined in comparable cultures set up in triplicate in glass tubes (12 by 75 mm), by the use of a commercially available kit (Schwarz/Mann) (*13*), the cells being washed thoroughly before assay. Complete (70 to 100 percent) inhibition of ^{3}H-labeled thymidine incorporation was associated with concentrations of added nucleotide that produced more than a twofold increase in intracellular cyclic AMP, and substantial inhibition was associated with smaller increases in cyclic AMP. Controls in each assay included the following: (i) all media used were tested for interference with the assay; (ii) known amounts of ^{3}H-labeled cyclic AMP were exposed to the standard purification procedure (if extraction was less than 60 percent, the experiment was discarded); and (iii) the product was treated with phosphodiesterase (Sigma) to show the cyclic AMP activity assayed was completely destroyed.

In further experiments, concentrated, purified IDS was added to similar cultures at a final concentration of 50 percent either simultaneously with the addition of phytohemagglutinin or 16 hours later. In our system, DNA synthesis began at about 16 hours (*3*). Addition of IDS at the start of culture resulted in complete suppression of later DNA synthesis, as judged by the uptake of ^{3}H-labeled thymidine (Table 2). In comparable cultures assayed for intracellular cyclic AMP, an early increase was observed in all cultures stimulated with mitogen; a peak was reached at 2 minutes, with a return to baseline values in 20 minutes (Fig. 1). In cells exposed to IDS the twofold rise in intracellular cyclic AMP at 16 hours was more than enough to account for the inhibition of DNA synthesis observed; in cells not exposed to IDS, the cyclic AMP declined. This rise was sustained until the end of the culture period (48 hours). The addition of IDS at 16 hours produced an immediate rise in intracellular cyclic AMP (Fig. 1) and a prompt arrest of DNA synthesis (Table 2).

Table 1. Inhibition of DNA synthesis in PHA-stimulated rat lymph node cells by exogenous cyclic AMP and dibutyryl cyclic AMP: relation to intracellular cyclic AMP. Cultures of 5×10^5 cells, 1.0 μl of PHA in 0.2-ml volumes, were harvested at 28 hours after 4 hours of treatment with 1 μc of ^{3}H-labeled thymidine. The background radioactivity was 8749 count/min; the PHA-stimulated incorporation was 24,307 count/min. The percentage inhibition was calculated as 100 − (count/min), calculated as the percentage of the number of counts per minute in PHA-stimulated cultures. The standard deviation of cyclic AMP assay was always less than 2.0 pmole/10^7 cells.

Molarity	Intracellular cyclic AMP (pmole/10^7 cells)	Inhibition of DNA synthesis (%)
	Dibutyryl cyclic AMP	
10^{-3}	50	92.3
5×10^{-4}	48	94.4
	Cyclic AMP	
10^{-2}	50	84
5×10^{-3}	48	84
2.5×10^{-3}	46	80
10^{-3}	49	72
	Dibutyryl cyclic AMP	
2.5×10^{-4}	28	62
10^{-4}	27	65
5×10^{-5}	25	41
	Cyclic AMP	
5×10^{-4}	27	56
	Dibutyryl cyclic AMP	
2.5×10^{-5}	27	12
	Cyclic AMP	
2.5×10^{-4}	20	33
10^{-4}	17	0
	Cells only	
	19–21	

Our results suggest that increased intracellular cyclic AMP may mediate (directly or indirectly) the inhibitory effect of IDS on DNA synthesis. The measurements recorded in Table 1 may have been inaccurate if any added (exogenous) nucleotide remained adherent to the surface of the cells. Such an error, however, would not negate the suggestion made here, since the rise in cyclic AMP, produced by exposing the cells to IDS (Fig. 1) exceeded the maximum measured in cells exposed to cyclic AMP or dibutyryl cyclic AMP. That the activity measured is actually cyclic AMP was verified by its sensitivity to phosphodiesterase. The marked reduction in blast cells in the cultures exposed to inhibitory concentrations of nucleotide rules out the possibility that reduced uptake of ^{3}H-labeled thymidine may have been an artifact resulting from diminished transport of the exogenous thymidine (*14*).

An unexplained finding was that intracellular cyclic AMP rose only at 16 hours, whether IDS was added to the system at 0 or 16 hours. This agrees with the fact that the inhibitory activity of IDS remains completely reversible until 16 hours (*3*). This finding implies some

Table 2. Inhibition of DNA synthesis in PHA-stimulated rat lymph node cells by IDS added at different times. The cultures (5×10^5 cells; 1 μl of PHA in an 0.2-ml volume) were treated with 1 μc of ^{3}H-labeled thymidine for the final 4 hours before harvesting. Duplicate values are shown.

Time of cell harvest (hr) after addition of PHA	Uptake of ^{3}H-labeled thymidine (10^3 count/min)				
	Addition at 0 hours		Addition at 16 hours		Background (No PHA)
	IDS	Control	IDS	Control	
12	4.5	3.9			3.8
	3.7	5.3			4.1
16	4.8	4.8			4.3
	5.6	6.6			4.0
20	3.8	16.7	12.0	18.3	3.8
	3.6	14.2	10.4	18.4	4.3
24	4.7	82.5			3.3
	5.5	78.5			3.1
48	7.9	100.2	9.4	89.7	3.0
	6.4	97.0	8.6	94.5	3.7

change in receptivity of the cell associated with the initiation of DNA synthesis, possibly expressed as the appearance of new receptors for IDS or a change in permeability of the cell membrane with altered accessibility of the cell's adenylate cyclase. Models for these alternatives have been described in other systems. For example, synchronized cultures of melanocytes show the appearance of specific cell surface receptors for melanocyte-stimulating hormone (which activates adenylate cyclase) at a precisely defined time during the G2 phase of growth (*15*). Added cyclic AMP, however, can act at any time during the cell cycle. Pardee has recently identified a "restriction point" during G1, in synchronized BHK cells; at this point nutritional factors either stimulate or inhibit cell proliferation in association with changes in intracellular cyclic AMP (*16*). Again, elevation of cyclic AMP, for example, by addition of $2 \times 10^{-3}M$ dibutyryl cyclic AMP, was effective at any time during the cycle.

BRIAN V. JEGASOTHY
ANDREW R. PACHNER
BYRON H. WAKSMAN

Department of Pathology,
Yale University School of Medicine,
New Haven, Connecticut 06501

References and Notes

1. J. F. A. P. Miller and G. F. Mitchell, *Transplant. Rev.* **1**, 3 (1969); H. N. Claman and E. A. Chaperon, *ibid.*, p. 92; R. K. Gershon, *Contemporary Topics in Immunobiology*, M. D. Cooper and N. L. Warner, Eds. (Plenum, New York, 1974), chap. 1; G. Moller, Ed., *Transplant. Rev.* **26** (1975).
2. M. Feldmann, *J. Exp. Med.* **136**, 737 (1972); ——— and A. Basten, *ibid.*, p. 49; B. H. Waksman and Y. Namba, *Cell Immunol.* **21**, 161 (1976).
3. Y. Namba and B. H. Waksman, *Inflammation* **1**, 5 (1975).
4. ———, *J. Immunol.* **115**, 1018 (1975).
5. B. T. Spofford, R. A. Daynes, G. A. Granger, *ibid.* **112**, 2111 (1974).
6. Y. Namba, B. V. Jegasothy, B. H. Waksman, in preparation.
7. J. Otten, G. S. Johnson, I. Pasten, *Biochem. Biophys. Res. Commun.* **44**, 1192 (1971); J. R. Sheppard, *Nature (London) New Biol.* **236**, 14 (1972); W. L. Ryan and M. L. Heidrick, *Adv. Cyclic Nucleotide Res.* **3**, 81 (1974).
8. H. J. Wedner and C. W. Parker, *Prog. Allergy* **20**, 195 (1976).
9. J. W. Smith, A. L. Steiner, W. M. Newberry, Jr., C. W. Parker, *J. Clin. Invest.* **50**, 432 (1971a).
10. R. Hirschhorn, *Cyclic AMP, Cell Growth and the Immune Response* (Springer, New York, 1973).
11. J. Watson, R. Epstein, M. Cohn, *Nature (London)* **246**, 405 (1973).
12. H. J. Wedner, R. Danker, C. W. Parker, *J. Immunol.* **115**, 1682 (1975).
13. A. L. Steiner, D. M. Kipnis, R. Utiger, C. Parker, *Proc. Natl. Acad. Sci. U.S.A.* **64**, 367 (1969).
14. P. V. Hauschka, L. P. Everhart, R. W. Rubin, *ibid.* **69**, 3542 (1972).
15. J. M. Varga, A. DiPasquale, J. Pawelek, J. S. McGuire, A. B. Lerner, *ibid.* **71**, 1590 (1974).
16. A. B. Pardee, *ibid.*, p. 1286.
17. Supported by grants AI 06112 and AI 06455 and by contract CB 43926 from the National Institutes of Health and a fellowship from the Dermatology Foundation to B.V.J.

2 February 1976

Physical Dependence on Opiate-Like Peptides

Abstract. *Methionine-enkephalin and β-endorphin, endogenous peptides with activities similar to those of opiates, were infused for 70 hours into the periaqueductal gray–fourth ventricular spaces of the rat brain. When challenged with a naloxone, a specific opiate antagonist, these animals manifested a typical morphine-like withdrawal syndrome. These results show that such peptides can cause physical dependence.*

Physical dependence on opiates is generally characterized by abstinence behavior when opiate intake is abruptly terminated or when an opiate antagonist is administered (*1*). A number of investigators using bioassays in mouse vas deferens and guinea pig ileum and stereospecific binding to purified brain extracts have discovered brain and hypophyseal peptides with opiate-like activities (*2*). The amino acid sequences of several opiate-like peptides, namely methionine- and leucine-enkephalin and α- and β-endorphin, are known and have been synthesized (*3*). Synthetic methionine-enkephalin has some central analgesic activity of short duration (*4*), whereas β-endorphin, which contains methionine-enkephalin as part of its first five NH_2-terminal residues, is, on a molar basis, at least ten times more active centrally than morphine (*5*). In considering the endogenous functions of these peptides and the development of these peptides as analgesics, it is important to determine whether opiate-like peptides can cause physical dependence (*6*). We describe here a novel method of long-term, localized, drug infusion into the brain which has enabled us to demonstrate that long-term exposure to methionine-enkephalin and β-endorphin can result in physical dependence.

Male Sprague-Dawley rats (220 to 380 g) were anesthetized with sodium pentobarbital (50 mg/kg) injected intraperitoneally; L-shaped steel cannulas, made from 21-gauge disposable needles filed to a predetermined length, were implanted into the frontal cortex or periaqueductal gray region of the rat brain (*7*). The implanted cannula, filled previously with distilled water, was secured to the skull with dental cement. To deliver drugs into the brain an osmotic minipump was utilized (*8*). The minipump, a system capable of delivering a small volume at a constant rate, was filled with

Table 1. Chronic infusion of morphine sulfate into the rat brain and the development of physical dependence.

Brain area	Morphine sulfate infusion (μg/μl)	Total estimated dose delivered in 70 hours* (μmole)	*N*	Animals showing withdrawal sign (%)		
				Teeth chattering	Escape responses	Wet shakes
Periaqueductal gray (minipump not connected to brain)	10	1.928	8	0	0	0
Frontal cortex	10	1.928	6	83	0	0
Periaqueductal gray	10	1.928	4	100	100	75
Periaqueductal gray	2.5	0.482	7	100	86	29

*Minipump flow rates of 0.92 ± 0.06 μl/hour.

Table 2. Continuous infusion of opiate-like peptides into the periaqueductal gray of the rat brain and the development of physical dependence; Met, methionine.

Chemical	Concentration of chemical in infusion (μg/μl)	Total estimated dose delivered in 70 hours* (μmole)	*N*	Animals showing withdrawal sign (%)		
				Teeth chattering	Escape responses	Wet shakes
Distilled water			4	0	0	0
Morphine sulfate	1.64	0.481	8	100	100	37
Met-enkephalin	9.00	1.537	7	100	86	14
Met-enkephalin	0.83	0.140	8	62	37	0
β-Endorphin	0.67	0.019	7	100	86	0
β-Endorphin	0.10	0.003	9	67	11	22

*Minipump flow rates of 1.40 ± 0.04 μl/hour.