(5), it was concluded that the lower percentage of SL's in MLR's as compared to nonmixed cultures reflected a lower number of such cells.

After being exposed to ^{125}I-labeled antibody to Ig, cells from both mixed and nonmixed cultures were found to be labeled or Ig-positive (Ig+). In nonmixed cultures, more than 90 percent of the Ig+ cells were SL's, the remainder being LML's. As shown in Table 1, these Ig+ SL's and Ig+ LML's accounted for approximately 10 percent of the total population of SL's and 33 percent of the total population of LML's, respectively. In MLR's, Ig+ SL's were also present but, unlike nonmixed controls, Ig+ blasts (Fig. 1) together with Ig+ LML's comprised over half the total population of Ig+ cells. As indicated in Table 1, these Ig+ SL's, Ig+ blasts, and Ig+ LML's represented approximately 10 percent of all SL's, 20 percent of all blasts, and 53 percent of all LML's, respectively, that were present in MLR's. By considering the fact that, in MLR's as compared to nonmixed controls, the total population of SL's was less, and the total populations of blasts and LML's were greater, these percentage values indicate that (i) there were fewer Ig+ SL's in MLR's than in nonmixed cultures and (ii) the numbers of Ig+ blasts and the numbers of Ig+ LML's had increased in MLR's over the numbers present in nonmixed controls.

From the foregoing experiments it is evident that the response of SL's in the MLR resulted in the appearance of a significant number of Ig+ blast cells. Although the B or T cell origin of such blasts could not be determined by the methods used in this study, it seems reasonable to propose that they represented transformed B cells rather than transformed T cells for several reasons.

1) In MLR's containing Ig+ blast cells there was a lower proportion of Ig+ SL's than in nonmixed cultures. This observation may indicate that the population of Ig+ SL's became reduced as such cells became transformed into Ig+ blast cells.

2) Since B cells have the capacity to undergo blastogenesis, as in the response to pokeweed mitogen (*11*), it is not unreasonable to predict that such cells might also be able to transform in response to allogeneic cells.

3) For T cells to have become Ig+ blast cells, surface receptors for antibody to Ig must have been revealed or acquired during the process of blastogenesis. Although this possibility cannot be excluded, it is believed unlikely that the number of T cells which became labeled with antibody to Ig could account for the proportion of Ig+ blasts that we observed.

Table 1. Percentages of small lymphocytes (SL), large and medium lymphocytes (LML), and blast cells labeled with ^{125}I-antibody to Ig. Nonmixed cultures contained either Lewis or F_1 (Lewis × BN) TDL cells. Mixed cultures were prepared with equal numbers of Lewis and F_1 (Lewis × BN) TDL cells. All cultures were incubated for 72 hours at 37°C before being exposed to antibody to Ig.

Labeled cell	Percentage labeled	
	Nonmixed	Mixed
SL	10	11
LML	33	53
Blasts	0	20

Finally, the conclusion that Ig+ blasts arose from B cells is consistent with several other studies which have suggested that B cells respond in the MLR (*4*).

Unlike these previous studies, however, results of the present investigation indicated by means of direct visual observation that B cells from the TDL of rats transformed in the MLR and that such transformation made a significant contribution to the total MLR response.

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References and Notes

1. B. Bain, M. R. Vas, L. Lowenstein, *Blood* **23**, 108 (1964).
2. M. R. Schwarz, *Am. J. Anat.* **121**, 559 (1967).
3. J. M. Johnston and D. B. Wilson, *Cell. Immunol.* **1**, 430 (1970); D. Mosier and H. Cantor, *Eur. J. Immunol.* **1**, 459 (1971); W. T. Weber, *Cell. Immunol.* **12**, 487 (1974).
4. T. L. Vischer, *Clin. Exp. Immunol.* **11**, 523 (1972); P. F. Piguet and P. Vassalli, *J. Exp. Med.* **136**, 962 (1972).
5. P. Häyry, L. C. Andersson, S. Nordling, M. Virolainen, *Transplant. Rev.* **12**, 91 (1972).
6. J. F. A. P. Miller, A. Basten, J. Sprent, C. Cheers, *Cell. Immunol.* **2**, 469 (1971); E. M. Lance, *ibid.*, p. 383; M. C. Gelfand, *ibid.* **11**, 221 (1974).
7. R. W. Dutton, *J. Exp. Med.* **122**, 759 (1965).
8. W. D. Perkins, M. J. Karnovsky, E. R. Unanue, *ibid.* **135**, 267 (1972).
9. F. C. Greenwood, W. M. Hunter, J. S. Glover, *Biochem. J.* **89**, 114 (1963).
10. M. R. Schwarz and W. O. Rieke, *Anat. Rec.* **155**, 493 (1966).
11. G. Janossy and M. F. Greaves, *Clin. Exp. Immunol.* **9**, 483 (1971).
12. We thank S. Carlson for technical assistance and Professor N. B. Everett for critical analysis of this manuscript. Supported by PHS grant AI 07509.

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Induction of Neural Differentiation in Cultures of Amphibian Undetermined Presumptive Epidermis by Cyclic AMP Derivatives

Abstract. *Induction of neural differentiation in cultures of undetermined presumptive epidermis from three amphibian species was achieved by the addition of 1 millimolar dibutyryl adenosine 3′,5′-monophosphate, 8-bromoadenosine 3′,5′-monophosphate, or adenosine C′,E′-monophosphate together with theophylline. Adenosine 5′-monophosphate, adenosine 2′,3′-monophosphate, dibutyryl guanosine 3′,5′-monophosphate, and butyrate at 1 millimolar are ineffective. These results suggest that the action of the primary inductor or inductors may be mediated via adenosine 3′,5′-monophosphate.*

Since the report of Spemann (*1*) that the dorsal blastoporal lip of developing amphibian embryos acts as an inductor (primary organizer/primary inductor) to determine the differentiation of the neural plate, the identity(s) and mode(s) of action of the inductor(s) have been the subject of intensive research [for review, see (*2*)]. The dorsal lip as well as tissue extracts and numerous other substances have been shown to induce neural differentiation in explants and in cells in culture. However, the best evidence to date suggests that the primary inductors are likely to be proteins [for review, see (*3*)]. In this respect, these inductors resemble polypeptide hormones in that they are proteins that serve the function of intercellular messengers. Since the protein hormones are generally believed not to enter the target cell but to act only on the membrane of the target cells, and since adenosine 3′,5′-monophosphate (c-AMP) is known to be the second messenger of a variety of hormones, we undertook to test whether the primary inductor(s) also acts via the second messenger c-AMP. We wish to report here that dibutyryl adenosine 3′,5′-monophosphate (dibutyryl-c-AMP), 8-bromoadenosine 3′,5′-monophosphate (8-Br-c-AMP), and c-AMP with theophylline can indeed induce undeter-

mined cells to differentiate into derivatives of neural ectoderm and neural crest. Other related nucleotides, 5′-AMP, 2′,3′-AMP, cyclic dibutyryl guanosine monophosphate (dibutyryl-c-GMP), and butyrate are ineffective.

Explants of presumptive epidermis of three species of amphibians, *Xenopus laevis, Pleurodeles waltili,* and *Siredon mexicanum,* were excised as soon as the dorsal lip became visible. These explants were incubated essentially as described by Niu and Twitty (*4*) as modified by Barth and Barth (*5*). Explants were incubated in the following media: Niu-Twitty solution (NT) plus dibutyryl-c-AMP, 8-Br-c-AMP, c-AMP with or without theophylline, or theophylline alone. Control explants were incubated in the following media: NT alone, NT plus 5′-AMP, 2′,3′-AMP, dibutyryl-c-GMP, or butyrate. All these agents were added at the concentration of $10^{-3}M$ as sodium salt. Incubation was carried out at room temperature of approximately 20° to 23°C.

The results obtained varied somewhat from one species to another. However, in all cases, neural differentiation was observed only in the experimental explants and not in the control explants except for rare occasional formation of simple neurons (Table 1). These results are illustrated in Fig. 1. Figure 1, a and b, shows typical control cultures in NT. Clusters of cells as shown in Fig. 1a were seen in all species, whereas cell clusters as shown in Fig. 1b were observed only with some cultures derived from *S. mexicanum.*

In the experimental cultures, a number of cell types of characteristic morphologies were observed. In general, the following recognizable cell types were observed with increasing duration of incubation (Table 1): simple "motor neurons" (Fig. 1c), astrocytes (Fig. 1d), melanophores (Fig. 1e), and central nervous system neurons (Fig. 1f). In addition, structures resembling nerve bundles were also observed in some cultures of *S. mexicanum* cells (Fig. 1g). The rate of formation of these cell types varies with the animal used, being much more rapid with *X. laevis* explants than with those of *P. waltlii* or *S. mexicanum* (Table 1). An estimated percentage of cells that developed into neural ectoderm derived cells varied from 10 to 50. Formaldehyde-induced fluorescence of biogenic amines (*6*) was used to support morphological identification of cells. Those identified as neurons are positive, while those identified as glial cells are negative.

The results obtained, illustrated in

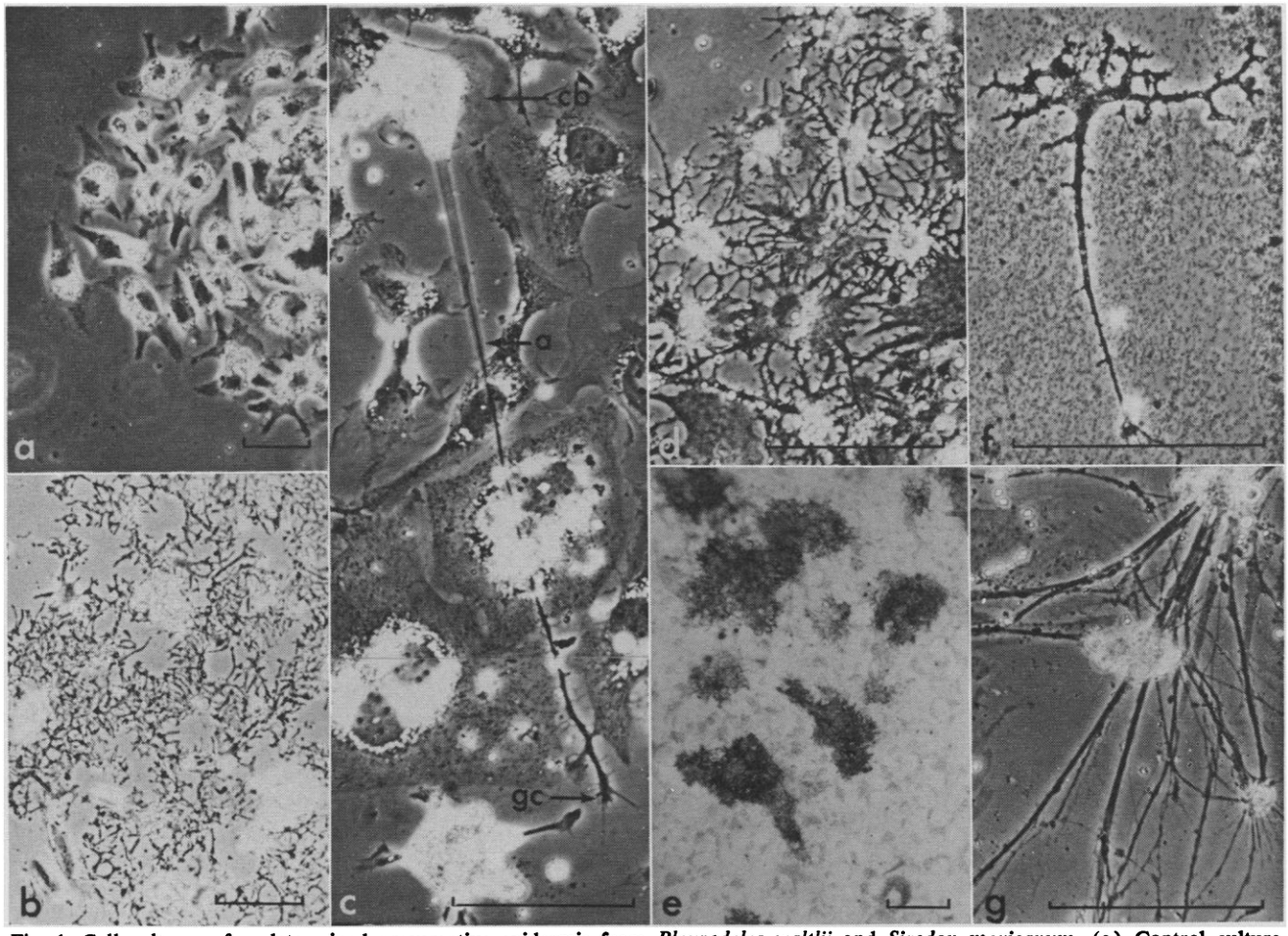

Fig. 1. Cell cultures of undetermined presumptive epidermis from *Pleurodeles waltlii* and *Siredon mexicanum.* (a) Control culture with epithelial-like cells (*P. waltlii.*) (b) Control culture with "chicken wire" cells (*S. mexicanum*). (c) Culture incubated with dibutyryl-c-AMP ($10^{-3}M$) demonstrating simple motor neuron (*P. waltlii*), cell body (*cb*), axon (*a*), and growth cone (*gc*). (d) Culture incubated with 8-Br-c-AMP ($10^{-3}M$) demonstrating astrocytes (*S. mexicanum*). (e) Culture incubated with dibutyryl-c-AMP ($10^{-3}M$) demonstrating melanophores (*P. waltlii*). (f) Culture incubated with dibutyryl-c-AMP ($10^{-3}M$) demonstrating central nervous system–type neuron (*P. waltlii*). (g) Culture incubated with dibutyryl-c-AMP ($10^{-3}M$) demonstrating nerve bundles (*S. mexicanum*). Bright areas in phase-control micrographs represent yolk platelets. All micrographs were taken with phase-contrast optics except (b), which was taken with bright-field optics. Bars represent 100 μm.

Table 1. Neural differentiation by derivatives of adenosine 3′,5′-monophosphate (c-AMP). The notations used are as follows: Th, theophylline; NaBu, sodium butyrate; dbc-AMP and dbc-GMP, dibutyryl derivatives of c-AMP and c-GMP, respectively; At, attachment of explant to surface of culture dish; S, spreading and "flattening" of attached explants; M, migration of cells out from the explants to form "monolayers" as illustrated in Fig. 1a; N and Ns, some and many neurons, respectively; A and As, some and many astrocytes, respectively; NB, nerve bundles; G and Gs, some and many oligodendroglia cells, respectively (some with gliosomes); F, most cells rounded up, detached from surface and floating in medium; Nt, network of cells as shown in Fig. 1b, not seen in all experiments; and As?, cells resembling astrocytes but may be altered cells from Nt, not seen in all experiments. All the agents were added as sodium salt and at a final concentration of $10^{-3}M$. Lower concentrations of sodium butyrate do induce neuron formation. The neurons that are formed quickly are predominately motor neurons (Fig. 1c). These neurons "disappear" after a few days, probably through a combination of detachments from culture dish surface and axon retraction (preliminary observations). The neurons that appear later and persist for long periods of time are mostly central nervous system neurons as illustrated in Fig. 1f. Dibutyryl-c-GMP and 5′-AMP appear to prevent (in most experiments) the migration of the cells away from the explants. This table represents the results of eight separate experiments where little or no autoneurulation was observed. In two other experiments with *S. mexicanum*, extensive autoneurulation occurred in basal medium within 7 days and the experiments were terminated.

Agent	Days of culture									
	1	2	3	4	5	6	7	8	9	10
					Siredon mexicanum					
dbc-AMP	At	S	S	S	M, N	Ns, As	Ns, NB, As	Ns, NB, As	Ns, NB, As, G	NB, As, G
8-Br-c-AMP	S	S	S	M	M	M	Ns, As	As	As	As
c-AMP + Th	At	S	S	S	M	N	Ns	Ns	Ns	Ns
NaBu	At	S	S	S	S	M	M	M, F	M, F	F
Th	At	At	S	S	S	M	M, F	M, F	M, F	M, F
2′,3′-AMP	At	At	S	S	S	M	M	M	M, F	M, F
5′-AMP	At	At	At	At	At	S	S, F	F	F	F
Basal	At	S	S	S	M	M, (Nt)	M, (Nt)	M, (Nt)	M, (Nt)	M, (As?)
					Pleurodeles waltlii					
dbc-AMP	At	S	S	S	M	N	Ns, As	Ns, As, G	Ns, As, Gs	
8-Br-c-AMP	At, S	At, S	At, S	At, S	M, N	Ns	Ns, As	Ns, As	Ns, As	
c-AMP + Th	At	S	S	M	N	Ns, As	Ns, As	Ns, As	Ns, As	
NaBu	At	S	S	S	S	S	S, F	S, F	F	
Th	At	S	S	S	M	M, N	M, N	M, N	M, N	
dbc-GMP	At	At	At	S	S	S	S	S	S, F	
Basal	At	S	S	S	M	M	M	M	M	
					Xenopus laevis					
dbc-AMP	S	M	Ns, As	Ns, As	Ns, As					
8-Br-c-AMP	S, M	Ns	Ns	Ns, As	Ns, As					
NaBu	At	At	At	At	At					
dbc-GMP	At	At	S	S	S					
Basal	S	M	M	M	M					

part in this report, indicate that dibutyryl-c-AMP, 8-Br-c-AMP, and c-AMP plus theophylline can indeed induce the differentiation of these cells to form neurons, melanophores, and glial cells. In contrast, 5′-AMP, 2′,3′-AMP, butyrate (degradation product of dibutyryl-c-AMP), and dibutyryl-c-GMP at the same concentration ($10^{-3}M$) are totally ineffective. Instead, these compounds either do nothing, cause cell disintegration, or prevent the migration of cells from the explants. Theophylline alone induced limited neural differentiation only in the case of *P. waltlii*. Cyclic AMP alone gave variable results, usually with limited neural differentiation (data not shown because of variability from one experiment to another).

The inductive effects of dibutyryl-c-AMP could be questioned, as butyrate at $10^{-4}M$ did cause some neural differentiation [see legend to Table 1 and (*7*)]. In the experiments using dibutyryl-c-AMP, this agent was added at the beginning of the experiments, and the concentration of butyrate in the incubation mixture during the days of culture was not determined. However, the observation that 8-Br-c-AMP and c-AMP plus theophylline can, while dibutyryl-c-GMP cannot, induce neural differentiation indicates that the inductive effects of dibutyryl-c-AMP are probably due to its being similar to c-AMP and not due to the release of butyrate.

It should be pointed out that the effects of 8-Br-c-AMP are not exactly the same as those of dibutyryl-c-AMP. In two experiments with *S. mexicanum*, 8-Br-c-AMP caused the formation of large numbers of astrocytes with few simple motor neurons and practically no central nervous system neurons, whereas dibutyryl-c-AMP caused the formation of both types of neurons as well as astrocytes (Table 1).

In spite of the differences between dibutyryl-c-AMP, 8-Br-c-AMP, and c-AMP plus theophylline shown in Table 1, it can be stated that all these agents induce neural differentiation, while other related nucleotides do not. It should be admitted, however, that these results do not per se prove that c-AMP is the normal agent mediating the effects of the primary inductors. Further work is needed to determine whether there is a rise in c-AMP level in embryos undergoing neural differentiation. In this relationship, it might be mentioned that during mesoderm segmentation, the c-AMP level increases in various regions of the chick embryo (*8*). Whether the increased c-AMP level is related to induction processes is not clear.

It should also be mentioned that Barth and Barth (*9*) showed recently that transient treatment, with high concentrations of cations, of monolayer cell cultures derived from presumptive epidermis of *Rana pipiens* resulted in the sequential appearance of cells characteristic of neural fold origin. The relationship between the cation induction and the c-AMP induction of neural differentiation remains to be determined (*10*).

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References and Notes

1. H. Spemann, *Arch. Entwicklungsmech. Org.* **48**, 533 (1921).
2. L. Saxen and S. Toivonen, *Primary Embryonic Induction* (Logos, London, 1962), p. 271.
3. H. Tiedemann, in *Biochemistry of Animal Development*, R. Weber, Ed. (Academic Press, New York, 1967), vol. 2, pp. 4–55.
4. M. C. Niu and V. C. Twitty, *Proc. Natl. Acad. Sci. U.S.A.* **39**, 985 (1953).
5. L. G. Barth and L. J. Barth, *Dev. Biol.* **20**, 236 (1969).
6. B. Falck, N. A. Hillarp, G. Thieme, A. Torp, *J. Histochem. Cytochem.* **10**, 348 (1962).
7. The mechanism by which butyrate induces neural differentiation is unknown. However, it has been shown [K. N. Prasad, K. Gilmer, S. Kumar, *Proc. Soc. Exp. Biol. Med.* **143**, 1168 (1973)] that butyrate stimulates c-AMP production by neuroblastoma cells in culture. It seems possible that butyrate at $10^{-4}M$ induces some neural differentiation via c-AMP.
8. M. Reporter and G. C. Rosenquist, *Science* **178**, 628 (1972).
9. L. G. Barth and L. J. Barth, *J. Embryol. Exp. Morphol.* **7**, 210 (1959).
10. Since this manuscript was submitted, D. McMahon [*Science* **185**, 1012 (1974)] has discussed a "hypothesis" that cyclic nucleotides may play a determinant role in embryonic differentiation.

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Besnoitia Species (Protozoa, Sporozoa, Toxoplasmatidae): Recognition of Cyclic Transmission by Cats

Abstract. *Isosporan oocysts, measuring 13 by 16 micrometers, from a cat in Hawaii produced* Besnoitia *cysts in tissues of mice and rats. Feeding these cysts to cats led to oocyst shedding after 11 to 13 days, continuing for a mean of 11 days. This indicates a two-host cycle for* Besnoitia, *adding an intestinal phase and oocyst production by a carnivore to the already known tissue stages. Thus a representative of* Besnoitia, *similar to other species in cattle, horses, reindeer, impala, other mammals, and reptiles, has been shown to be a coccidian of cats, capable of being spread by fecal contamination.* Besnoitia *is the fourth mammalian tissue parasite, together with* Toxoplasma, Hammondia, *and* Sarcocystis, *found to produce isosporan-type oocysts.*

During studies on coccidia of cats (*1*), typical *Besnoitia* cysts (Fig. 1, C and E) were observed in the omentum and mesentery and on the serosal surfaces of viscera of mice treated with cortisone acetate and inoculated with an unidentified isosporan-type oocyst (Fig. 1, A and B) from the feces of a stray cat in Hawaii. These observations are of interest because the life cycle of *Besnoitia*, a sporozoan parasite of veterinary and biologic importance, has been obscure.

Of 23 cats fed carcasses of mice infected with the oocyst, 17 developed patent infections. Times to oocyst shedding ranged between 11 and 13 days (average, 12 days) and the patent periods ranged between 5 and 12 days (average, 11 days). Several *Besnoitia* cysts isolated from the omentum and mesentery of infected mice were fed intact to three kittens 3 days old, resulting in oocyst shedding after 16 days in one kitten; two kittens died earlier from a viral infection. Single isolated cysts were fed to four cats, two of which developed a 16-fold increase in antibody, although no oocysts were detected.

Schizonts were found both in intestinal epithelial cells and in the lamina propria of the ileum (Fig. 1F). Macrogametes (Fig. 1G), measuring 10 to 13 μm, were larger than those of *Toxoplasma gondii*, *Hammondia hammondi* (*2*), and *Sarcocystis muris* (*3*). Macrogametocytes were found only in goblet cells of the small intestine. Three out of five cats shed oocysts after a second meal of cyst-infected mice and one cat that received a third meal also shed oocysts. Prepatent periods remained between 12 and 13 days, but oocyst shedding lasted only 3 to 7 days. Likewise, a cat previously infected with *T. gondii* and another infected with *S. muris* shed *Besnoitia* oocysts after consuming *Besnoitia* cysts. The skeletal muscles of two cats were fed to other cats and to mice, but no infections resulted.

The *Besnoitia* oocyst is shed unsporulated (Fig. 1A) and contains a finely granular, light brown sporont, which initially fills the oocyst almost completely and later shrinks in size; there is no micropyle or polar granule. Twenty unsporulated oocysts averaged 12 by 17 μm, ranging from 10 to 13 by 16 to 19 μm. When suspended in 1 or 2 percent sulfuric acid at room temperature (24° ± 1°C) and exposed to air, sporulation is complete between 48 and 96 hours. Each oocyst contains two ellipitical sporocysts, without oocyst residuum. Each sporocyst contains four sporozoites and a diffuse granular residuum; no Stieda body was observed (Fig. 1B). The wall of sporulated oocysts is smooth, about 0.5 μm thick, and appears to consist of two layers; the inner layer frequently collapses inward. The average measurement of 100 sporulated oocysts was 13 by 16 μm, ranging from 12 to 15 by 15 to 18 μm. Fifty sporocysts averaged 8 by 11 μm, with ranges from 7 to 8 by 10 to 11 μm. Sporozoites mea-

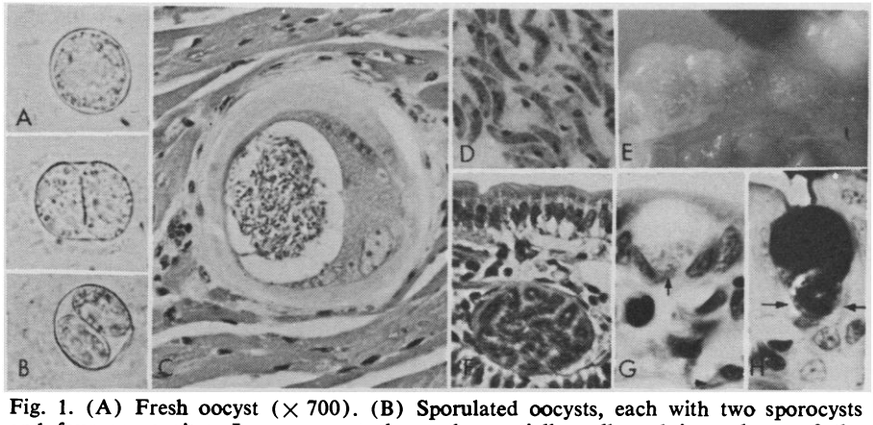

Fig. 1. (A) Fresh oocyst (× 700). (B) Sporulated oocysts, each with two sporocysts and four sporozoites. Lower oocyst shows the partially collapsed inner layer of the oocyst wall (right lower center) (× 700). (C) Section of cyst, in the myocardium of a mouse, with pale-staining cyst wall surrounding host cell, several nuclei of which are in the cytoplasm at the right, with the microorganisms in the cytoplasmic vacuole on the left [hematoxylin and eosin stain (H and E); × 280]. (D) Bradyzoites from cyst in impression film (Giemsa stain; × 700. (E) Cysts developing in intestinal wall of a mouse 48 days after the oral administration of oocysts (× 2). (F) Macroschizont from intestinal lamina propria of cat infected for 11 days (H and E stain; × 280). (G and H) Macrogametocytes in goblet cells of the small intestine of a cat, 16 days after infection. Each parasite (arrows) is at the base of the cells. The mucin remained unstained with H and E (G) but stained deeply with periodic acid—Schiff and hematoxylin (H), which renders the parasite (between arrows) as a series of granules (× 700).