Table 1. Comparison of x-ray-diffraction and titrimetric characteristics of the polarographically reducing crystalline substance isolated from dried range grasses with those of authentic *trans*-aconitic acid; stoichiometric 50-percent titration points, assuming a tricarboxylic acid.

Spacing (Å)		Relative intensity	pH at % titration		
d_1	d_2	$(I_2:I_1)$	50	150	250
		trans-*Aconitic acid*			
6.26	4.16	0.26	2.90	4.12	5.78
		Isolate			
6.24	4.17	.32	2.87	4.11	5.76

5 percent as *cis*-aconitate. Thus *trans*-aconitate may be a dominant form of aconitate in living corn plants, at least during certain stages of growth.

In order to determine the natural distribution of isomeric aconitates in our fresh samples of forage, we devised a charcoal column chromatographic procedure to separate *cis*-aconitate from *trans*-aconitate contained in aqueous extracts from macerated green grasses; the method caused no interconversion between *cis*- and *trans*-aconitate during maceration or subsequent processing. A fresh sample of indiscriminately mixed forage grasses having a relatively high concentration (2.9 percent) of total aconitate contained only 0.03 percent *cis*-aconitate and 2.9 percent *trans*-aconitate. Other samples of fresh grasses contained little *cis*-aconitate relative to *trans*-aconitate. *trans*-Aconitate thus appeared to be the dominating aconitate isomer in our young grasses.

Examination of fresh samples by silica-gel chromatography showed that fumaric, mesaconic, and syringic acids are also minor constituents (less than 0.1 percent each). These range grasses thus had approximately the same proportion of *trans*-aconitate to accompanying nonnitrogenous organic acids as solids of sugar cane juice (*3*).

We have found considerable differences in *trans*-aconitic acid concentration between different species of range grasses. Broad-leafed annuals generally have lower concentrations than grasses, but there may be some exceptions. Young filaree (*Erodium* spp.) growing among grasses showed less than 0.1 percent (dry weight basis) of polarographically reducible organic acid including *trans*-aconitate. Weather conditions seem to alter the amounts of *trans*-aconitate in grasses, judged from collections made from a single plot during February and March 1964; these collections reflected an inverse relation between the content of *trans*-aconitate and air temperature at the time of sampling. The samples were taken in late afternoon at intervals of 2 to 3 days, when temperatures varied between 5.5° and 14.5°C; corresponding extremes of *trans*-aconitate content were 1.83 and 1.13 percent.

Burt and Thomas (*7*) found significant reductions of magnesium and phosphate in the serums of heifer calves fed sodium citrate as a dietary supplement in concentrations equivalent to 1 percent citric acid. Consequently, contents of *trans*-aconitate in range grasses raise questions concerning the feeding quality of grass for ruminants when *trans*-aconitate is ingested in quantity comparable with or greater than the citrate concentration used by Burt and Thomas. Saffran and Prado (*5*), working with slices of rat kidney cortex, have shown that *trans*-aconitate causes accumulations of citrate while lowering respiratory rates of uptake of oxygen. The activity of certain microorganisms can be restricted by *trans*-aconitate: *Pseudomonas aeruginosa* may be completely inhibited by *trans*-aconitate at 1 mg/ml (*8*), whereas *Staphylococcus aureus* and two different psuedomonads utilize *trans*-aconitate (*9*).

Several nutritional disorders of ruminants grazing in early spring may appear or disappear rather suddenly, the number of affected animals varying considerably from year to year. Perhaps the three most important maladies are grass tetany (hypomagnesemia), nitrate poisoning, and phalaris staggers. We do not now propose that the high content of *trans*-aconitic acid in early spring grasses is definitely related to any of these disorders, but we do suggest that herbivores depending on grasses must be able to cope with *trans*-aconitic acid either directly or through rumen or intestinal flora. Indirectly, rumen flora must overcome the acid in performing normal digestive processes and in special cases of metabolizing nitrate to fully reduced forms or of denaturing alkaloids or other toxins that may occur in some grasses.

R. BURAU
P. R. STOUT
Department of Soils and Plant Nutrition and *Kearney Foundation of Soil Science, University of California, Davis*

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26 August 1965

Eimeria tenella: Cultivation of the Asexual Stages in Cultured Animal Cells

Abstract. Eimeria tenella, *an intracellular protozoan parasite of the cecal epithelium of chickens, developed asexually in monolayer cultures of mammalian fibroblasts, mammalian epithelial cells, and avian fibroblasts mintained under various mediums at 41°C. Sexual stages of the parasite were not seen. Established cell lines and secondary cell cultures were equally suitable for cultivation of the parasite.*

Until now it has not been possible to cultivate the endogenous stages of any coccidium outside its host's body, not even in tissue cultures (*1*). This is a report of the cultivation of the asexual stages of a coccidium of chickens, *Eimeria tenella*, in monolayer cultures of mammalian and avian cells. This highly specific obligate intracellular protozoan parasite completed at least one asexual generation in some cell types and part of an asexual generation in others. Mature merozoites (Fig. 1*F*), the terminal product of an asexual generation, were demonstrated in secondary cultures of bovine kidney cells, although cultures of a bovine kidney cell line (*2*) were equally suitable. Merozoites were also demonstrated in secondary cultures of fibro-

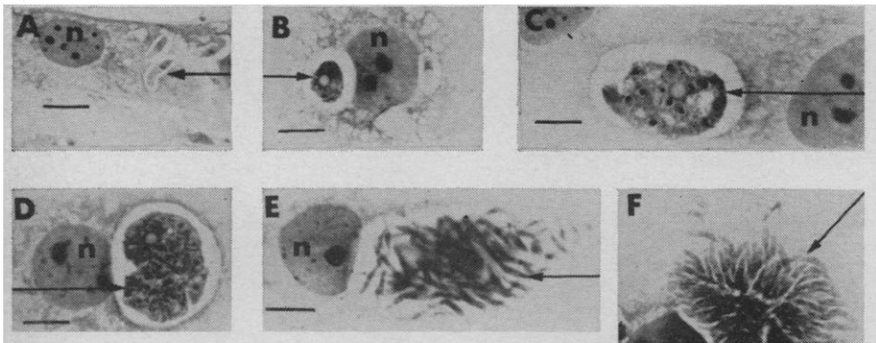

Fig. 1. Six stages in the asexual development of *Eimeria tenella* in bovine kidney cell cultures at 41°C. Arrows indicate the developing parasites. Host cell nuclei are identified (*n*). The scale in each photograph is 10 μ. (*A*) The initial infective form, sporozoites, after invasion of a host cell. (*B*) A cell with a dormant sporozoite and a trophozoite, the first stage beyond sporozoite and before division of the parasitic nucleus. (*C*) An immature schizont with 12 to 14 nuclei. The parasitic cytoplasm has not yet divided around the parasitic nuclei. (*D*) A dividing schizont. Individual nucleated parasites are ready to elongate into merozoites. (*E*) Merozoites ready for release from a host cell. (*F*) Merozoites leaving a host cell.

blast-like cells derived from 9-day embryos of Japanese quail (*Coturnix coturnix japonica*). Schizonts, the intermediate stages of asexual development, were demonstrated in fibroblasts derived from 9-day chicken embryos. Infected fibroblasts increased from 8 to 10 times their normal size. L cells (NCTC clone 929) supported development only to immature schizont stages. The absence of merozoites in the chick-embryo fibroblasts and the L cells may be due to the early degeneration of their cultures at 41°C. The variety of cell types which was found suitable for in vitro cultivation of the asexual stages of *Eimeria tenella* was unexpected since the parasite in the chicken is generally considered to require epithelial cells of the cecum (*3, 4*). Lotze *et al.* (*5*) have presented evidence which suggests that some species of *Eimeria* can survive and grow, at least for a limited time, in areas outside their well-known places of development.

Young growing cell cultures held at 41°C were used in all experiments. Cultures were inoculated with sporozoites as soon as monolayers were nearly complete. Sporozoites in the inoculum generally showed only occasional sluggish motility. More frequent and vigorous motility of sporozoites near the cells was observed repeatedly. The number of sporozoites eventually entering a cell ranged from one to twelve, with two to four being common (Fig. 1*A*). Very few of the intracellular sporozoites developed to any subsequent stages, regardless of the number of sporozoites placed in the culture vessels. However, the inoculation of large numbers of sporozoites assured the easy demonstration of trophozoites (Fig. 1*B*), immature schizonts (Fig. 1*C*), and mature segmenting schizonts (Fig. 1*D*) at 48 to 96 hours after inoculation. No schizonts were seen before 48 hours. The first-generation trophozoite, the early undivided asexual form, and first-generation schizonts in the chicken are characterized by a conspicuous eosinophilic globule (*3*). A markedly refractile globule was often seen in the asexual stages in cultured animal cells (Fig. 1, *B*, *C*, and *D*). Merozoites, ready for release from host cells (Fig. 1, *E* and *F*), were evident at 4 to 6 days, with the greatest prevalence at 5 days. In the chicken the parasite requires 3 days for the first generation of asexual multiplication (*4*). The second asexual generation is completed in another 1½ to 2 days. Most of the merozoites emerging from the second asexual generation initiate a sexual generation which completes the parasite's development in another 24 to 36 hours. The sexual stages were not observed in tissue culture. The merozoites observed in fixed, stained bovine kidney cell cultures were 7 to 8 μ in length. First-generation merozoites in the chicken are 3 μ long, and second-generation merozoites are 16 μ long (*4*).

Sporozoites were the only successful inoculum; intact oocysts or sporocysts were not infective for cell cultures. Goodrich (*6*) first showed that sporozoites of *Eimeria tenella* and *Eimeria stiedae* could be obtained by mechanically rupturing the oocyst wall and then treating the released sporocysts with trypsin. This method was later improved by using bile with trypsin (*7*). The suspension of sporozoites for each of my experiments was produced from 175×10^6 sporulated oocysts. Oocysts were sterilized in 5.25 percent sodium hypochlorite (*8*) by weight in water (that is, undiluted Clorox) for 15 to 30 minutes. They were resuspended in phosphate-buffered saline (*p*H 6.0) and ruptured in a motor-driven Teflon tissue grinder (diameter, 25 mm) mounted on a drill press (1300 rev/min). Ten minutes of grinding ruptured most of the oocysts without destroying many sporocysts. Sporozoites were then released from the sporocysts in 2 to 3 hours at 37°C with 0.2 percent trypsin and 5 percent chicken bile in saline A (*9*), the yield of sporozoites being increased by frequent gentle agitation. The sporozoites were washed three times in phosphate-buffered saline to remove bile and trypsin. Centrifugation at 1300*g* for 5 minutes was sufficient to sediment most of the sporozoites which were then resuspended in 20 ml of tissue culture medium. Two milliliters of this suspension was placed in each of ten Leighton tube cultures containing coverslips, 10.5 by 22 mm. After 3½ to 4 hours at 41°C, the sporozoite suspension was removed. Each culture was washed twice with medium, and then 2 ml of medium was added to each one. Ten uninoculated cultures were maintained in each experiment. An inoculated culture and an uninoculated culture were fixed, stained, and examined at 24-hour intervals after inoculation. Early and thorough washing of the inoculated cultures was necessary because the debris and shells from oocysts and sporocysts carried in the suspension of sporozoites were toxic for cell cultures. This is consistent with a toxic effect in rabbits inoculated with extracts of *E. tenella* oocysts (*10*).

The choice of medium did not influence the parasite's development in tissue culture. Basal HeLa-cell medium of Eagle (*11*) supplemented with 2, 5, and 10 percent bovine serum was used in five experiments; Hanks balanced salt solution supplemented with 10 percent bovine serum was used in two. Choice of serum percentage was gov-

erned by the anticipated needs of the cultures, according to the appearance of the cells.

The incubation temperature had a decisive effect on the parasite's development. Cultures incubated at 41°C after inoculation supported at least one complete asexual generation of the parasite in bovine kidney cell cultures. These cultures were also grown at 41°C prior to inoculation. Similar cultures held at 37°C both before and after inoculation showed only a few very immature schizonts which failed to develop further. A vacuole-like intracytoplasmic space around the parasite in fixed stained tissue cultures (Fig. 1, *A–E*) was useful in quickly locating the parasite in all its asexual stages. These vacuolar spaces are probably artifacts similar to those seen by Huff (*12*) in his study of malarial parasites in tissue cultures, by Hogan *et al.* (*13*) in their study of *Toxoplasma* in tissue cultures, and by Hogan *et al.* (*14*) in their study of *Toxoplasma* in mouse peritoneal macrophages. Coverslip cultures were fixed for 5 to 10 minutes in 10 percent formalin in Hanks balanced salt solution. Fixed cultures were then taken through a series of increasing concentrations of tertiary butyl alcohol mixed with decreasing concentrations of ethyl alcohol and water. Staining was done with 0.05 percent aqueous toluidine blue O (*p*H 6.2). The developing parasites (that is, beyond the sporozoite stage) always accepted the stain to a greater degree than did their host cells. This also aided in locating the parasite in all its asexual stages. Intracellular structures even remotely resembling the various parasitic stages of *Eimeria tenella* were never found in uninoculated cultures.

Finally, two points deserve emphasis: First, the many manipulations in preparing the inoculum resulted in loss of large numbers of oocysts, sporocysts, and sporozoites. Second, further inefficiency was incurred with the relatively small number of sporozoites which developed after invading the host cells. However, all of the stages in one complete asexual generation of the coccidium, *Eimeria tenella*, were clearly and repeatedly demonstrated in a variety of types of cultured animal cells.

W. H. PATTON

Department of Veterinary Science, Pennsylvania State University, University Park 16802

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Lymphoma Growth in vivo: Electronic Discrimination between Tumor and Stroma Cells

Abstract. *Moloney lymphoma cells are larger than normal cells of mouse spleen, lymph-node, and thymus. Use of electronic cell-size anaylsis in conjunction with specific immunocytolysis permits differential counting of host and tumor cells. Increase of lymphoma cells occurs first in spleen and then in lymph nodes; in thymus it occurs only during the terminal stages of tumor growth.*

Lymphoma cells, transplanted to genetically compatible mice, can be characterized by various means (histology, specific immunofluorescence, cytotoxicity, and so on). Such techniques, however, are not convenient for detection of tumor cells during the early stages of growth, or for enumeration of these cells within host organs (for example, for quantitative study of growth characteristics).

Electronic analysis of cell size, combined with specific immunocytolysis for differentiation between host and neoplastic cells, has indicated that the enlarged lymph nodes of mice carrying transplanted lymphomas induced by Moloney virus contain cells of two

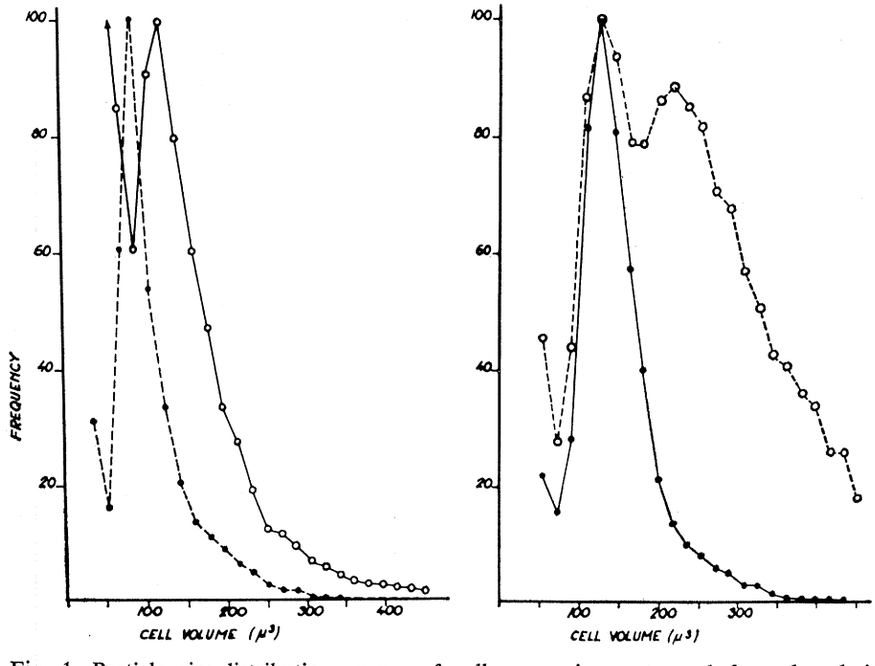

Fig. 1. Particle size-distribution curves of cell suspensions prepared from lymphoid organs of adult mice. Suspensions in BSS containing about 10^5 cells per milliliter were examined with model B Coulter counter. (Left) ●---● thymus, and ○—○ spleen, of normal mice. (Right) Lymph nodes from a normal mouse (●—●) and from a mouse inoculated with YHA lymphoma (○---○). Frequency is on an arbitrary scale.