imagery in the waking state usually, although not always, leads to a reduction of the alpha rhythm, it is difficult to reconcile the increased occipital alpha rhythm at the time of the eye bursts. The periodicity of respiration points to a trigger in the central nervous system more prosaic than that of dream content.

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Trans-Aconitic Acid in Range Grasses in Early Spring

Abstract. trans-*Aconitate ion, an inhibitor of the tricarboxylic acid cycle, was identified in range grasses as* trans-*aconitic acid, which was isolated in crystalline form. It occurs in surprisingly high concentrations in early-season forage grasses. Dry-weight concentrations of* trans-*aconitate vary with season and species; concentrations of between 1 and 2.5 percent are common in mixed pasture grasses, but are higher in certain species such as* Hordeum leporinum *(3.5 percent) and* Phalaris tuberosa *var.* stenoptera *(4.2 percent). Leaves of western larkspur* (Delphinium hesperium) *contain 12.2 percent* trans-*aconitate.* trans-*Aconitate may be partially responsible for nutritional disorders, such as grass tetany (hypomagnesemia), that occur in grazing cattle in early spring.*

During the early spring of 1964 and again in 1965, samples of range grass were collected for analysis in connection with especially severe seasonal outbreaks of grass tetany (hypomagnesemia) in range cattle of the lower Sierran foothills of central California. When applying a quantitative polarographic method to determine nitrate concentrations (*1*) in dried samples, we noted a surprisingly large amount of a soluble, polarographically reducing substance in charcoal-clarified aqueous extracts from the samples. The polarographic wave caused by this substance thoroughly masked the nitrate-diffusion current. Supporting electrolytes were devised to give well-defined, reproducible, polarographic waves from the unknown so that relative concentrations could be determined accurately. The polarograph was then used to follow the material through fractionation procedures that led to isolation and identification as *trans*-aconitic acid. At this point the polarographic method automatically became a quantitative means of determining *trans*-aconitate in plant materials.

The water-soluble fraction of the grasses was subjected to paper chromatography, a mixture of mesityl oxide, formic acid, and water being used for elution (*2*). The material moved with an R_F value essentially the same as that of fumaric acid, but unlike authentic fumaric acid it did not react in the presence of fumarase either directly or when extracted from the chromatographic paper. Consequently, 10 g of dried plant material was extracted with water, and the extract was clarified with charcoal, acidified, and further extracted with ether. The unknown reducible substance passed from the aqueous into the ether phase and crystallized when the ether was evaporated. Refined polarographic examinations of half-wave potentials of the crystalline material, in both acid and neutral supporting electrolytes, suggested that it was probably *trans*-aconitic acid. Spacings from x-ray powder-diffraction patterns and titrimetric curves of the crystalline material were identical with those of authentic *trans*-aconitic acid (Table 1). Melting points were not sharply defined, indicating slight impurity.

Powder-diffraction patterns (Cu-Kα) showed fewer distinguishable lines and greater background scattering than in the reference sample of *trans*-aconitic acid because of the lower degree of crystallinity of the isolated material; relative intensities of the principal lines were not identical but were in keeping with the lower degree of crystallinity (Table 1). Thus, polarographic reduction potentials, x-ray diffraction data, coincidence of titrimetric curves made with NaOH, melting-point data, and consideration of the chemistry of extraction determined that the material isolated from samples of dry grass was slightly impure *trans*-aconitic acid.

Polarographic analyses were made of other dried samples of mixed forage grasses from ranges where animals were prone to grass tetany. These samples, which had been collected in early spring from new growth and dried for 48 hours at 70°C, revealed a consistently high content of 1.5 to 2.5 percent *trans*-aconitate. Lawn clippings from outside our laboratory yielded much less (0.05 percent). Certain dried samples of individual wild and cultivated species have yielded as much as 3.5 and 4.2 percent *trans*-aconitic acid equivalent (*Hordeum leporinum* and *Phalaris tuberosa* var. *stenoptera*, respectively) in early spring, whereas dried mature grass left from the preceding fall contained no detectable aconitate. Dried leaves of *Delphinium hesperium* (western larkspur), a member of the same family as the aconites, contained 12.2 percent aconitate, mostly as *trans*-aconitate.

There are relatively few references to the occurrence of high concentrations of aconitic acid in plant materials. Roberts and Martin (*3*) report concentrations of 2 percent aconitate in solids from dried sugar cane juice, with an additional 1 percent distributed among fumaric, mesaconic, succinic, glycolic, citric, malic, syringic, and oxalic acids. Our drying procedure for samples (48 hours at 70°C) was found to change aconitate from the *cis* to the *trans* isomer; conversion rates increase with temperature and with increasing acidity or alkalinity (*4*).

The high concentration of *trans*-aconitate in these samples of grass suggested interesting biochemical questions relative to the *trans*-aconitate content of the growing plants, as well as to possible consequences for grazing animals. Because *trans*-aconitate is a competitive inhibitor of aconitase in the tricarboxylic acid cycle (*5*), it was important to ascertain the relative distributions of *cis*- and *trans*-aconitate in fresh, green plants.

MacLennan and Beevers (*6*) showed that corn plants synthesize *trans*-aconitate when they are fed acetate labeled with C^{14} at position 1; of the aconitate-C^{14} generated from the acetate, 95 percent was found as *trans*-aconitate and

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) d_1	Spacing (Å) d_2	Relative intensity (I_2:I_1)	pH at % titration 50	pH at % titration 150	pH at % titration 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.

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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-