or puromycin or cycloheximide was added.

For about 3 days cultures containing up to 1 μg of actinomycin D per milliliter or 100 μg of cycloheximide per milliliter maintained large muscle fiber resting potentials (50 to 70 mv) and the ability to contract upon electrical stimulation. Cultures containing 10 μg of puromycin per milliliter usually lasted less than 2 days. Since nearly complete development of ACh sensitivity requires about 6 days, most experiments involved cutting the phrenic nerve and, 1 to 4 days later, transferring the denervated diaphragm to culture.

Acetylcholine sensitivity was measured by recording transmembrane potentials with an intracellular micropipette while ACh was applied iontophoretically to the outer surface of the muscle fiber membrane from a micropipette containing 3*M* AChCl solution (*4*). Sensitivity is expressed as millivolts of depolarization per nanocoulomb (nC) of iontophoretic current. Iontophoretic current pulses of 5×10^{-9} to 2×10^{-7} amp and durations of 1 to 100 msec were employed. Iontophoretic pulses were adjusted to yield maximum values of ACh sensitivity. Latency between onset of iontophoretic current and beginning of depolarization was always less than 10 msec. An ACh sensitivity of 0.01 mv/nC (0.2 mv depolarization produced by an iontophoretic current pulse of 2×10^{-7} amp for 100 msec) was considered the minimum detectable ACh sensitivity in any experiment. When no ACh sensitivity could be detected, the accurate placement of micropipettes was confirmed by advancing the ACh micropipette into the fiber, passing current, and recording electrotonic pulses (*4*). Measurements were made on four to eight fibers at positions 1, 2, 3, and 4 mm from the area of neuromuscular junctions. Separation of ACh and recording micropipettes was usually less than 200 μm. Acetylcholine sensitivity around individual end plates was also mapped. After electrophysiological analysis the location of end plates was verified by staining acid-fixed preparations with thiolacetic acid–lead reagent (for cholinesterases) (*5*) and Schiff reagent (for the phrenic nerve) (*6*).

The development of extrajunctional ACh sensitivity in organ-cultured rat diaphragm follows a time course similar to that in vivo. Fibers rarely have a sensitivity greater than 0.1 mv/nC 48 hours after denervation, and virtually all fibers have a sensitivity of 2 to 20 mv/nC by 72 hours (Fig. 1A). Actinomycin D (1 μg/ml) added to organ cultures of 0-, 1-, or 2-day denervated rat diaphragms prevents the rise of extrajunctional ACh sensitivity (Fig. 1B). However, actinomycin D has no effect upon the ACh sensitivity at the neuromuscular junction or upon established extrajunctional ACh sensitivity. One microgram of actinomycin D per milliliter retards the rate of RNA synthesis by 80 percent (measured as incorporation of 5-^{3}H-uridine into trichloroacetic acid–precipitable, alkali-labile material), while the rate of protein synthesis (incorporation of ^{14}C-leucine into trichloroacetic acid–precipitable, hot trichloroacetic acid–stable material) declines only 15 percent in 48 hours.

Cycloheximide (1 to 100 μg/ml) or puromycin (10 μg/ml) added to diaphragm cultures likewise halts the development of extrajunctional ACh sensitivity (Fig. 1C). Neither inhibitor has any effect upon end-plate ACh sensitivity or upon established extrajunctional ACh sensitivity. Puromycin is quite toxic, and most cultures become electrically inexcitable after 48 hours' exposure. Cycloheximide is much less toxic and at 10 μg/ml immediately retards the rate of protein synthesis by 95 percent while the rate of RNA synthesis declines slowly to about 40 percent of control levels in 48 hours. Concentrations of actinomycin D and of cycloheximide which do not block RNA or protein synthesis (0.01 μg/ml) have no effect upon the increase in ACh sensitivity.

While it remains possible that unknown side effects of the inhibitors caused the observed results, some tentative conclusions can be drawn regarding the molecular basis of ACh sensitivity and its regulation. First, since ACh sensitivity remains undiminished for 3 days in the virtual absence of protein synthesis, I conclude that the rate of turnover of ACh receptors is probably very slow. Second, since inhibition of RNA or protein synthesis will prevent the rise of extrajunctional ACh sensitivity in denervated muscle fibers, I conclude that new species of RNA and protein are required for the expression of new ACh sensitivity. Perhaps these requirements reflect the synthesis of ACh "receptors" from newly activated genetic information. A corollary of the second conclusion is that the neuronal regulation of ACh sensitivity probably involves regulation of gene activity in muscle fibers.

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1,3-Bis(p-chlorobenzylideneamino)guanidine Hydrochloride (Robenzidene): New Poultry Anticoccidial Agent

Abstract. *At 66 parts per million in the feed, robenzidene is highly effective in preventing chicken coccidiosis caused by any one of eight* Eimeria *species. Three times this dose is safe for broiler chickens. In dogs and rats, the toxicity was relatively low over a period of 90 days. In laboratory trials, the drug completely prevents oocyst production by seven species and greatly reduces oocyst production by* Eimeria maxima.

Over the past 20 years anticoccidial drugs have been added to feed for chickens to help prevent coccidial diseases. Nevertheless, coccidiosis continues to be one of the three most prevalent poultry diseases (*1*). Reports of drug resistance in field strains of the protozoa responsible, *Eimeria* species, have appeared with increasing frequency, sometimes soon after introduction of new agents (*2*). The recommended concentrations of anticoccidial agents for continuous preventive use in feed are effective in reducing economic losses, but do not completely prevent production of oocysts and thereby permit

Table 1. Efficacy of 1,3-bis(*p*-chlorobenzylideneamino)guanidine hydrochloride at a concentration of 66 ppm in feed against six individual species and a mixture of eight species of *Eimeria* in chickens. Cockerels were 9 or 10 days old when inoculated. Mean weight gains from the day of inoculation until test termination 6 or 7 days later are shown in grams for uninoculated, unmedicated controls (UUC), and as percentages of UUC gains for inoculated, unmedicated controls (IUC) and the inoculated, medicated groups (IM). For each treatment there were 20 birds except for *E. tenella* and *E. necatrix*, for which 40 were used.

Eimeria species	UUC (g)	Weight gain		Gross lesions (%)		Mortality (%)	
		IUC (% of UUC)	IM (% of UUC)	IUC	IM	IUC	IM
tenella	118	39	100	100	0	50	0
necatrix	98	34	99	100	0	70	0
acervulina	101	61	98	100	0	0	0
brunetti	95	25	104	100	0	0	0
maxima	82	81	101	100	0	0	0
mivati	118	73	99	100	0	0	0
Mixture of eight *	118	56	100	100	0	10	0

* Above species plus *E. hagani* and *E. praecox* given as a live avian coccidiosis vaccine at 50 times recommended immunizing dose (*6*).

the continued transmission of coccidiosis (*3*). The new anticoccidial compound robenzidene [1,3-bis(*p*-chlorobenzylideneamino)guanidine hydrochloride] is a highly effective prophylactic agent against the eight species of chicken coccidia tested. It is safe for chickens when included in the diet at 66 parts per million. This dosage prevented oocyst production in birds subjected to severe laboratory infections with seven species, and it greatly reduced oocyst production with *E. maxima*. The ninth species, *E. mitis*, one of the least pathogenic, has not been tested (*4*).

This compound was ineffective against the protozoan *Histomonas meleagridis* in turkeys and the nematode *Capillaria obsignata* in chickens. In experimental protozoan infections in mammals (*4*), robenzidene was somewhat effective against the asexual stages of *Plasmodium berghei* in mice but was ineffective against *Trichomonas vaginalis, Trypanosoma equiperdum*, and *Entamoeba histolytica* in rats. Robenzidene was also inactive (*4*) against experimental infections in mice with the trematode *Schistosoma mansoni*, the cestode *Hymenolepis nana*, the nematodes *Nematospiroides dubius, Aspiculuris tetraptera*, and larvae of *Ascaris suum*.

The nitrate salt of robenzidene was prepared first by addition of a concentrated aqueous solution of 1,3-diaminoguanidine nitrate, containing a few drops of the free mineral acid, to a hot solution of *p*-chlorobenzaldehyde (2.2 equivalents) in ethanol. The mixture was kept at room temperature for several hours, and the precipitate was then collected, washed with a little ethanol, and recrystallized from the same solvent. The white crystalline 1,3-bis(*p*-chlorobenzylideneamino)guanidine nitrate (m.p. 198°C with decomposition) had acceptable analyses for C, H, N, and Cl.

The methyl sulfate (m.p. 256° to 259°C with decomposition, after partial melting at 217° to 224°C and resolidification) and the hydrochloride (m.p. 289° to 290°C with decomposition) salts were prepared in a similar manner (*4*).

The hydrochloride salt (robenzidene), at 66 parts per million in feed, is the preferred form for general use, though each of the above salts has equivalent activity in terms of their base content.

Anticoccidial activity in the feed was tested in broiler-type cockerels; customary methods and criteria of efficacy were used (*5*). Robenzidene was included in the diet fed freely from 1 to 2 days before the sporulated oocysts were inoculated until 6 or 7 days afterward, when the birds were killed, and autopsies were performed to evaluate gross intestinal lesions.

When robenzidene at a concentration of 66 ppm was tested against six species of *Eimeria* individually and against a mixture of eight species of *Eimeria* (*6*) (Table 1), it prevented retardation of body weight gain, appreciable gross intestinal lesions, and mortality due to coccidiosis.

In other trials, robenzidene was equally effective in 3-day-old and 11-week-old chickens and in chickens receiving 12 or 24 hours of light daily, as well as in tests of longer duration. It was effective against a strain of *E. acervulina* partially resistant to sulfanilamides (*7*) and against some strains of five species which were incompletely controlled by one or more of the following chemically diverse anticoccidial agents: quinolones, amprolium plus ethopabate, clopidol, or 7-bromo-6-chlorofebrifugine. Forty-one recent field collections of chicken coccidia obtained from diagnostic laboratories throughout the United States, many with multiple *Eimeria* species, were fully responsive to robenzidene in severe laboratory challenges, except for some strains of *E. maxima*. With the latter, there was also high clinical efficacy, but greatly reduced oocyst production persisted.

Uninoculated chickens reared in floor pens and fed 198 parts per million of robenzidene continuously for 8 weeks had weight gains and feed intakes equal to those of the unmedicated controls. Birds fed 330 parts per million weighed 7 percent less than the unmedicated controls ($P < .01$) (*4*).

No significant pathological changes (grossly, microscopically, or in clinical chemistry) were found in dogs and rats that received in feed about 20 mg of compound per kilogram of body weight per day for 90 days.

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