Our results confirm the view that the rate of dehydroxylation of kaolinite (and probably of other similar hydroxyl-containing minerals) depends sensitively on vapor pressure because of chemisorption of water on the mineral surfaces.

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Puromycin Analogs: Action of Adrenocorticotropic Hormone and the Role of Glycogen

Abstract. *The effect of the injection into rats of analogs of puromycin, 6-dimethylaminopurine, and the aminonucleoside of puromycin on the stimulation of steroidogenesis by adrenocorticotropic hormone was compared with that of puromycin and cycloheximide. This stimulation was blocked only by the antibiotics, which also inhibited adrenal protein synthesis. Glycogenolysis is not associated with the primary mechanism of the adrenocorticotropic hormone stimulation of steroid hormone biosynthesis in rats.*

Adrenocorticotropic hormone (ACTH) stimulates steroid biosynthesis by a mechanism involving protein synthesis (*1–3*). The injection into rats of inhibitors of protein synthesis, puromycin or cycloheximide, blocked the ACTH stimulation of steroidogenesis in vivo. These studies suggested that this action of ACTH might be caused by activating the synthesis of protein undergoing rapid turnover (*3*). In that the injection of puromycin into mice resulted not only in an inhibition of protein synthesis, but also a depletion of hepatic glycogen (*4*), it has been suggested that the puromycin inhibition of ACTH-stimulated steroidogenesis could be explained primarily by its glycogenolytic effect (*5*).

We have attempted to clarify this issue and conclude that the antibiotics inhibited the ACTH stimulation by blocking protein synthesis.

Albino rats (200 g) were hypophysectomized from 2 to 6 hours before the start of the experiment. The effect of ACTH on corticosterone production by rat adrenals in vivo and the incorporation of radioactive amino acids into adrenal protein were determined as described (*3*). Glycogen concentration in liver and adrenal was determined by the Anthrone method (*6*).

The analogs of puromycin, 6-dimethylaminopurine, and the aminonucleoside of puromycin do not inhibit protein synthesis but, like puromycin, cause a decrease in hepatic glycogen concentration (*4*). To observe the effect of these compounds on the stimulation of steroidogenesis by ACTH, they were injected intraperitoneally into hypophysectomized rats; 15 minutes later there was a marked decrease in liver glycogen, but there was no change in adrenal glycogen (Table 1). However, the stimulation of steroidogenesis by ACTH was inhibited only by those antibiotics that blocked adrenal protein synthesis—cycloheximide and puromycin. The aminonucleoside of puromycin and 6-dimethylaminopurine, which did not inhibit either protein synthesis or steroidogenesis, caused an even greater fall in hepatic glycogen concentration than puromycin.

These antibiotics failed to stimulate hepatic glycogen depletion in anesthetized animals (*4*); therefore, the effect of inhibitors of protein synthesis on ACTH action under these conditions was studied. Thirty minutes after hypophysectomized rats were anesthetized with pentobarbital, puromycin or cycloheximide was adminstered intraperitoneally. Although hepatic or adrenal glycogen concentration was not diminished, puromycin and cycloheximide inhibited adrenal protein synthesis and still blocked the ACTH stimulation of steroidogenesis (Table 2).

To demonstrate further that the antibiotics affect the ACTH stimulation of steroidogenesis by their action on protein synthesis, the experiment described in Table 3 was performed. When adrenal glands were incubated for 30 minutes to 1 hour in Krebs-Ringer bicarbonate buffer, the concentration of adrenal glycogen fell markedly, in agreement with the observations of Vance *et al.* (*7*). However, the ACTH stimulation of steroidogenesis was not impeded in these glycogen-depleted glands. Furthermore, when

Table 1. Effect of glycogenolysis and inhibition of protein synthesis on the ACTH stimulation of steroidogenesis. Recently hypophysectomized rats were injected intraperitoneally with 10 mg of cycloheximide, 30 mg of aminonucleoside of puromycin, or 14 mg of 6-dimethylaminopurine; 15 minutes later the animals were killed, and glycogen in the liver and adrenals was measured (optical density, per 100 mg, at 620 mμ, as recorded in a Coleman spectrophotometer). Results are means ± standard error (seven animals in each group). In similarly treated rats, ACTH was administered intravenously 15 minutes after treatment with the antibiotics. Corticosterone was assayed from the adrenal-vein blood obtained from 7 to 10 minutes after ACTH treatment (*3*). Simultaneously with the injection of ACTH, that is, 10 min. before the animals were killed, 4μc of C^{14}-labeled algal protein hydrolyzate was injected intravenously; when the animals were killed, the adrenals were removed immediately, and the incorporation of radioactive amino acids into protein was determined (*3*). Results are means ± standard rror determination of four different animals.

Treatment	Glycogen		Cortiscosterone (μg/3 min)	C^{14} incorporated (dpm)*
	Liver (OD/100 mg)	Adrenal (OD/100 mg)		
Adrenal baseline (no ACTH)		1.7 ± .3	0.2 ± .02	4900 ± 300
ACTH + no antibiotic	4.3 ± 0.9	1.7 ± .2†	1.3 ± .2	4978 ± 210
ACTH + puromycin	1.6 ± 0.5	1.8 ± .1	0.2 ± .1	686 ± 52
ACTH + cycloheximide	0.4 ± 0.2	1.7 ± .1	.3 ± .02	200 ± 60
ACTH + aminonucleoside of puromycin	.4 ± .01	1.9 ± .2	1.7 ± .2	6363 ± 697
ACTH + 6-dimethylaminopurine	.2 ± 0.1	1.7 ± .1	1.6 ± .4	4265 ± 124

* Disintegrations per minute per 100 mg of adrenal tissue. † Only six adrenals tested in this group.

Table 2. Effect of the inhibition of protein synthesis on the ACTH stimulation of steroidogenesis after preliminary treatment with pentobarbital anesthesia. The experiments and assays were performed exactly as in Table 1, except that all animals were anesthetized with 8 mg of pentobarbital intraperitoneally 30 minutes before the intraperitoneal injection of 30 mg of puromycin or 10 mg of cycloheximide. The number of rats is indicated by parentheses.

Treatment	Glycogen		Corticosterone (μg/3 min)	C^{14} incorporated (dpm)*
	Liver (OD/100 mg)	Adrenal (OD/100 mg)		
Adrenal baseline (No ACTH)		1.7 ± .2 (6)	0.2 ± .01 (4)	3800 ± 186 (4)
ACTH	2.3 ± .6 (5)	1.8 ± .3 (7)	1.6 ± .2 (4)	3210 ± 227 (4)
ACTH + puromycin	2.3 ± .5 (8)	1.7 ± .1 (7)	0.24 ± .2 (4)	786 ± 59 (4)
ACTH + cycloheximide	2.4 ± .5 (7)	1.9 ± .2 (6)	0.14 ± .02 (4)	83 ± 14 (4)

* Disintegrations per minute per 100 mg of adrenal tissue.

these glands were incubated with the purine analog 6-dimethylaminopurine, which markedly activates hepatic glycogenolysis, the ACTH stimulation of steroid biosynthesis was unaffected. As Ferguson showed (*1*), the ACTH stimulation of steroidogenesis in vitro was blocked by the antibiotics which inhibited adrenal protein synthesis (Table 3). He also demonstrated that the aminonucleoside of puromycin failed to inhibit the ACTH stimulation in vitro (*1*).

The observation that puromycin and its analogs caused hepatic glycogen depletion prompted our investigations. The mechanism of this antibiotic effect is not known, nor is the inhibition of this phenomenon by anesthesia understood. However, this effect doesn't appear to be generalized since glycogen concentrations of muscle (*8*) and adrenals are unaffected by these compounds, and this action on liver glycogen does not correlate at all with the effect of inhibitors of protein synthesis on the stimulation of steroidogenesis. Indeed, the data demonstrate that the antibiotics inhibited the ACTH stimulation of steroid hormone synthesis only when they also blocked protein synthesis, regardless of their effect on glycogen concentrations.

The possibility that adrenal glycogenolysis was even involved in the ACTH stimulation of steroid hormone production was brought forth by the investigations by Haynes *et al.* (*9, 10*), who showed that ACTH caused an increased concentration of cyclic 3, 5-adenosine monophosphate (AMP) which activated phosphorylase to degrade glycogen in bovine adrenal slices. This, it was postulated, resulted in an increased availability of intermediates which stimulated the production of reduced nicotinamide-adenine dinucleotide phosphate (NADPH) required for steroid biosynthesis.

Although Noble and Papageorge (*11*) and Greenberg and Glick (*12*) demonstrated that ACTH caused a decreased glycogen concentration in the adrenals of the rat, their observations were made 3 hours or more after the administration of ACTH to intact rats, whereas ACTH starts stimulating steroidogenesis within 2 minutes and a maximum is reached 10 minutes after administration. Furthermore, unlike the findings with bovine adrenals, ACTH did not activate phosphorylase in rat adrenals (*1, 13*); and when rats adrenals were incubated with glucose-1-C^{14}, the specific activity of the $C^{14}O_2$ was not diminished after the addition of ACTH in concentrations which markedly stimulated steroidogenesis (*7*). This would have occurred, however, if ACTH caused glycogen breakdown to the intermediates of the hexose monophosphate shunt, the pathway proposed by Haynes for the ACTH stimulation of NADPH. Even though Greenberg and Glick demonstrated an increase in the amount of active phosphorylase in certain specific areas of the adrenal cortex, it was shown that this was not the result of an increase in the activation of inactive phosphorylase (*14*). This finding is different from that of Haynes *et al.* who demonstrated such an activation in bovine adrenals in response to ACTH (*10*). Furthermore, interpretation of the studies of Greenberg and Glick remains unclear because the adrenals were again examined as long as 3 hours after ACTH treatment, and the areas of the adrenal cortex which showed an increase in phosphorylase could not be correlated with the areas where they had observed glycogenolysis (*12*). Whenever a decrease in adrenal glycogen concentration was detectable by the elegant studies of Greenberg and Glick (that is, 3 hours after ACTH treatment), it was also easily shown by chemical methods (*11*) similar to those used in our investigation. However, in our study, and in that of Vance *et al.* (*7*), no decrease in the glycogen concentration of hte rat adrenal occurred within 10 minutes after ACTH treatment (that is, when steroidogenesis was markedly stimulated). Of course, this does not rule out the possibility that, even at this early time after ACTH treatment, glycogen breakdown had started but was not sufficient to be detectable. Nevertheless, there is no evidence to indicate that glycogenolysis, whether it occurs at this time or not, is a primary event in the ACTH stimulation of steroidogenesis in the rat.

Even though ACTH does not act primarily through phosphorylase in the rat (*1, 13*), the possibility is not excluded that ACTH may still act through cyclic-AMP (*10*), which apparently activates several enzymes and cellular processes in addition to activating phosphorylase (*15*). Furthermore, even though ACTH does not activate phosphorylase in the rat, cyclic-AMP still stimulates adrenal steroidogenesis in vivo and in vitro (*10, 16*), just as ACTH does in these animals. Even in bovine adrenals where cyclic-AMP activates phosphorylase, puromycin prevents the

Table 3. Glycogen concentrations and steroid secretions by rat adrenals in vitro. The adrenal glands obtained from 200-g rats were quartered and incubated at 37°C in Krebs-Ringer bicarbonate buffer containing 200 mg of glucose per 100 ml (*17*). Thirty minutes after this incubation, the media were removed, the buffer was replaced, and the vessels were distributed into groups in which the following additions were made: 0.2 unit of ACTH; 0.2 unit of ACTH plus 2 μM cycloheximide; 0.2 unit ACTH plus 2 μM puromycin; 0.2 unit ACTH plus 2 μM 6-dimethylaminopurine; controls—no additions. The vessels were incubated for an additional 2 hours at 37°C. The media were extracted with methylene chloride and assayed for corticosterone (*18*) and the adrenal glands were assayed for glycogen (*6*). The glycogen concentration of the incubated glands was also compared with that of control adrenal glands which were not incubated.

Prior incubation (min)	Glycogen (OD/100 mg adrenal)	Corticosterone per hour (μg/100 mg)
	No addition	
0	1.8 ± .3 (7)*	
30	0.5 ± .02 (7)	3.0 ± 0.5 (7)
	ACTH added	
30	0.5 ± .10 (7)	9.0 ± 1.3 (7)
	Puromycin plus ACTH	
30	0.4 ± .01 (7)	3.4 ± .5 (7)
	6-Dimethylaminopurine + ACTH	
30	0.4 ± .06 (7)	12.0 ± 1.4 (7)
	Cycloheximide + ACTH	
30	0.4 ± 0.3 (7)	2.4 ± .6 (7)

* Parentheses signify the number of rats.

stimulation of steroidogenesis without blocking this enzyme activation (*1*). The possibility that the ACTH stimulation of steroidogenesis is mediated through the activation of protein synthesis by cyclic-AMP, therefore, is not incompatible with our findings (*3*).

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Endrin: Use of Concentration in Blood To Diagnose Acute Toxicity to Fish

Abstract. *Channel catfish,* Ictalurus punctatus *(Rafinesque), were exposed to continuously renewed solutions of endrin in water. Analyses of the fish blood by gas chromatography revealed a well-defined threshold concentration of endrin in the blood, approximately 0.30 microgram per gram, that, if exceeded, results in death. Fish exposed to lethal concentrations of endrin in water for periods of time insufficient to cause death had blood-endrin concentrations markedly lower than those that died from exposure to the same water. There was little overlap in range of endrin concentration in blood between dead and living exposed fish.*

Probably no other single group of chemicals has been accused more frequently of causing fish kills than the modern pesticides. There is a serious need for techniques that will give additional proof that pesticides are the cause of death of fish or wild animals. During the 1963–64 investigations of large fish kills on the lower Mississippi River, a direct relationship was observed between endrin concentration in blood and intoxication of channel catfish, *Ictalurus punctatus* (Rafinesque). These observations, plus additional ones obtained more recently, are presented in this report.

The concentrations of pesticides in various organs of animals have been measured frequently, but the purpose was often for survey data and not for toxicological studies. Among those who have tried to relate concentrations in tissue with intoxication, Brown *et al.* reported that in the blood of man and dogs there is a critical concentration of dieldrin above which clinical signs of intoxication appear (*1*); and Burdick *et al.* reported a relatively close relationship between the DDT content of eggs and fry mortality in lake trout (*2*).

Dale *et al.* found a relationship between the degree of illness and the concentration of DDT in rat brains, and they also noted a sharp rise in the concentration of DDT in plasma after administration of the insecticide (*3*). The plasma concentration, however, remained approximately constant from the slight tremor stage through convulsions and death. In our efforts to detect acute endrin toxicity, we used the concentration of endrin present in the blood of fish that had been exposed to endrin dispersed in water. We reasoned that blood was the vehicle by which endrin reached the target organ and that the concentration of endrin in the blood should be related to intoxication. This work was begun before the paper by Brown *et al.* (*1*) was available.

Channel catfish, weighing from 200 to 500 g, were exposed to 15 different lethal and sublethal concentrations of endrin in water. The test water was renewed continuously by a serial dilution apparatus (*4*) from a water-supply system similar to the one described by Lemke and Mount (*5*). Renewal of the endrin was necessary to maintain known and constant concentrations. Tap water was passed through a 1.6-m^3 activated carbon filter as a safety precaution to remove traces of pesticides and then to a 375,000-liter pond reservoir where it was detained 60 to 90 days before use. This detention period minimized daily variations in chemical characteristics. Tests were made at 15° and 22°C ± 1°C in stainless-steel troughs 30 by 30 by 180 cm. All water lines and valves were made of plastic or stainless steel; after the introduction of endrin, contact of the test water with plastic was minimized, for many plastics readily sorb endrin. Three fish were placed in each trough, and the rates of water flow were controlled so as to give approximately 1 ml of water per 2 g of fish per minute. Flow rates were not adjusted during a given test when one or two fish died.

Fish were obtained from four locations (one in Arkansas and three in Ohio); before exposure, all contained less than 0.03 μg/g of endrin in the blood. Blood was obtained by drying and severing the caudal peduncle and allowing the blood to drip into an acetone-rinsed vial; the sample was then kept frozen until analysis. Fish were removed at the time of death; those that died during the night were discarded.

The blood samples were assayed by the procedure described by Schafer, Busch, and Campbell (*6*). If less than a 10-g sample of blood was used (usually 2 to 3 g was available), the sample weight was adjusted to 10 g with distilled water. Reagent blanks were consistently negative for endrin or other interferences. After saponification of the blood sample with alcoholic KOH, the chlorinated hydrocarbons were extracted into hexane. Measured volumes of the hexane extract were assayed with a gas chromatograph equipped with an electron-capture detector. Chromatograms of samples were compared with chromatograms of standard solutions of recrystallized analytical reference standard endrin (Shell). Some of the sam-