ions agree very closely with the experimental values. Also, the agreement is good even though the ratio r varies from anion to anion because of the increasing valency as predicted by Eq. 1. The measured potential as well as the citrate distribution is unaffected by KCN.

It is possible to test further the applicability of Eq. 1 and the accompanying assumption that the anionic distribution is imposed by a Donnan effect brought about by a positive internal fixed charge. The Donnan ratio r of Eq. 2 (*12*) for the univalent ion would be a function of the positive charges (z_pP) per concentration of fixed or impermeable molecules (P) and the anion

$$r = \frac{z_pP}{2A_o} + \left[\left(\frac{z_pP}{2A_o}\right)^2 + 1\right]^{1/2} \quad (2)$$

present in the outside medium (A_o). An increase in A_o would lead to a drop in r and consequently (see Eq. 1) to a drop in the measured potential. Alternatively, a decrease in z_pP brought about by osmotic swelling of the mitochondria should also decrease r.

With the mean state-4 potential of +9.8 mv, Eq. 1 leads to the calculation of an r of 1.5 for the case of a monovalent anion. With an external anion concentration of 1 mM (either pyruvate or acetate at 1 mM) the z_pP of Eq. 2 corresponds to approximately 0.8 mM.

Figure 1 illustrates an experiment in which the external tris(hydroxymethyl)-aminomethane (tris) acetate concentration is varied from 1 mM to 50 mM. The values calculated for the membrane potential at different acetate concentrations (with Eqs. 1 and 2 at z_pP = 0.8 mM) are shown by the lower dashed line. The upper line represents the observed curve where the potentials were measured directly with the microelectrodes. Although the two curves are not superimposable, the observed potentials are not too distant from the predicted values. Acetate causes little or no swelling under these conditions as determined in two independent experiments by the photometric technique of Tedeschi and Harris (*13*). The maximum difference between the control and the experimental values was negligible throughout the range used.

When osmotic swelling is induced (table 2 in *1*) by suspending the mitochondria in a hypotonic medium (109 milliosmolal), the potential drops by an average of 6.7 mv from the control potential (in a 450 milliosmolal solution). Since the amount of z_pP is presumably fixed, a fourfold increase in volume would reduce the concentration to one-fourth of its original value. It is possible to calculate from Eqs. 1 and 2 that the potential should drop approximately 7.5 mv, in agreement with the measured value.

The results support the hypothesis that the measured potential is across the mitochondrial semipermeable membrane. The properties of the potential suggest that it is the result of the distribution of anions imposed by a Donnan effect.

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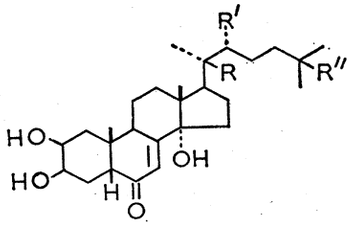
Ecdysone Analog: Conversion to Alpha Ecdysone and 20-Hydroxyecdysone by an Insect

Abstract. *The tritium-labeled synthetic ecdysone analog Δ^7-5β-cholestene-2β,3β,14α-triol-6-one terminated diapause when injected into diapausing tobacco hornworm pupae and was converted into tritium-labeled α-ecdysone and tritium-labeled 20-hydroxyecdysone. About half of the crystalline α-ecdysone and 20-hydroxyecdysone isolated from the tobacco hornworms 7½ to 8½ days after injection was derived from endogenous steroid precursors and half from the transformation of the synthetic ecdysone analog.*

Certain synthetic ecdysone analogs exhibit molting-hormone activity in ligated abdomens of *Diptera* (*1*) and severely inhibit larval growth and ovarian maturation in several species of insects (*2*, *3*). In addition to these effects, injection of the synthetic ecdysone analog Δ^7-5β-cholestene-2β,3β,-14α-triol-6-one (**1**, triol) terminated pupal diapause in the tobacco hornworm *Manduca sexta* (Johannson). In association with this physiological phenomenon we report our results on the conversion of the [^{3}H]triol to [^{3}H]α-ecdysone (**2**) and [^{3}H]20-hydroxyecdysone (**3**) in this insect.

The 1α[^{3}H]triol was prepared from 1α[^{3}H]cholesterol according to procedures used for the synthesis of the unlabeled compound (*4*); [observable specific activity, 1757 disintegrations per minute (dpm)/μg (*5*)]. Male, diapausing, tobacco hornworm pupae (669) collected from the field were injected in the ventral intersegmental membrane between the fifth and sixth abdominal segments with a microsyringe. The [^{3}H]triol was administered at 10 μg per gram of body weight (1 μg/μl, in 60 percent acetone solution). Initially we observed that doses of 1 to 10 μg per gram of body weight terminated diapause in the treated organisms and that the externally visible character of eye pigmentation (*6*) served as a valid and reliable indicator for ascertaining termination of diapause or spontaneous development, or both, in the intact organism. With eye pigmentation as the criterion, we harvested 621 insects (1.99 kg, 3.5×10^7 dpm) 7½ to 8½ days after injection. To determine the metabolic fate of the triol, we extracted the hornworms as described (*7*). The crude extractive (2.0 g, 1.9×10^7 dpm) was first fractionated on silicic acid (*8*) (Table 1) because this column permitted the separation of the unmetabolized triol from the ecdysones and more polar metab-



1, R = R′ = R″ = H

2, R = H, R′ = R″ = OH

3, R = R′ = R″ = OH

olites. Fraction 1 (Table 1) contained the unmetabolized triol, and analysis by thin-layer chromatography showed that only one-half of the radioactivity present in the fraction was associated with the triol. These findings indicate that over 98 percent of the administered triol was metabolized by the hornworm. Approximately 14.6 percent of the administered dose and 92 percent of the total biological activity (*9*) was eluted in fractions 4 through 7 (207.8 mg, 5.0×10^6 dpm), which are known to elute α-ecdysone and 20-hydroxyecdysone.

Fractionation of this material on alumina (see *10*) with methanol as the eluant reduced the mass to one-third, without any loss of either radioactivity or biological activity. The methanol eluate (68 mg, 5.15×10^6 dpm) was subjected to 60 transfers in a countercurrent distribution system of cyclohexane, butanol, and water (5 : 5 : 10) with 10 ml of each phase. Analyses of the fractions both by radioassay and ultraviolet spectroscopy showed two major peaks. The relative distribution of the radioactive peaks was 71.9 percent in tubes 9 through 31 and 28.1 percent in tubes 32 through 50 for the polar and the apolar compounds, respectively. The radioactive peaks closely paralleled the peaks obtained by ultraviolet spectroscopy. However, quantitative analysis by ultraviolet spectroscopy indicated a quantity of ecdysones 2 to 2.5 times greater than that indicated by radiometric analysis. After silicic acid chromatography (*7*) and crystallization from ethyl acetate, the more polar compound (10.6 mg; 3.54×10^6 dpm), tubes 13 through 26, yielded 2.82 mg of 20-hydroxyecdysone [specific activity, 760 dpm/μg (theoretical, 1581 dpm/μg)]. After fractionation and crystallization from ethyl acetate, the apolar compound (5.0 mg; 1.3×10^6 dpm), tubes 36 through 48, yielded 0.54 mg of α-ecdysone [specific activity 802 dpm/μg (theoretical, 1636 dpm/μg)]. The physical properties, including nuclear magnetic resonance and mass spectra, of the crystalline 20-hydroxyecdysone and α-ecdysone from the hornworm were identical to those of authentic standards. The isolated crystalline [^{3}H]ecdysones were radiochemically pure, as determined by a number of thin-layer and column chromatographic systems.

Table 1. Fractionation on silicic acid column of crude extractive isolated from tobacco hornworms injected with tritium-labeled triol.

Fraction	Volume (ml)	Weight (mg)	Total dpm (×10^5)	Housefly units (*9*) (% of total)
	Benzene-methanol (95:5)			
1	3550	82.4	15.0	0.6
2	1775	45.1	1.6	0.1
3	1775	99.1	0.7	0.1
	Benzene-methanol (90:10)			
4	940	74.7	7.5	12.0
5	940	54.3	23.0	50.0
6	940	50.2	15.0	25.0
7	940	28.6	4.5	5.0
8	940	38.5	4.4	1.5
9	940	28.6	4.5	0.5
10	940	25.2	3.3	0.2
	Benzene-methanol (75:25)			
11	940	134.2	28.0	2.0
	Methanol			
12	940	1195.0	30.0	2.0
	Water			
13	940	132.3	3.0	1.0

The specific activity of the crystalline compounds is about 50 percent of theoretical, an indication that approximately one-half of both α-ecdysone and 20-hydroxyecdysone was derived from the [^{3}H]triol and that the other half was derived from endogenous sterol precursors. This was further substantiated in the housefly assay (*9*). Therefore in diapausing hornworm pupae, the triol or its metabolites trigger the biosynthetic mechanism for the syntheses of the ecdysones from endogenous steroid precursors, and the compound also serves as a precursor for the molting hormones.

We postulated that certain of the synthetic ecdysone analogs including the triol may be similar to or identical with intermediates involved in ecdysone biosynthesis (*2*). Our results with the hornworm strongly support this hypothesis. The administered [^{3}H]triol readily enters the steroid pool in the tobacco hornworm and is efficiently converted to [^{3}H]α-ecdysone and [^{3}H]20-hydroxyecdysone. In addition to these two major insect molting hormones, we have evidence by countercurrent distribution and bioassay of the more polar fractions for the presence of [^{3}H]20,-26-dihydroxyecdysone, the third molting hormone isolated from the tobacco hornworm (*11*). The report that [^{3}H]-α-ecdysone is converted to 20-hydroxyecdysone in an insect (*12*), taken together with our finding that the three known hornworm ecdysones are biosynthesized from a common precursor, confirms our premise (*11*) that these three steroids are metabolites in the biosynthetic-degradative scheme of the ecdysones. Our data also point to the triol as a probable intermediate in the biosynthesis of the ecdysones in the tobacco hornworm.

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