Table 1. Diffusion coefficient values (D) measured intracellularly compared to values for dilute solution diffusion (25°C).

Solute	$D \times 10^{-5}$ (cm²/sec)		
	Intracellular	Dilute solutions	
Water	2.42 ± 0.28 [16]*	2.51 ± 0.01 (spin echo)	(4)
		2.28 (spin echo)	(5)
		2.25 ± 0.02 (^{3}H-tracer)	(6)
Urea	1.87 ± .15 [22]	1.36	(6)†
Glycerol	1.25 ± .25 [9]	0.95	(6)†

* Number in bracket indicates number of experiments. † Measured at 20°C and converted to 25°C by a factor 3 percent/°C = + 15 percent.

tion is questioned (*1*) and various authors have suggested a slower rate of intracellular diffusion, but few precise measurements have been attempted (*2*). We measured quantitatively the diffusion coefficient of radioactively labeled water, urea, and glycerol in the cytoplasm of the muscle fiber of the giant barnacle *Balanus nubilus* and then compared these results with the standard values measured in dilute solution.

The size of the muscle fiber (2 by 30 mm) allows dissection of individual fibers, therefore eliminating the potential difficulties of extracellular constituents and their contribution to diffusion. Each cell was tied by a fine thread at the tendonous end and hooked at the open end. It was then extended to normal resting length on a screw clamp, and placed vertically in a diffusion chamber with the open end immersed in the experimental solution. This apparatus allowed single dimensional diffusion from an essentially infinite volume of a dilute iso-osmotic solution containing labeled water, urea, or glycerol. The remaining part of the cell above the solution level was covered with a light oil to prevent drying and contamination via evaporation and condensation. After 90 minutes, the cell was removed, frozen, and sliced into 5-mm segments. Each segment was dissolved, its solute concentration was measured by liquid scintillation counting, and its diffusion coefficient was calculated.

The solution to Fick's law of diffusion for the nonsteady state of one dimensional diffusion from a source of constant concentration is given by the equation of Höber (*3*)

$$C_x = C_0\left(1-\frac{2}{\sqrt{\pi}}\int_0^y e^{-t^2}dt\right)$$

where $y^2 = x^2/4\,DT$; C_x is the concentration of the diffusion molecule at distance x from the source of concentration C_0 and at time T. C_x is assumed to be 0 at T_0; D is the diffusion coefficient expressed in square centimeters per second. Error function tables greatly simplify the calculations.

Our results, together with values obtained by others (*4–7*), are given in Table 1. The intracellular diffusion coefficient for water was $2.42 \pm .28 \times 10^{-5}$ cm²/sec, which is midway between the two values for the self-diffusion of water. Similarly, the experimental value for glycerol of $1.25 \pm .25 \times 10^{-5}$ cm²/sec does not differ from the measured value for diffusion in water. The value for urea of $1.87 \pm .15 \times 10^{-5}$ cm²/sec is somewhat higher than expected.

At least two possible complicating factors are present but, if significant, would tend to slow intracellular diffusion, not hasten it. Approximately 40 percent of the barnacle water is bound, and presumably binding could occur with the test molecules (*7*). The results suggest that the unbound water behaves, from a diffusional standpoint, as a dilute solution. Second, the concentration of barnacle cytoplasm is not dilute but is approximately 1*M*. However, the diffusion coefficient is proportional to the activity coefficient, and if activity coefficients change significantly with concentration they are lowered with increasing concentration.

The purpose of these experiments was to test the hypothesis that diffusion proceeds more slowly intracellularly than in dilute aqueous solution. This hypothesis was rejected for all three solutes. It would appear from calculation of permeability coefficients that one may reasonably assume that the rates of intracellular diffusion are equal to those in dilute solution.

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References and Notes

1. D. A. T. Dick, *Int. Rev. Cytol.* **8**, 87 (1959); G. N. Ling, M. M. Ochsenfeld, G. Karreman, *J. Gen. Physiol.* **50**, 1807 (1967).
2. A. Krogh, *J. Physiol.* **52**, 291 (1919); A. L. Hodgkin and R. D. Keynes, *ibid.* **119**, 513 (1953); *ibid.* **131**, 592 (1956); S. Løvtrup, *J. Theor. Biol.* **5**, 34 (1963).
3. R. Höber, *Physical Chemistry of Cells and Tissues* (Blakiston, Philadelphia, 1945), p. 7.
4. N. J. Trappenier, C. J. Gerritsma, P. H. Oosting, *Phys. Lett.* **18**, 256 (1965).
5. L. Devell, *Acta Chem. Scand.* **16**, 2177 (1962).
6. *International Critical Tables*, vol. 5, p. 63, (1929).
7. S. G. A. McLaughlin and J. A. M. Hinke, *Can. J. Physiol. Pharmacol.* **44**, 837 (1966).

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Diphtheria Toxin Subunit Active in vitro

Abstract. *Exposure of diphtheria toxin to dithiothreitol (and similar thiols) resulted in a subunit which was active in catalyzing the adenosine diphosphate–ribosylation of mammalian aminoacyl-transferase II in the presence of nicotinamide adenine dinucleotide. At the same time there was a marked increase in total ADP–ribosylation activity. A molecule which was apparently identical to the derived subunit in size and activity was detected in partially purified preparations of toxin.*

Diphtheria toxin has been crystallized in several laboratories. It is a simple protein of molecular weight about 65,000 (*1–3*). That the toxin molecule may be composed of more than one polypeptide chain is suggested by the observations that the sedimentation coefficient decreases from 4.2*S* to about 2*S* after treatment with sulfite (*3*) and that more than one equivalent of amino-terminal amino acid is present per 65,000 daltons of toxin (*4*). We now report the identification in supernatants from cultures of *Corynebacterium diphtheriae* of a molecular species that is approximately half the size of native toxin and exhibits the specific catalytic activity in vitro attributed to native toxin (*5, 6*). In addition we have derived a subunit of apparently identical size and high activity by exposure of native toxin to thiols such as dithiothreitol (DTT). Such treatment of native toxin also results in an increase in catalytic activity.

Diphtheria toxin inhibits protein synthesis in mammalian cells and cell-free systems derived therefrom (*7, 8*). This inhibition requires the presence of nicotinamide-adenine dinucleotide (NAD) and is effected through specific inactivation of aminoacyl-transferase

II (*8, 9*), one of two supernatant enzymes involved in the transfer of amino acids from transfer RNA to ribosomes (*10*). Honjo *et al.* (*5*) and Gill *et al.* (*6*) have elucidated the mechanism of inactivation of transferase II by demonstrating that toxin catalyzes the covalent attachment of the adenosine diphosphate ribose moiety of NAD to the transferase, with the concomitant release of nicotinamide. We have obtained evidence to support this mechanism by showing that toxin catalyzes the transfer of virtually 100 percent of the label from NAD-(^{14}C-adenosyl) to acid-precipitable material in the presence of excess transferase II. This reaction system has been used to measure toxin activity in our experiments.

Toxin was prepared by growing *C. diphtheriae,* strain PW-8 (SM-1 variant) according to the method of Yoneda (*11*). After the cells were removed by centrifugation the supernatant was fractionated with ammonium sulfate. The fraction precipitating between 45 and 60 percent saturation was dissolved in buffer containing tris-HCl (50 mmole/liter), *p*H 7.5, and EDTA (0.5 mmole/liter), and chromatographed on a column of Sephadex G-100 equilibrated with the same buffer. The fractions obtained were assayed for adenosine diphosphate (ADP)–ribosylation activity in the presence of NAD-(^{14}C-adenosyl) plus a protein fraction from rabbit reticulocytes containing transferase II. Two peaks of activity were detected (Fig. 1). The protein from peak I had a sedimentation coefficient of about 4*S* in the analytical ultracentrifuge and flocculated rapidly in the presence of horse diphtheria antitoxin; we concluded, therefore, that this peak corresponded to the 65,000-dalton species of toxin previously described. The fact that peak II was retarded to a greater extent than peak I on Sephadex G-100, thus corresponding to a species of lower molecular weight, led us to suppose that this peak might represent a catalytically active subunit of the toxin. Data obtained subsequently have all been in accord with this hypothesis.

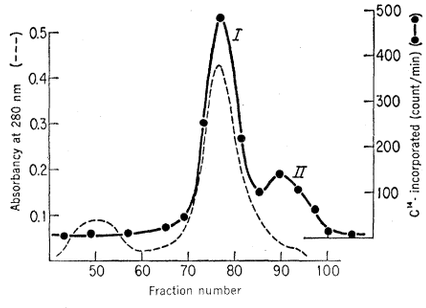

Fig. 1. Fractionation of crude diphtheria toxin on Sephadex G-100. Twenty-one milligrams of protein precipitated from a culture supernatant between 45 and 60 percent saturation with ammonium sulfate were dissolved in 10 ml of buffer (50 m*M* in tris-HCl, *p*H 7.5, and 0.5 m*M* in EDTA) and fractionated on a column of Sephadex G-100 (2.5 by 84 cm) equilibrated with the same buffer. Fractions (3 ml) were collected, and 5 μl samples from the fractions indicated were added to reaction mixtures containing 12 μmole of tris-HCl, *p*H 7.5; 0.25 μmole of EDTA; 1.25 μmole of DTT; and 190 μg of protein (an ammonium sulfate precipitate at 40 to 60 percent saturation) from rabbit reticulocytes containing transferase II (*9*). Then NAD-(^{14}C-adenosyl) (1200 count/min; specific activity 410 mc/mmole) was added to each mixture to give a final volume of 0.25 ml; after 15 minutes at 37°C, 1 ml of 5 percent trichloroacetic acid (TCA) was added to stop the reaction. The precipitates were then collected on 2.5-cm Whatman GF/C glass-fiber disks, washed with 5 percent TCA, and dried; the radioactivity was counted in a thin-window gas-flow counter.

After chromatographing peaks I and II separately on Sephadex G-100, we studied the sedimentation behavior of each in sucrose density gradients. In Fig. 2, a and b, it can be seen that each component sedimented as a single, virtually symmetrical peak. From the relative positions of the peaks in this figure, and from other similar experiments, we have calculated that the ratio of the sedimentation coefficient of the smaller component to that of the larger is 0.59 ± 0.03. Thus, given the value of $s_{20,w}$ (sedimentation coefficient) of 4.2*S* for the 65,000-dalton component (*2*), we estimate an $s_{20,w}$ of 2.5 ± 0.15*S* for the subunit. The positions of marker proteins in identical gradients (bovine serum albumin, ovalbumin, chymotrypsinogen, and myoglobin) correlate well with this estimate.

Bizzini *et al.* (*12*) have shown that the 4.2*S* toxin contains four half-cystine residues per 64,500 daltons but that it contains no free sulfhydryl groups; they concluded, therefore, that there were two disulfide bridges per toxin molecule. If either or both of the disulfide bridges formed an interchain linkage within the molecule, then rupture of the bridge or bridges by reduction might lead to dissociation of the constituent chains. We incubated the 4.2*S* toxin with 0.25*M* DTT and found that the ADP-ribosylation activity then sedimented at a rate apparently identical to that of the 2.5*S* subunit isolated from culture supernatants (Fig. 2c). Hereafter we refer to the latter as the 2.5*S* (native) subunit to distinguish it from the 2.5*S* (derived) subunit obtained by treatment of 4.2*S* toxin with DTT. There was no apparent change in the sedimentation behavior of the 2.5*S* (native) subunit after exposure to DTT (Fig. 2d).

The possibility that the observed change in sedimentation coefficient might result from a conformational change, or unfolding, of the 4.2*S* toxin

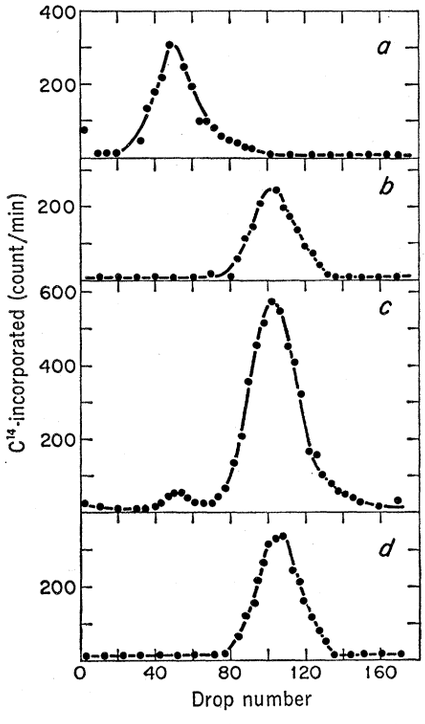

Fig. 2. Sucrose-gradient sedimentation analysis of peaks I and II from Sephadex G-100. Samples from peak I (10 μg of protein) or from peak II (11 μg protein) were incubated for 2 hours at 30°C in a final volume of 50 μl (peak I) or 100 μl (peak II) in the presence of 10 m*M* tris-HCl, *p*H 7.5, and 0.1*M* EDTA. In addition, to one of the two samples from each Sephadex peak, DTT was also added to give a final concentration of 0.25*M*. After incubation each sample was layered on a separate 12-ml, 10 to 25 percent linear sucrose gradient which was 50 m*M* in tris-HCl, *p*H 7.5; 50 m*M* in KCl; and 0.5 m*M* in EDTA. The DTT (5 m*M*) was also present in gradients used for samples which had been previously incubated with DTT. The gradients were centrifuged at 40,000 rev/min, 5°C, for 63 hours in a Spinco SW-41 rotor, and selected single-drop fractions were assayed for ADP-ribosylation activity as described under Fig. 1. (a) Sephadex activity peak I, incubated without DTT; (b) peak II, incubated without DTT; (c) peak I, incubated with DTT; (d) peak II, incubated with DTT. The top of the gradients is to the right.

rather than a dissociation into subunits, seems virtually excluded by the observation that the 2.5*S* (derived) subunit is retarded on Sephadex G-100 relative to the 4.2*S* species. Moreover a conformational change sufficient to cause an alteration of this magnitude in the sedimentation coefficient would be tantamount to denaturation and would not be likely to yield an active product.

In addition to demonstrating a marked change in sedimentation behavior, the data in Fig. 2 suggest that an increase in total ADP-ribosylation activity also occurs in the presence of DTT. Thus the total area under the activity curve of the 4.2*S* toxin (Fig. 2a) is less than half that of the 2.5*S* species (Fig. 2c) derived by treatment of an equivalent amount of 4.2*S* toxin with DTT. We have demonstrated this effect more clearly by assaying the ADP-ribosylation activity of 4.2*S* toxin and the 2.5*S* (native) subunit after varying periods of incubation in the presence of DTT. A net increase in activity of approximately twofold occurred over a 60-minute period when 4.2*S* toxin was incubated at 37°C in the presence of 2.5 m*M* DTT (Fig. 3). The activity of the control without DTT declined about 25 percent, while the activity of the 2.5*S* (native) subunit declined over 50 percent under the same conditions, suggesting a greater thermolability of the latter. The decline in activity of the 2.5*S* subunit was only slightly affected by the presence of DTT. Thus, DTT has a strikingly different effect on the activity of the 4.2*S* toxin.

Inasmuch as transferase II requires the presence of thiols to maintain its enzymatic activity (*13*), we routinely added small amounts of DTT to the reaction mixtures when assaying for ADP-ribosylation activity. In view of the effect of DTT on ADP-ribosylation activity shown above, one would expect to observe a progressive increase in the rate of ADP-ribosylation during the course of incubation of an assay mixture containing the 4.2*S* toxin. Indeed, a kinetic lag is observed at low DTT concentrations, and the lag can be abolished by addition of higher concentrations of DTT (about 50 mmole/liter) to the assay mixture, or by prior incubation of the toxin with DTT (*14*). A certain percentage of the activity of native 4.2*S* toxin samples must, therefore, be attributed to activation during the assay. While exact figures on the maximum increase in specific activity are not yet available, some data indicate that an enhancement of at least fivefold can be produced by exposure of 4.2*S* toxin to DTT (*14*). It is possible that the 4.2*S* toxin which has not been exposed to thiols may in fact be devoid of ADP-ribosylation activity.

A clear correlation between the processes of dissociation of 4.2*S* toxin into subunits and increase in ADP-ribosylation activity is not possible from the data presented here. The increase in activity apparently parallels the degree of dissociation of the 4.2*S* toxin, and dissociation appears to be requisite for the increase in activity (*14*). Thus the 2.5*S* subunit may well be the molecular species through which the intoxication of cells is effected.

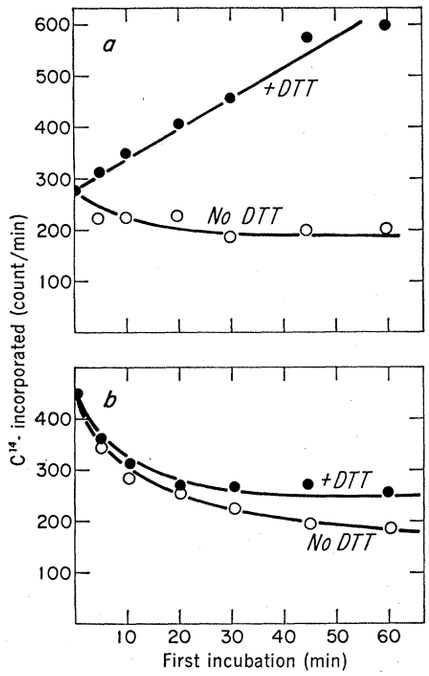

Fig. 3. Effect of DTT on the ADP-ribosylation activity of 4.2*S* toxin and the 2.5*S* (native) subunit. Toxin from Sephadex peak I (4 μg of protein) or peak II (3.5 μg of protein) was incubated at 37°C, in a final volume of 500 μl containing 5 μmole of tris-HCl, *p*H 7.5; 0.5 μmole of EDTA; 50 μg of bovine serum albumin; and 1.25 μmole of DTT. Control mixtures lacked DTT. At the indicated times 25-μl samples were withdrawn from each mixture and added to assay mixtures containing 12.5 μmole of tris-HCL, *p*H 7.5; 0.25 μmole of EDTA; and 190 μg of reticulocyte protein [fraction precipitated with $(NH_4)_2SO_4$, 40 to 60 percent saturation]. Dithiothreitol (62.5 μmole) was immediately added to control mixtures to bring the concentration to the same level as those containing toxin samples previously incubated with DTT. Then NAD-^{14}C was added to give a final volume of 0.25 ml, and the samples were incubated, and radioactivity was counted as described in Fig. 1; (a) 4.2*S* toxin; (b) 2.5*S* (native) subunit.

Goor (*15*) has demonstrated the presence of a form of toxin with a sedimentation coefficient of 6.8*S* in a preparation of toxin obtained from the Massachusetts Department of Public Health. He reported an apparent conversion of this form into 4.2*S* toxin upon exposure to DTT and concluded that the 6.8*S* form was a dimer of 4.2*S* toxin. We subjected a sample of toxin obtained from the same source to sucrose density gradient analysis and observed an active component with a sedimentation coefficient close to 7*S*. However, an identical sample treated with DTT exhibited activity only in the 2.5*S* region of the gradient. We are unable at present to explain this discrepancy. The 6.8*S* form has not been detected in our own preparations of toxin.

Gabliks and Falconer (*16*) have reported that toxin which has been exposed to sensitive human cells in culture exhibits an increased diffusion rate through dialysis membranes in parabiotic culture chambers and an increased potency when tested on mouse cells, which are normally highly resistant to toxin. The increased diffusion rate may be explained if sensitive cells are able to dissociate 4.2*S* toxin into highly active 2.5*S* subunits. In addition, we suggest that the normal resistance of mouse cells may be the result of an inability to dissociate 4.2*S* toxin.

From sedimentation data we have estimated the molecular weight of the 2.5*S* subunit as being about 29,000 and from gel-filtration data, as about 26,000. The probable error in these estimates, however, together with our lack of information on the possible heterogeneity of the component subunits, prevents our drawing conclusions concerning the number of 2.5*S* subunits per toxin molecule.

The 2.5*S* subunit found in culture supernatants may either be released from cells as such or may arise through dissociation of the 4.2*S* toxin after its release into the medium. The possibility that the subunit arises only at some step in the purification procedure is highly unlikely, since we have detected significant amounts of the subunit in sucrose gradient analyses of unprocessed culture supernatant.

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References and Notes

1. C. G. Pope and M. F. Stevens, *Brit. J. Exp. Pathol.* **40**, 410 (1959); I. Kato, H. Nakamura, T. Uchida, J. Koyama, T. Katsura, *Jap. J. Exp. Med.* **30**, 129 (1960).
2. E. H. Relyveld and M. Raynaud, *Ann. Inst. Pasteur* **107**, 618 (1964).
3. M. Raynaud, B. Bizzini, E. Relyveld, *Bull. Soc. Chim. Biol.* **47**, 261 (1963).
4. J. Iskierko, *Med. Dosw. Mikrobiol.* **17**, 217 (1965).
5. T. Honjo, Y. Nishizuka, O. Hayaishi, I. Kato, *J. Biol. Chem.* **243**, 3553 (1968).
6. D. M. Gill, A. M. Pappenheimer, Jr., R. Brown, J. T. Kurnick, *J. Exp. Med.* **129**, 1 (1969).
7. N. Strauss and E. D. Hendee, *ibid.* **109**, 145 (1959).
8. R. J. Collier and A. M. Pappenheimer, Jr., *ibid.* **120**, 1019 (1964).
9. R. J. Collier, *J. Mol. Biol.* **25**, 83 (1967); R. S. Goor and A. M. Pappenheimer, Jr., *J. Exp. Med.* **126**, 899 (1967).
10. E. Gasior and K. Moldave, *J. Biol. Chem.* **240**, 3346 (1965).
11. M. Yoneda, *Brit. J. Exp. Pathol.* **38**, 190 (1957).
12. B. Bizzini, R. O. Prudhomme, A. Turpin, M. Raynaud, *Bull. Soc. Chim. Biol.* **45**, 925 (1963).
13. R. P. Sutter and K. Moldave, *J. Biol. Chem.* **241**, 1698 (1966).
14. R. J. Collier, M. Maler, J. Gerstl, H. A. Cole, in preparation.
15. R. S. Goor, *Nature* **217**, 1051 (1968).
16. J. Gabliks and M. Falconer, *J. Exp. Med.* **123**, 723 (1966).

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Puparium Formation in Flies: Contraction to Puparium Induced by Ecdysone

Abstract. *Larvae of the fly* Calliphora erythrocephala *(Meigen) were deprived surgically of their ring glands at an age prior to the appearance of ecdysone in the blood, and then injected with ecdysone. They contracted into the typical barrel-shaped puparium, before the onset of tanning. This proved that ecdysone controls the puparial contraction as well as tanning.*

Pupariation (*1*) in flies proceeds in two steps: (i) the larva contracts to the barrel shape of the puparium, and (ii) the cuticle becomes dark and hard by a process of phenolic tanning induced by a hormone (*2*), ecdysone. Ecdysone appears in the blood of larger flies (for example, *Calliphora, Phormia, Sarcophaga*) about 15 hours before the beginning of the puparial contraction, a process which lasts about 30 minutes. The effect of ecdysone on tanning has been studied frequently (*3*), but the control of the puparial contraction has been ignored.

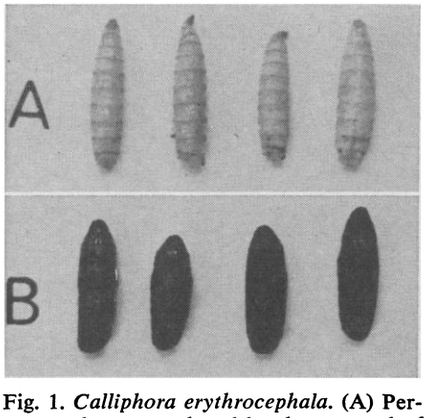

Fig. 1. *Calliphora erythrocephala.* (A) Permanent larvae produced by the removal of the ring gland in mature larvae. (B) Puparia in contracted state formed after the injection of ecdysone into permanent larvae.

Ecdysone originates from the ring gland situated above the brain. It was long considered impossible to study the effect of extirpating the gland because of its close proximity to the brain and the intrinsic difficulties of operating on a small soft-bodied organism. However, Possompès (*4*) reported a method by which the ring gland was removed without causing other damage to the larva; he showed that puparium formation was prevented when the extirpation was accomplished before a critical period but that normal puparia were formed when ring glands were subsequently implanted into operated specimens. This operation was much improved by Berreur (*5*).

The existence of a technique for the extirpation of the larval ring gland in larvae, and the availability of pure ecdysone now makes it possible to investigate the effect of ecdysone on contraction to the puparium as distinct from its effect on tanning. The fact that implantation of ring glands produces normal puparia (*4*) does not by itself prove that ecdysone alone is involved in the process.

The ring glands were removed (*5*) from 5-day-old larvae of *Calliphora erythrocephala.* This operation resulted in "permanent larvae" with otherwise normal activity. Four or five days later each larva was injected with 1 μl of a saturated aqueous solution of ecdysone (Schering). Dosages were calculated from the stated solubility of ecdysone in water (300 μg/ml). Only a few puparia had been formed 12 hours after the injection (Table 1). Those which had not pupariated were injected a second time 24 hours after the first injection. A large percentage of pupariation resulted overnight. Altogether 29 puparia were formed out of 39 permanent larvae injected with ecdysone, whereas only one puparium ensued from 30 control larvae which had received two injections of water.

Permanent larvae side by side with puparia formed from permanent larvae after the injection of ecdysone are shown in Fig. 1. The larva had contracted into the typical barrel shape with rounded ends and straight walls, exactly as in normal puparium formation, proof that the function of ecdysone is not limited to tanning but applies also to the preceding puparial contraction.

It was puzzling to note that two injections of ecdysone at intervals of 24 hours were required for inducing pupariation. According to Ohtaki *et al.* (*6*) ecdysone is rapidly inactivated in mature fly larvae so that at any time prior to puparium formation the ecdysone titer in the hemolymph is too low to induce pupariation. This suggested that pupariation is induced not so much by accumulation of ecdysone in the hemolymph beyond a critical concentration, but by a cumulative effect of small doses on the cuticle. It would appear that in our experiment ecdysone was quickly inactivated after a single dose, and a second injection 1 day later found the cuticle in a more receptive state.

Table 1. The effect of the injection of synthetic ecdysone into mature larvae of *Calliphora erythrocephala* which had been deprived of their ring glands (permanent larvae) on the formation of the puparium. A second injection was applied 24 hours after the first in those larvae which by then had not pupariated. The controls received two injections of water.

Larvae injected (No.)	First injection ecdysone (μg/larva)	Puparia formed after 12 hours (No.)	Second injection ecdysone (μg/larva)	Puparia formed after 12 hours (No.)	Puparia in controls (No.)
10	0.3	2	0.6	4	1/10
10	0.6	2	0.6	5	0/10
19	0.6	2	0.6	14	0/10