

## DECREASED FORMATION OF GOLGI BODIES IN AMEBAE IN THE PRESENCE OF RNA AND PROTEIN SYNTHESIS INHIBITORS

CHARLES J. FLICKINGER. From the Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, Colorado 80302. Dr. Flickinger's present address is the Department of Anatomy, School of Medicine, University of Virginia, Charlottesville, Virginia 22903

### INTRODUCTION

Golgi bodies in amebae depend upon the nucleus for maintenance of their normal form and numbers. They decline in size and number following enucleation and appear to be absent from cells enucleated for longer than 3 days (Flickinger, 1968). If a new nucleus is introduced by nuclear transfer into an ameba previously enucleated for 5 days, Golgi bodies are regenerated within hours (Flickinger, 1969). Thus, the formation of many Golgi bodies can be brought about rapidly and reproducibly by experimental manipulation.

This system can be used to study some of the requirements for the formation of Golgi bodies by exposing renucleated amebae to metabolic inhibitors. The size and number of Golgi bodies formed in the presence of an inhibitor are compared with the size and number of those formed in untreated renucleated amebae. In the present study, the effects of inhibitors of RNA and protein synthesis upon the formation of Golgi bodies are reported.

### MATERIALS AND METHODS

#### *Cultures*

Cultures of *Amoeba proteus* were maintained at 21°C in Prescott's medium (Prescott and Carrier, 1964) with daily feedings of *Tetrahymena*.

#### *Microsurgery*

Enucleated amebae were obtained by cutting normal amebae in two with the tip of a braking pipette (Flickinger, 1968). The enucleates were maintained at 21°C for 5 days. They were then renucleated by transfer of a nucleus from a donor ameba by means of the technique of Jeon (Jeon and Lorch, 1968; Jeon, 1970) as in previous experiments (Flickinger, 1969). The recipient renucleated amebae were maintained at 21°C until they were fixed for electron microscopy.

To test the effects of protein synthesis inhibition, renucleated amebae were placed in  $1 \times 10^{-4}$  M emetine hydrochloride (Eli Lilly & Co., Indianapolis, Ind.), an antiparasitic drug. Inhibition of protein synthesis by emetine has been demonstrated in mam-

malian, yeast, and plant cells (Grollman, 1966, 1968), and in *Amoeba proteus* (D. M. Prescott and M. Lauth, personal communication). One of the more widely used protein synthesis inhibitors such as puromycin or cycloheximide was not used because concentrations that are sufficiently high to inhibit protein synthesis significantly result in the death of the amoebae within hours (D. M. Prescott, personal communication).

The effects of inhibiting RNA synthesis were studied by exposing renucleated amoebae to solutions of actinomycin D (Mann Research Labs Inc., New York), which inhibits RNA synthesis by binding to guanine in DNA (Reich, 1963). Higher concentrations of actinomycin are needed to suppress RNA synthesis in *Amoeba proteus* than are commonly used with other cell types (Stevens, 1967; Rao and Prescott, 1970). In the initial experiments, a concentration of 100  $\mu\text{g}/\text{ml}$  was used because previous studies had indicated that this was sufficient to block RNA synthesis completely (Stevens, 1967). Later the possibility arose that RNA synthesis may not have been inhibited completely or with sufficient speed. Consequently, other experiments were carried out with a concentration of 1  $\text{mg}/\text{ml}$ . This concentration has been shown to inhibit all detectable RNA synthesis in amoebae within 18 min (Rao and Prescott, 1970).

Control renucleated amoebae were maintained in normal amoeba medium. Experimental renucleates were placed in either emetine or actinomycin immediately after the operation. Samples of each group were fixed 6 and 24 hr after renucleation. To test the ability of renucleated amoebae to recover from the effects of an inhibitor, some renucleated amoebae were placed in one of the inhibitors for 6 hr and then were transferred to normal amoeba medium free of the drug for 18 hr before fixation. In other experiments, the nucleus donor amoebae were treated with actinomycin for 1 or 2 days before the operation, and the recipients were then placed in actinomycin for 6 or 24 hr before fixation.

#### *Preparation for Electron Microscopy*

Amoebae were fixed for 1 hr at room temperature in Karnovsky's fixative, containing 4% formaldehyde and 5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.3 (Karnovsky, 1965). After aldehyde fixation, the cells were rinsed overnight in water and were embedded in a small cube of agar as described previously (Flickinger, 1969) to prevent their dispersion during subsequent processing. The amoebae, embedded in agar, were postfixed for 1 hr in 1%  $\text{OsO}_4$  in 0.1 M cacodylate buffer at pH 7.3. The specimens were rinsed briefly in water, dehydrated in a graded series of ethanols followed by propylene oxide, and embedded in Araldite. Silver-to-pale gold sections were obtained with a Porter-Blum MT-1 microtome

equipped with a diamond knife. The sections were mounted on uncoated copper grids and were stained with lead citrate (Reynolds, 1963). The specimens were examined in a Philips EM-300 electron microscope operated at 60 kv.

#### *Method of Examination of Specimens*

In many cases, differences in the size and number of Golgi bodies under the different experimental conditions were large enough to be obvious on examination of sections. A semiquantitative technique was devised, however, to aid in estimating the magnitude of differences between the various treatments. For each grid, the total number of profiles of amoebae, the number of profiles containing Golgi bodies, and the size of the organelles were determined. This permitted an estimate of the per cent of amoeba profiles that contained Golgi bodies as well as visualization of their size and morphology. This method is only semiquantitative, but it provides a rough comparison of the frequency with which Golgi bodies were encountered in the different specimens.

The numbers of cells upon which the results are based were limited by the number of enucleates that remained alive after 5 days and by the time required to perform the renucleation operation. Samples usually consisted of between 15 and 40 amoebae. A total of about 50 cells treated with emetine, and 150 amoebae treated with actinomycin were examined. In this and a previous study several hundred untreated renucleated amoebae have been examined.

## RESULTS

### *Normal Amoeba Golgi Apparatus*

Normal amoebae contain multiple Golgi bodies or dictyosomes, distributed throughout the cytoplasm. Each Golgi body is composed of a stack of six to eight curved, centrally flattened cisternae (Fig. 1). A few smooth vesicles  $\sim 400$  A in diameter are distributed about the periphery of the stacks. The cisternae are 1-2  $\mu$  in diameter or lateral extent and average 1.2  $\mu$  in this dimension (Flickinger, 1968).

### *Untreated Renucleates*

A previous study (Flickinger, 1969) has shown that small Golgi bodies first appear  $\frac{1}{2}$ -1 hr after renucleation of amoebae previously enucleated for 5 days. Golgi bodies increase progressively in size and number in samples fixed at increasing intervals after the introduction of the nucleus, and some full-sized examples are present by 6 hr after the operation. In the present study, Golgi

bodies were encountered in 60–70% of profiles of untreated renucleated amoebae both 6 and 24 hr after the operation. In the 6 hr sample most of the Golgi bodies were smaller than normal, but by 24 hr after renucleation the majority of the organelles were of normal size (Fig. 2). These untreated renucleated amoebae serve as a control with which renucleated cells exposed to an inhibitor will be compared.

#### *Emetine Treatment*

In renucleated amoebae exposed to  $1 \times 10^{-4}$  M emetine, the formation of Golgi bodies was almost completely blocked. None of the profiles of amoebae fixed 6 hr after renucleation contained Golgi bodies. Only 0–17% (mean, 8.5%) of profiles of amoebae fixed 24 hr after the operation contained Golgi bodies. The few Golgi bodies present were small, averaging about  $0.5 \mu$  in diameter. This suppression of the formation of Golgi bodies by emetine was reversible by removing the cells from the inhibitor. Some renucleates were exposed to emetine for 6 hr and then placed in normal amoeba medium free of the drug for 18 hr before fixation. In this group 72% of profiles contained Golgi bodies, many of which had attained a normal diameter in excess of  $1 \mu$  (Fig. 3).

#### *Actinomycin Treatment*

Exposure of renucleated amoebae to actinomycin D reduced the size of Golgi bodies that were formed, and they were encountered less frequently than in untreated renucleates. No difference was detected, however, between the two different concentrations of actinomycin in ability to inhibit the formation of Golgi bodies, nor did pretreatment of nucleus donor amoebae with actinomycin for 1 or 2 days before operation affect the formation of Golgi bodies.

In amoebae exposed to actinomycin for 6 hr after renucleation, 0–17% (mean, 13%) of profiles contained Golgi bodies, a decrease to less than one-third that of the untreated control renucleates. In samples exposed to actinomycin for 24 hr after renucleation, an average of 26% of profiles contained Golgi bodies. The proportion containing Golgi bodies was always less than in untreated renucleated amoebae, but it varied considerably from one experiment to another. In six of eight experiments the per cent of profiles

with Golgi bodies was reduced at least to one-half that of the control. Most of the Golgi bodies formed in actinomycin-treated renucleates were smaller than normal (Figs. 4 and 5).

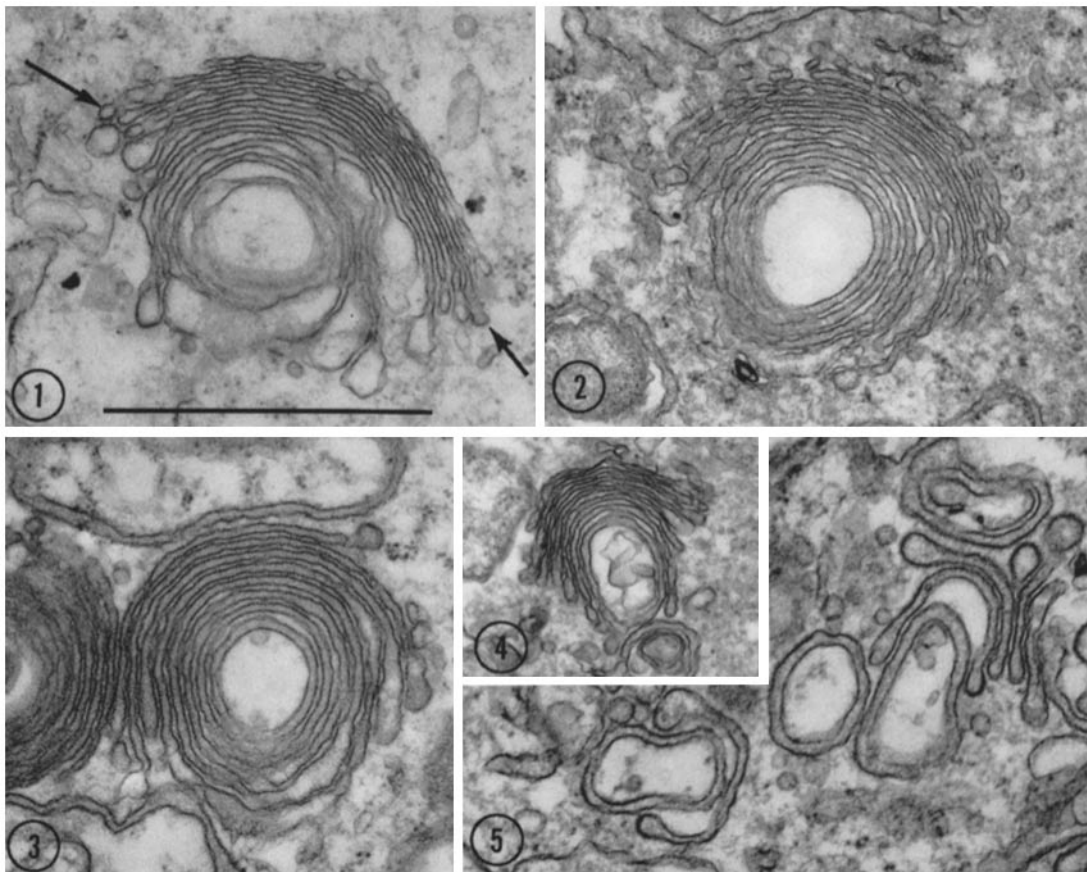
The extent to which renucleated amoebae can recover the ability to form Golgi bodies if removed from actinomycin is uncertain since 42% of profiles contained Golgi bodies after 6 hr of actinomycin treatment followed by 18 hr in normal amoeba medium. This value is greater than that for renucleated cells treated with actinomycin for 6 hr, but it is less than in untreated renucleated amoebae, and it overlaps with values for renucleated cells maintained in actinomycin continuously for 24 hr.

The results are summarized in Table I, which lists the per cent of amoeba profiles with Golgi bodies in the different experiments. It seems worth emphasizing that these data are semi-quantitative in nature and should be regarded only as a rough guide to the relative frequency with which Golgi bodies were encountered under various conditions. The figures probably do not accurately reflect the per cent of amoebae that actually contained Golgi bodies because some profiles in random sections may by chance not include any Golgi bodies even though Golgi bodies may be present elsewhere in the cell.

#### DISCUSSION

The results indicate that the formation of Golgi bodies was almost completely blocked by emetine, which inhibits protein synthesis in a variety of eukaryotic cells (Grollman, 1966, 1968) including *Amoeba proteus* (D. M. Prescott and M. Lauth, personal communication), apparently by inhibiting the aminoacyl-sRNA transfer reaction (Grollman, 1966). The precise means by which the inhibition of protein synthesis affects the formation of Golgi bodies, however, is not known.

When renucleated amoebae were exposed to actinomycin D, the size of Golgi bodies and the proportion of profiles in which Golgi bodies were detected were decreased in comparison with untreated renucleates, suggesting that after the insertion of the nucleus, new RNA synthesis is necessary for the development of Golgi bodies to proceed at its normal pace. Since the Golgi bodies formed in the presence of actinomycin, however, were smaller than normal, this size change would decrease the probability of their being detected and may account for at least some of the apparent



FIGURES 1-5 Ameba Golgi bodies. To facilitate size comparisons, all micrographs are the same magnification, 43,000. The length of the bar is 1  $\mu$ .

FIGURE 1 A Golgi body from a normal ameba is composed of a stack of six to eight curved cisternae and a few smooth vesicles. The diameter of normal Golgi bodies (between the arrows) averages 1.2  $\mu$ .

FIGURE 2 Renucleated ameba maintained in ameba medium with no inhibitor for 24 hr. Golgi bodies are regenerated after the introduction of a nucleus into previously enucleated cells. By 24 hr after renucleation, most of the Golgi bodies have attained a normal size.

FIGURE 3 Renucleated ameba treated with emetine for 6 hr and then placed in ameba medium for 18 hr before fixation. Emetine blocks the formation of Golgi bodies in renucleated amebae. The inhibition is reversible, since cells removed from the inhibitor are able to form normal-sized Golgi bodies.

FIGURE 4 Renucleated ameba maintained for 24 hr in actinomycin D (100  $\mu$ g/ml). Golgi bodies formed in the presence of actinomycin D are smaller than normal. This example contains a normal number of cisternae, but the diameter of the organelle is about about 0.5  $\mu$ .

FIGURE 5 Renucleated ameba maintained for 24 hr in actinomycin D (1 mg/ml). Some Golgi bodies formed in the presence of actinomycin D contain stacks of only two to four curved cisternae and single circular cisternae.

reduction in number. In any event, actinomycin appeared to be less effective than emetine in suppressing the formation of Golgi bodies, because at both 6 and 24 hr after the operation the

average number of Golgi bodies formed in the presence of actinomycin exceeded the average number formed in the presence of emetine.

Although reduced in size, some Golgi bodies

TABLE I

Experiment No.	Time after operation		Inhibitor	Profiles with Golgi bodies
	<i>Hr</i>			%
1	6		None	60
2	6		None	66
3	24		None	63
4	24		None	69
5	6		Em	0
6	24		Em	0
7	24		Em	17
8	24		Em 6 hr + None 18 hr	72
9	6		Act 100	17
10	6		Act 100*	13
11	6		Act 100‡	17
12	6		Act 1000	0
13	6		Act 1000*	17
14	24		Act 100	50
15	24		Act 100*	0
16	24		Act 100‡	30
17	24		Act 1000	20
18	24		Act 1000	35
19	24		Act 1000*	0
20	24		Act 1000*	44
21	24		Act 1000*	25
22	24		Act 1000 6 hr + None 18 hr	42

\*. ‡ indicates donor cells pretreated with actinomycin before operation for 1 or 2 days, respectively.

Em, emetine  $1 \times 10^{-4}$  M.

Act 100, actinomycin D 100  $\mu$ g/ml.

Act 1000, actinomycin D 1000  $\mu$ g/ml.

were formed in the presence of a concentration of actinomycin that is sufficient to inhibit all detectable RNA synthesis in *Amoeba proteus* (Rao and Prescott, 1970). This suggests that the new nucleus brings with it a pool of some preformed factor that is capable of promoting the formation of a reduced but significant number of small Golgi bodies in the absence of new RNA synthesis. The nature of this factor is unknown. Since pretreatment of nucleus donor amoebae with actinomycin for 1 or 2 days did not alter the formation of Golgi bodies in actinomycin-treated recipient cells, the nuclear factor may be some substance other than RNA or it may be a stable RNA which persists for longer than 2 days. The existence of such a long-lived RNA is plausible, since enucleated amoebae are capable of a limited amount of protein synthesis for at least 3 days after removal of the nucleus (Mazia and Prescott, 1955),

which implies that some messenger RNA can also persist for at least the same length of time.

#### SUMMARY

Golgi bodies in amoebae decline in size and number after enucleation. Following the introduction of a new nucleus by nuclear transfer, Golgi bodies are regenerated and increase progressively in size and number. Emetine, which inhibits protein synthesis, almost completely blocks the formation of Golgi bodies. In renucleated amoebae exposed to actinomycin D to inhibit RNA synthesis, the size and possibly the number of Golgi bodies is also decreased. The results suggest that the formation of Golgi bodies in amoebae requires protein synthesis. RNA synthesis is required for the development of Golgi bodies to proceed at the normal rate, but the production of some small Golgi bodies can take place in the absence of

new RNA synthesis, suggesting that the nucleus contains a pool of a factor that promotes the formation of Golgi bodies.

The author wishes to acknowledge the technical assistance of Mrs. Heather Taylor.

This study was supported by a research grant from the American Cancer Society (E-500), Program Project HD-02282 of the National Institutes of Health, and Health Sciences Advancement Award FR-02084 from the National Institutes of Health. The author is the recipient of a Research Career Development Award from the National Institute of General Medical Sciences.

Received for publication 31 August 1970, and in revised form 6 November 1970.

#### REFERENCES

- FLICKINGER, C. J. 1968. The effects of enucleation on the cytoplasmic membranes of *Amoeba proteus*. *J. Cell Biol.* **37**:300.
- FLICKINGER, C. J. 1969. The development of Golgi complexes and their dependence upon the nucleus in amebae. *J. Cell Biol.* **43**:250.
- GROLLMAN, A. P. 1966. Structural basis for inhibition of protein synthesis by emetine and cycloheximide based on an analogy between ipecac alkaloids and glutarimide antibiotics. *Proc. Nat. Acad. Sci. U.S.A.* **56**:1867.
- GROLLMAN, A. P. 1968. Inhibitors of protein biosynthesis. V. Effects of emetine on protein and nucleic acid biosynthesis in HeLa cells. *J. Biol. Chem.* **243**:4089.
- JEON, K. W. 1970. Micromanipulation of amoeba nuclei. *Methods Cell Physiol.* **4**:179.
- JEON, K. W., and I. J. LORCH. 1968. New simple method of micrurgy on living cells. *Nature (London)*. **217**:463.
- KARNOVSKY, M. J. 1965. A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. *J. Cell Biol.* **27**(2):137A (Abstr.)
- MAZIA, D., and D. M. PRESCOTT. 1955. The role of the nucleus in protein synthesis in *Amoeba*. *Biochim. Biophys. Acta.* **17**:23.
- PRESCOTT, D. M., and R. F. CARRIER. 1964. Experimental procedures and cultural methods for *Euplotes eurystomus* and *Amoeba proteus*. *Methods Cell Physiol.* **1**:85.
- RAO, M. V. N., and D. M. PRESCOTT. 1970. Inclusion of predivision labeled nuclear RNA in post division nuclei in *Amoeba proteus*. *Exp. Cell Res.* **62**:286.
- REICH, E. 1963. Biochemistry of Actinomycins. *Cancer Res.* **23**:1428.
- REYNOLDS, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* **17**:208.
- STEVENS, A. R. 1967. Machinery for exchange across the nuclear envelope. In *The control of nuclear activity*. L. Goldstein, editor. Prentice-Hall, Inc., Englewood Cliffs, N.J. 189.