STUDIES ON THE MODE OF ACTION OF DIPHTHERIA TOXIN

II. Protein Synthesis in Primary Heart Cell Cultures*

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The inhibition of protein synthesis by diphtheria toxin in tissue culture systems is well documented (1). The toxin has also been shown to exert its inhibitory effect in cell free protein synthesizing systems (2) and appears to act by inactivating a transferase enzyme (3). While these data provide compelling evidence that inhibition of protein synthesis is important in a consideration of the mode of action of diphtheria toxin, one should remain aware that tissue culture systems do not necessarily reflect the in vivo situation. This being the case, a recently completed investigation was designed to ask two important questions relative to the action of diphtheria toxin: (*a*) how pertinent is inhibition of protein synthesis in vivo; and (*b*) if it is a significant biochemical lesion, what are the tissues or target organs involved? By two independent methods of analysis (microdensitometry of tissue radioautograms and radioactivity of tissue proteins), the effect of crystalline diphtheria toxin on the incorporation of tritiated leucine in vivo was evaluated in guinea pigs and mice. It was established that inhibition of protein synthesis is not a widespread metabolic effect of diphtheria toxin but rather seems to be restricted primarily to cardiac tissues (4).

When the diphtheria-sensitive guinea pig was used as the experimental animal, the toxin inhibited protein synthesis of heart tissues by 75%. Protein synthesis in all other guinea pig tissues examined, however, remained essentially normal. No inhibition of protein synthesis was seen in the cardiac tissues (or other tissues) of the mouse, a species comparatively resistant to diphtheria toxin. This rather surprising biochemical observation, which pointed to a cardiac specificity for the action of the toxin, is not completely unexpected when viewed in the light of past clinical observations made in cases of severe or fatal diphtheria infections of man. The older literature contains many descriptions of cardiac failure as the cause of death of patients convalescing from severe diphtheria, and pathologists have described a variety of both parenchymal and interstitial lesions of the heart found at necropsy (5).

The present paper describes the results of experiments in which primary heart cell cultures, derived from embryonic guinea pigs and neonatal rats,

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were exposed to diphtheria toxin and examined with respect to morphological changes and protein synthesis. The rationale for the experiments was to determine if species sensitivity or resistance was reflected when the cardiac tissues were removed from neural and endocrine influences and before complete dedifferentiation had occurred. It was considered important that dedifferentition be considered since established cell lines more often than not bear little resemblance to the tissues from which they originally were derived. The data presented corroborate the findings of the previous in vivo experiments (4) in that species sensitivity or resistance to diphtheria toxin was exhibited by the primary heart cell cultures. They show further that the toxin exerts a direct toxicity to cardiac tissues of a sensitive animal, and that the effects of the toxin on the intact heart are not secondary to neural or endocrine factors.

Materials and Methods

Diphtheria Toxin and Antitoxin—Crystalline dipththeria toxin was obtained from the Wellcome Research Laboratories, Beckenham, Kent, England. Each milliliter of reconstituted toxin contained 324 Lf units/mg (approximately 20,000 guinea pig MLD). Diphtheria antitoxin was obtained from hyperimmunized rabbits. Crude nonfractionated antiserum and purified gamma globulin fractions of the whole sera were used in the experiments. Purification of the antisera was accomplished by ammonium sulfate precipitation followed by G-25 Sephadex (Pharmacia Fine Chemicals, Uppsala, Sweden) column chromatography. Purity and potency of the antitoxin preparations were determined by immunoelectrophoresis.

Animals.—Pregnant guinea pigs (English strain) were supplied by a local animal dealer, and Wistar rats were obtained from a breeding colony of the Department of Laboratory Animal Medicine, University of Cincinnati College of Medicine. For each experiment either one or two pregnant guinea pigs were used, depending on the size of the litters. Pregnancy was terminated between 40 and 45 days of gestation. It was found that embryos obtained at this stage of development provided suitable heart tissues for establishing primary cell cultures. Full term embryos apparently are too well developed for this purpose since they did not yield viable heart cell cultures. The litter of one rat was used for each experiment. Neonatal animals (1–3 days old) were used for establishing the rat heart cell cultures. Since rats are not as well developed as guinea pigs at birth, newborn rats can be used successfully for the primary cell cultures.

Primary Heart Cell Cultures.—Primary cell cultures were established by a modification of the method described by Harary and Farley (6). All manipulations were carried out under aseptic conditions. The embryos were removed quickly from the mother and the heart tissues minced and placed in Eagle's minimal essential medium (MEM) (7), containing 0.25% trypsin (Difco Laboratories, Detroit, Mich.). After 15 min of trypsinization at 37°C, the tissues were centrifuged at low speed and the supernatant discarded. The sedimented tissues were resuspended and trypsinized for an additional 45 to 60 min. The heart cells were then collected by centrifugation and suspended in MEM containing the appropriate serum. Guinea pig heart cells were collected in MEM plus guinea pig serum (final concentration of 8%), and MEM plus calf serum at a final concentration of 10% was employed with the rat heart cells. In both cases, the cells were suspended to an optical density of 0.06 in the appropriate tissue culture fluid. Monolayers of the heart cells were obtained by growth either on cover slips (three per 60 mm Petri dish) or on the surface of 60 mm plastic dishes (Falcon Plastics, B-D Laboratories, Los Angeles, Calif.) Tissue culture fluids were changed every 48 hr of incubation

at 37°C under an atmosphere of 95% air and 5% CO_2. Confluent monolayers were achieved between 5 and 7 days after initiating the cultures. Beating centers of heart cells were seen routinely in the rat heart cell cultures beginning approximately 2 days after the cultures were established. Guinea pig heart cells which exhibited rhythmic contractions were seen occasionally but not in all cultures. Two distinct morphological types of cells could be identified in the cultures; one is identified as an endothelioid cell derived from the capillaries of the heart muscle and the second type is the heart muscle cell (8). The endothelioid cell can be identified by its clear cytoplasm, large nucleus containing multiple nucleoli, and absence of myofibrils; a typical myocardial muscle cell is characterized by a dense cytoplasm containing numerous sarcosomes, a well-defined small nucleus containing one nucleolus and surrounded by a double membrane-like structure (Fig. 1). Typical myofibrils of the heart cells could be visualized, although not with uniform success by silver nitrate staining (Fig. 2).

Incorporation of Tritiated Leucine into Cell Proteins.—The primary heart cell monolayers were divided into three groups for each experiment: (*a*) incubated with diphtheria toxin; (*b*) incubated with a toxin-antitoxin mixture; and (*c*) incubated with tissue culture medium (controls). The heart cell cultures were exposed to concentrations of toxin between 10 and 100 MLD/ml of fluid for various lengths of time which ranged between 3 and 48 hr. After incubation with the toxin, the tissue culture fluids were replaced with medium containing tritiated leucine-^{3}H (L-leucine, 4, 5, T, specific activity 500 mc/mM) at a concentration of 5 μc/ml. After an exchange period of 3–4 hr, the cell cultures were rinsed thoroughly with MEM and the proteins precipitated with cold 0.5 M perchloric acid. The precipitates were washed twice in perchloric acid before protein assays and radioactivity determinations.

Protein Assay.—Total protein of the heart cell culture precipitates was measured by the method of Lowry et al. (9) modified by Oyama and Eagle (10) for tissue culture.

Radioactivity Measurements.—The protein precipitates were digested in minimum volumes of 0.1 N NaOH and prepared for liquid scintillation counting in a dioxane-toluene-napthalene mixture (11). The samples were counted in a model 574 Tri-Carb liquid scintillation spectrometer (Packard Corporation, Downers Grove, Ill.). All values of radioactivity are expressed as counts per minute per milligram protein (cpm/mg).

Staining and Microscopy.—The heart cell cultures were stained either with a Giemsa stain (12) or a modification of the Rio-Hortega silver stain (13). Unstained preparations were examined by phase microscopy with a Zeiss research photomicroscope.

EXPERIMENTAL RESULTS

Effect of Diptheria Toxin on Rat Heart Cells.—Rat heart cultures were found to be refractory to the action of diphtheria toxin. The typical rhythmic beating cells were unaffected and morphology was unchanged even after a prolonged incubation period of 42 hr with concentrations of the toxin as high as 100 MLD/ml of tissue culture medium. In addition, protein synthesis by the rat heart cells as measured by the incorporation of tritiated leucine remained essentially normal in the presence of the toxin (Table I). Concentrations of toxin of 10, 25, and 100 MLD/ml had no significant effect on protein synthesis. It was seen that toxin-antitoxin mixtures using unpurified rabbit antiserum caused an inhibition of protein synthesis but the inhibition was ablated when the crude antitoxin was replaced by a purified rabbit globulin preparation. Presumably the rabbit antisera contained materials which were inhibitory for the rat heart cells in culture. Although tissue cultures usually require serum for optimum

growth the cells can at times be extremely sensitive to undefined toxic factors present in some batches of either homologous or heterologous sera (14). These data would suggest, therefore, that the species resistance of the rat to diphtheria toxin is reflected by the resistance of the primary heart cells to the action of the toxin.

Effect of Diphtheria Toxin on Guinea Pig Heart Cells.—In contrast to the rat heart cell cultures, guinea pig heart cells in primary cultures were found to be extremely sensitive. Guinea pig heart cells incubated with 25 MLD/ml of diphtheria toxin incorporated leucine to a much lesser extent than did the normal untreated heart cells. Incubation with the toxin for periods between 3 and 48 hr resulted in inhibition of protein synthesis between 37 and 53% (Table

TABLE I

*Incorporation of Tritiated Leucine into Proteins of Primary Rat Heart Cell Cultures** (*Neonatal Rat*)

Toxin	Time incubated with toxin	Radoactivity		
		Normal	Toxin	Toxin-antitoxin
MLD/ml	*hr*	*cpm* × 10^{-3}/*mg protein*		
10	18	30.5	27.9	22.9‡
10	42	30.5	31.7	—
25	18	24.0	22.1	14.1‡
25	42	24.0	19.3	—
100	6	178.8	183.2	178.6§
100	18	178.8	174.5	182.8§

Composite data of three experiments.

* Cell monolayers incubated with toxin for specified number of hours and tissue culture fluids (MEM), then replaced with fluids containing 5 μc/ml of leucine-^{3}H for 4 hr.

‡ Whole hyperimmune rabbit serum used as antitoxin.

§ Purified gamma globulin fraction of hyperimmune rabbit serum used as antitoxin.

II). Increasing the concentration of toxin to 100 MLD/ml did not result in an inhibition of protein synthesis greater than that observed with the smaller amounts of toxin (Table III). These results are in agreement with the observations made in vivo where protein synthesis in cardiac tissues was inhibited equally in animals injected either with 20 or 1000 MLD intramuscularly (4). It should be noted also that purified antitoxin reversed the inhibition completely.

The cytopathic effect of diphtheria toxin was also manifested in the primary guinea pig heart cell cultures. The cells exposed to the toxin exhibited increased granularity and vacuolization with time, and after 72 hr the monolayers were usually destroyed (Figs. 3 and 4). Thus the primary guinea pig heart cells reflected the typical species sensitivity to diphtheria toxin with respect to both inhibition of protein synthesis and direct cytotoxicity.

DISCUSSION

The apparent specificity of cardiac tissues as the site of action of diphtheria toxin is substantiated by the results of this investigation. Heart cells derived from the embryonic guinea pig, in primary culture, were found to be extremely sensitive to the toxin. The inhibition of protein synthesis and the cytopathic effects noted in the absence of neural or endocrine stimuli show that diphtheria

TABLE II

*Incorporation of Tritiated Leucine into Proteins of Primary Guinea Pig Heart Cell Cultures (Embryonic Guinea Pig)**

Toxin	Time incubated with toxin	Radioactivity		
		Normal	Toxin	Inhibition
MLD/ml	*hr*	*cpm* × 10^{-3}/*mg protein*		%
25	3	171.8	108.2	37
25	6	145.9	79.2	46
25	24	135.5	64.4	53

Composite data for four experiments.

* Monolayers incubated with leucine-^{3}H (5 μc/ml) for 3 hr after incubation with diphtheria toxin for specified number of hours.

TABLE III

*Effect of Diphtheria Toxin on Protein Synthesis by Guinea Pig Embryo Primary Heart Cell Cultures**

Treatment	Radioactivity			
	3 hr toxin	Inhibition	6 hr toxin	Inhibition
	cpm × 10^{-3}/*mg protein*	%	*cpm* × 10^{-3}/*mg protein*	%
Normal	195	—	195	—
Toxin (100 MLD/ml)	89.7	54	97.8	50
Toxin-antitoxin‡	—	—	188.4	3.4
Antitoxin‡	—	—	181.3	7.1

* Cell cultures incubated with toxin and/or antitoxin before incubation with tritiated leucine (5 μc/ml) for 3 hr.

‡ Purified gamma globulin fraction of rabbit hyperimmune serum used as antitoxin.

toxin exerts a direct toxicity to these muscle cells. The direct effect on heart cells in culture suggest further that the action of the toxin on cardiac tissues in the intact guinea pig is not due to secondary or terminal effects. Finally, it can be concluded that the deleterious effects on the heart is manifested only in those mammalian species which are not endowed with a natural resistance to the protein toxin.

A question which arises is why the extent of inhibition of protein synthesis

in the case of the guinea pig heart cell cultures should be less than that observed in vivo. A possible explanation is the relative proportion of true heart muscle cells which grow out in any given culture as compared to the endothelioid cells. It may be that the muscle cells are sensitive while the endothelioid cells are comparatively resistant to the toxin. If this were the case, then the process of dedifferentiation with the concomitant proliferation of nonheart cells may yield cultures which become refractory to the effects of diphtheria toxin. Since it has not as yet been possible to prevent the growth of the endothelioid cells in culture, this possibility has not been substantiated experimentally. An experimental approach which might circumvent this problem is the use of organ culture of heart tissues, since this may delay or prevent dedifferentiation.

At this juncture it is necessary to speculate on the mode of action of diphtheria toxin. Are the results obtained with the intact guinea pig and the primary heart cell cultures compatible with the findings using established tissue cultures and cell free systems? The current hypothesis advanced by Collier (3) and Goor (15) is that the toxin acts by binding a transferase through nicotinine adenine dinucleotide (NAD) and thereby prevents the formation of the polypeptide chain. Since transferase enzymes are presumably present in all types of mammalian cells (16) one would expect that inhibition of protein synthesis would be apparent in all tissues and not be restricted to the heart as our experiments suggest. However, it may be that the toxin binds specifically to cardiac tissues and is localized in high concentration at that site because of a peculiar tissue affinity for the protein toxin. Although this is an unlikely possibility we are now testing it experimentally by means of fluorescent antibody methods. If tissue affinity for the toxin proves not to be the answer, then it will be necessary to seek an explanation of cardiac specificity by examining in detail the biochemical machinery of the heart tissues. It is an intriguing situation since heart tissues are composed almost exclusively of structural proteins which are replenished at a relatively slow rate. One would expect a priori that tissues, such as the small intestine which turn over at a fast rate, would be the likely sites at which the toxin exerts its action. No such inhibition however could be detected in our in vivo experiments (4). If it were possible to detect a unique feature in the structural components or the enzymatic makeup of the protein-synthesizing machinery of cardiac tissues, then it may be possible to explain in molecular terms why diphtheria intoxication appears to manifest itself as a heart disease.

SUMMARY

Primary heart cell cultures of embryonic guinea pigs and the neonatal rat were established and incubated with purified diphtheria toxin. The rat heart cell cultures were refractory to the effects of the toxin; protein synthesis proceeded normally as measured by the incorporation of tritiated leucine into cell

proteins; beating heart cells continued to contract; and the cell monolayers remained intact after exposure to the toxin for periods as long as 72 hr. These findings are compatible with the species resistance of the rat to diphtheria toxin. The guinea pig heart cell cultures were found to be extremely sensitive to the toxin. Protein synthesis was inhibited by approximately 50% after incubation with small quantities of toxin for 3 hr. Increasing the concentration of the length of exposure to the toxin did not increase this inhibition significantly. In addition, diphtheria toxin exerted a true cytopathic effect on the guinea pig heart cells. Monolayers were destroyed when incubated with the toxin for 2 to 3 days.

The results show that the heart cells reflect species resistance or sensitivity to diphtheria toxin in the absence of neural or endocrine influences and suggest further that the toxin exerts a direct toxicity to muscle cells of the heart. It is not yet possible to explain in biochemical terms why the toxin seems to act specifically on cardiac tissues.

BIBLIOGRAPHY

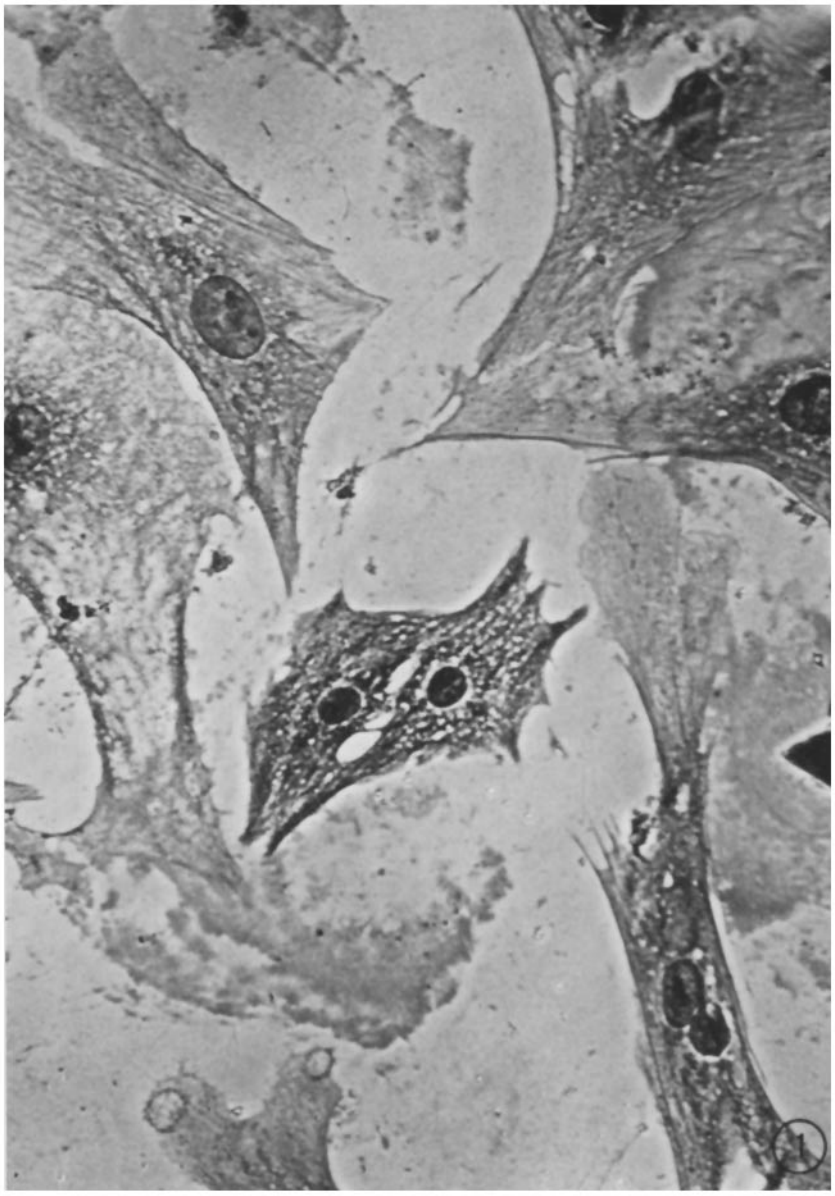
1. Strauss, N. and E. D. Hendee. 1959. The effect of diphtheria toxin on the metabolism of HeLa cells. *J. Exptl. Med.* **109**:145.
2. Collier, R. J. and A. M. Pappenheimer, Jr. 1964. Studies on the mode of action of diphtheria toxin. II. Effect of toxin on amino acid incorporation in cell-free systems. *J. Exptl. Med.* **120**:1019.
3. Collier, R. J. 1967. Effect of diphtheria toxin on protein synthesis: Inactivation of one of the transfer factors. *J. Mol. Biol.* **25**:83.
4. Bonventre, P. F. and J. G. Imhoff. 1966. Studies on the mode of action of diphtheria toxin. I. Protein synthesis in guinea pig tissues. *J. Exptl. Med.* **124**:1107.
5. Andrewes, F. W., W. Bullock, S. R. Douglas, G. Dreyer, A. D. Gardner, P. Fildes, J. C. G. Ledingham, and C. G. L. Wold. 1923. Diphtheria, its Bacteriology, Pathology, and Immunology. Monograph. Medical Research Council, London.
6. Harary, I. and B. Farley. 1960. In vitro studies of single isolated beating heart cell. *Science.* **131**:1674.
7. Eagle, H. 1959. Amino acid metabolism in mammalian cell cultures. *Science.* **130**: 432.
8. Mark, G. E., and F. F. Strasser. 1966. Pacemaker activity and mitosis in cultures of newborn rat heart ventricle cells. *Exptl. Cell Res.* **44**:217.
9. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265.
10. Oyama, V. I., and H. Eagle. 1956. Measurement of cell growth in tissue culture with a phenol reagent (Folin-Ciocolteau). *Proc. Soc. Exptl. Biol. Med.* **91**:305.
11. Tye, R., and J. D. Engle. 1965. Liquid scintillation counting of carbon-14 in aqueous digests of whole tissues. *Anal. Chem.* **37**:1225.
12. Armed Forces Institute of Pathology, Manual of Histologic and Special Staining Technics. 1960. McGraw-Hill Book Co., Inc., New York. 2nd edition. 109.
13. Penfield, W., and W. V. Cone. 1950. Neuroglia and microglia (the metallic

methods). *In* McClung's Handbook of Microscopical Technique. R. McClung Jones, editor. Paul B. Hoeber, Inc., New York. 3rd edition. 426.
14. Paul, J. Media for culturing cells and tissues. *In* Cell and Tissue Culture. 1965. Williams and Wilkins Co., Baltimore. 67.
15. Goor, R. 1967. Doctoral Thesis, Harvard University, Cambridge.
16. Gasior, E., and K. Moldave. 1965. Resolution of aminoacyl-transferring enzymes from rat liver by molecular sieve chromatography. *J. Biol. Chem.* **240**:3346.

EXPLANATION OF PLATES

PLATE 81

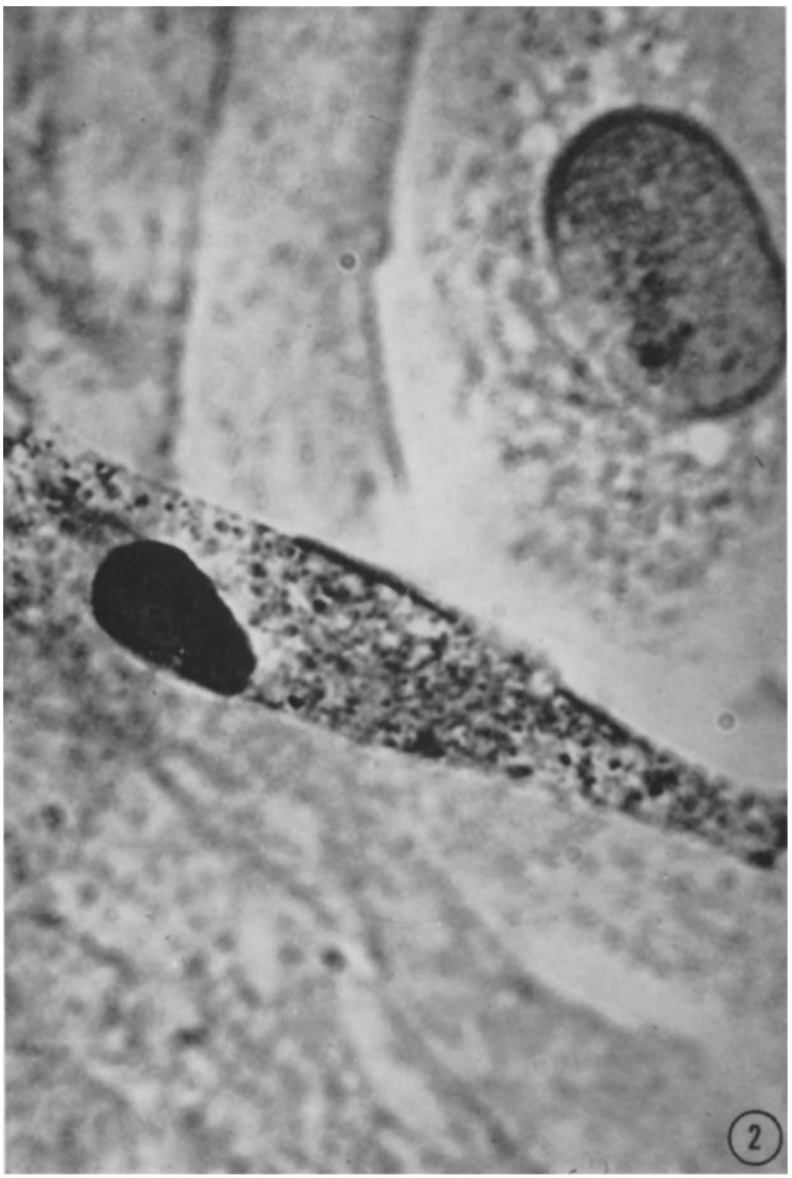
FIG. 1. Primary heart cell culture established from the heart tissues of neonatal rats. Two distinct morphological types of cells are visible. Two typical heart cells are seen in the center of the field. They are characterized by a dense cytoplasm containing numerous sarcosomes, a small nucleus containing one nucleolus, and a double membrane-like structure surrounding the nucleus. An endothelioid cell is seen in the upper left hand corner; it is characterized by a clear cytoplasm, a large nucleus with numerous nucleoli, and an absence of myofibrils. (Phase contrast × 160.)



(Bonventre and Imhoff: Protein synthesis in heart cell cultures)

PLATE 82

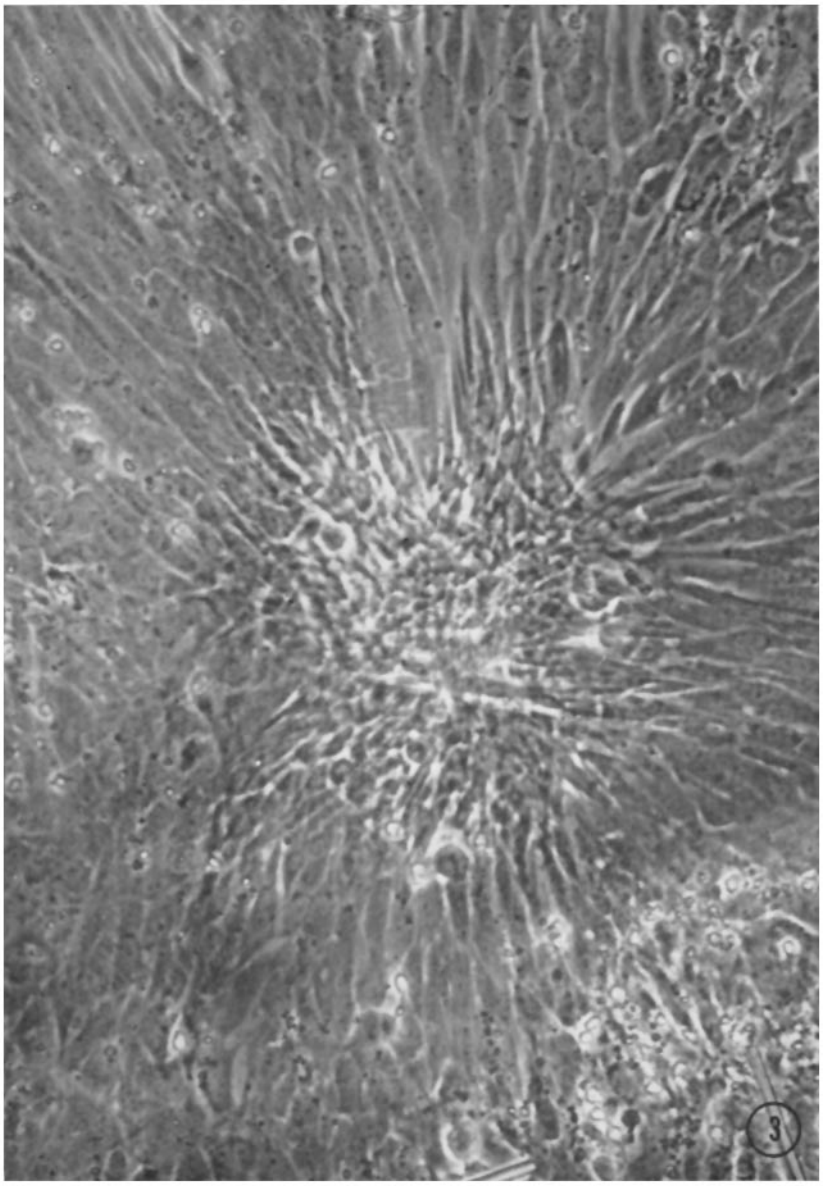
FIG. 2. Primary rat heart cell culture showing two distinct morphological types. The elongated cell in the center of the field is a heart muscle cell possessing characteristic myofibrils. An endothelioid cell is seen in the upper right hand corner. (Silver nitrate stain × 400.)



(Bonventre and Imhoff: Protein synthesis in heart cell cultures)

PLATE 83

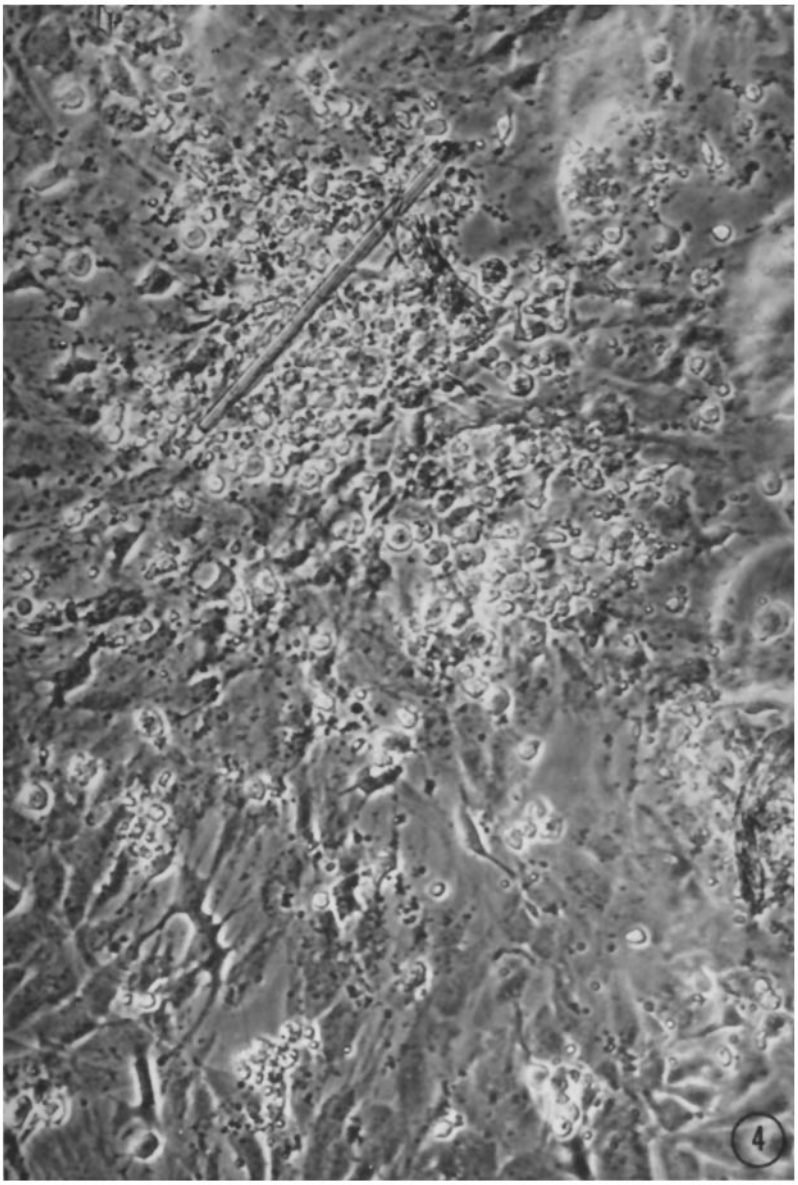
FIG. 3. Primary cell culture of embryonic guinea pig heart tissues. Normal monolayer 7 days old with a synchronous contracting cell mass in the center of the field. (Phase contrast × 40.)



(Bonventre and Imhoff: Protein synthesis in heart cell cultures)

PLATE 84

FIG. 4. Primary cell culture of embryonic guinea pig heart tissues. Culture incubated with purified diphtheria toxin (25 MLD/ml) for 48 hr. Note the extensive alteration in cell morphology and the virtual destruction of the monolayer. (Phase contrast × 100.)



(Bonventre and Imhoff: Protein synthesis in heart cell cultures)