T_3 BACTERIOPHAGE PENETRATION OF HUMAN EPITHELIAL CELLS IN THE PRESENCE OF DIMETHYL SULFOXIDE

Thesis for the Degree of M. S.
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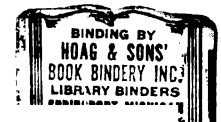
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ABSTRACT

T_3 BACTERIOPHAGE PENETRATION OF HUMAN EPITHELIAL CELLS IN THE PRESENCE OF DIMETHYL SULFOXIDE

By William Charles Wallen

Dimethyl sulfoxide, a chemical solvent which penetrates cell membranes, rapidly mediated the uptake of T_3 bacteriophage into a foreign host. Cytotoxic studies on the effect DMSO has on the AU human epithelial cell line showed that 10% DMSO was the most effective concentration to use. DMSO, in concentrations of 10% or less, was shown to have no effect on the infectious titer of the T_3 bacteriophage. Electron microscope autoradiographs of the beta tracks demonstrated the location of the tritium labeled phage within the cytoplasm and, in a few cases, the nucleus of the cells.

T_3 BACTERIOPHAGE PENETRATION OF HUMAN EPITHELIAL CELLS IN THE PRESENCE OF DIMETHYL SULFOXIDE

By

William Charles Wallen

A THESIS

Submitted to
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in partial fulfillment of the requirements
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1968

Dedication

To my wife, in appreciation of her kindness, understanding, and support which contributed immensely to the completion of this work.

ACKNOWLEDGEMENTS

The author expresses his appreciation to Dr. Walter N. Mack for his guidance and encouragement throughout the completion of this work.

I also wish to thank Dr. L. F. Wolterink for his guidance; Dr. William Deal and Dr. Peter Hirsch for their critical review of this manuscript; Mrs. June P. Mack of the Electron Microscope Laboratory, Biology Research Center, for performing the electron microscopy; Dr. Gordon C. Spink for guidance and assistance with the autoradiography; and Mrs. Radene B. Winterton for her valuable technical assistance.

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T_3 BACTERIOPHAGE PENETRATION OF HUMAN EPITHELIAL CELLS IN THE PRESENCE OF DIMETHYL SULFOXIDE

INTRODUCTION

Dimethyl Sulfoxide (DMSO), a by-product of paper manufacturing, was first produced by Alexander Saylzeff in 1866. The DMSO molecule is composed of sulfur, oxygen, and two methyl groups located at the "corners." The sulfur-oxygen bond being highly dipolar and aprotic, results in DMSO being an excellent solvent (22). DMSO has been shown to penetrate most cells, apparently without destroying the integrity of the membrane (18). Its penetrant and solvent properties make it useful in the topical application of drugs (20). DMSO has also been used to enhance the absorption of drugs in vivo (5), and to preserve cells in the frozen state (9, 4). The penetrating capacity of DMSO has been used to mediate the uptake of molecules varying in size from D_2o (28), sugars (10), and proteins such as insulin (16) to large polypeptides of molecular weight in excess of 300,000 (17), and infectious viral nucleic acid

into susceptible host cells (2, 30). Amstey (2) used infectious polivirus RNA and African Green Monkey Kidney cell monolayers to demonstrate that a significant amount of the nucleic acid was absorbed into the cell within the first few minutes after exposure to DMSO. The mechanism of action of DMSO is unclear as yet, but its effect is apparently to increase the rate of movement of certain molecules across membranes into the cell. This effect increases as the size of the molecule being transported increases (3).

The present study utilized DMSO as a mediator of bacteriophage penetration into a foreign host, cultured human epithelial cells. A continuous human epithelial cell line isolated by Wheeler (31) was used as the host. The T_3 bacteriophage was the virus used because of its foreignness for the host, its small size, and its ease and accuracy in titration.

Brief studies were initially performed to determine the toxicity level for the cells when exposed to varying concentrations of DMSO. Plaque reduction studies were then performed to determine if there was any significant reduction of phage in the medium in the presence of DMSO and the foreign host cells. Electron microscope autoradiographs

were prepared to demonstrate that the tritium labeled phage had penetrated the foreign host and to localize the position of the viral particles within the epithelial cells.

Electron microscope autoradiography permitted direct visualization of the radiation emitted by a radioisotope. The emitted beta rays effect the silver salts in photographic emulsion in a manner similar to visible light rays. Upon development, the salts that were sensitized by the beta rays are reduced to metallic silver grains and appear black. The isolated sensitized grains in the emulsion or, if the beta ray was sufficiently energetic, the tracks of grains made by the beta rays are readily photographed. Thin layer nuclear emulsions of 5μ or less provide the best resolution when used with a beta emitter or low energy. The use of H_3 as the beta emitter improves resolution because its low energy limits its effects to within 1μ of the labeled particle (14).

The T_3 bacteriophage was labeled with H_3 thymidine. The labeled nucleotide is incorporated into the nucleic acid of the bacterium and then during the phage replication cycle, the radioisotope is incorporated into the DNA of the bacteriophage. The labeling efficiency of a radioisotope

incorporated into the nucleic acid of a bacterium depends on the specific activity to the isotope, the dilution of the labeled compound with nonlabeled intracellular pools, and the bacteria's capacity to metabolize the labeled compound (15).

MATERIALS AND METHODS

The T_3 bacteriophage is a small, DNA virus which specifically attacks the bacterium Eacherichia coli, strain B. Its dimensions are: 47x47 mμ head, 10x15 mμ tall, and a DNA content of $0.9x10^{-16}$ grams/particle (27). The experimental host cell was the human epithelial cell (AU) isolated in cell culture by Wheeler (1956) (31). The AU cells were maintained on a medium of lactalbumin hydrolysate, (LAH) (24), with 2% inactivated calf serum. Monolayers of AU cells were grown in French square bottles to a concentration of approximately $1x10^5$ cells/ml for plaque reduction tests. The monolayers were grown in milk dilution bottles to a concentration of $1.2x10^6$ cells/ml for the autoradiographic studies.

Cellular toxicity studies were performed by exposing three day old cell culture monolayers to varying concentrations of DMSO. The concentration of DMSO (reagent grade) was varied from 5% to 100%. The DMSO was mixed with the maintenance medium (LAH-2% inactivated calf serum) and added to the cell monolayers. The cells were then placed

in the incubator (37C) for 60 minutes. The DMSO media was then replaced with maintenance media lacking DMSO and the monolayers were allowed to grow for 24 hours. The cells were then compared under light microscope examination for apparent alterations that occurred 24 hours after exposure to DMSO as compared to controls not receiving DMSO. The selection of the optimal concentration of DMSO was based on light microscope examination of the cells to determine which concentration caused the least apparent alteration in metabolism or cell appearance due to exposure for periods up to 60 minutes.

After determining that 10% DMSO did not demonstrate toxicity to the AU cells plaque reduction studies were performed by exposing cell monolayers to 10% DMSO and T_3 bacteriophage simultaneously for varying lengths of time. Controls involved (a) T_3 phage exposed to DMSO only and (b) T_3 phage exposed to AU cells only. After appropriate time periods of exposure, the phage was assayed on E. coli B by the soft agar technique (19). The assay by Gratia (1936) (12), described by Adams (1950) (1), and modified by Lawrence (1957)(19), was performed in the following manner: 0.5ml of a 24 hour suspension of growing E. coli B cells

was placed into a test tube containing 2.5ml of melted soft agar (7.5 g agar in 1 liter of water) at 45 C. Then 0.1ml of an appropriate dilution of the T_3 phage sample was placed in the bacteria-agar mixture. The soft agar-phage-bacteria mixture was poured over a plate of nutrient agar, spread uniformly over the plate surface, and incubated at 37 C. Plaques, clear areas in the bacterial growth due to lysis of the bacteria appeared in about 6 hours. They were counted after 14 to 16 hours of incubation. The number of viable phage particles per ml. was determined by counting the number of plaques, multiplying by 10 to compensate for the 0.1ml inoculum, and then multiplying by the dilution factor. Luria, et al. (1951) determined that this method was extremely accurate for the titration of phage and that one plaque corresponded to one infectious phage particle (21). All phage dilutions were ten fold dilutions performed in 2% nutrient broth (Difco).

Tritiated (H^3) thymidine labeled T_3 bacteriophage and AU cells were prepared for autoradiography in the following manner: A thymine deficient E. coli B mutant was selected by the aminopterin solid medium technique (8). The bacteria was grown overnight in a defined medium lacking thymine. Approximately 10^8 bacterial cells were spread

over a plate containing supplemented A medium consisting of (in grams per liter): K_2HPO_4, 10.5; KH_2PO_4; Sodium citrate $5H_2O$, 0.47; MgSO4, 0.05; (NH_4) SO_4, 1.0; agar, 15; casamino acids, 5; adenosine, guanine, cytocine, thymidine, and tryptophane, 0.1 each; aminoterin, 0.5; glucose, 2; and thiamine, 0.001. The cultures were incubated for 24 hours at 37 C. Several of the resulting colonies were selected and tested for thymine dependence. Those mutant colonies which could not grow without exogenous thymidine were maintained on refrigerated aminopterin plates.

Tritiated thymidine (29) (spec. act. > 99%; 12c/ml. diluted to 120mc/ml) was substituted in fresh aminopterin plates for the non-labeled thymidine. The E. coli B was incubated for 16 hours at 37 C on the solid medium to label its nucleic acid pool. The phage was mixed thoroughly with the labeled E. coli and incubated for an additional 16 hours at 37 C. The labeled phage lysate was harvested from the plates with 2-3 ml of sterile H_2O and collected in a sterile 20ml. syringe. The labeled phage was then clarified by two cycles of centrifugation (5,000 RPM; 15 minutes International PR-1). It was then filtered through a sterile HA millipore filter (Millipore Corporation, Bedford, Mass.).

The lysate was dialyzed against 200ml of 2% nutrient broth. The dialysate was changed at one hour intervals and samples (20λ each) were collected to determine the rate of removal of dialyzable H^3-thymidine label from the phage suspension. The samples were individually counted on a Mark IV Scintillator in a toluene based scintillation fluid.

The 50ml of phage suspension was concentrated to 5ml of material in an ultracentrifuge (Spinco; Type 50 rotor, 50,800xg, 70 min.). The pellet was resuspended with 5ml of sterile distilled water, low speed centrifuged (5,000 RPM, 15 min. International PR-1), and the resultant 10ml supernatant fluid was concentrated to 2ml of suspension by ultracentrifugation (Type 50 rotor, 57,900xg, 60 min.). Biological (pfu/ml) and Radiological (cpm/20λ) activities were determined on the approximately one hundred fold concentrated by volume phage suspension. A 0.1ml phage inoculum was used on each monolayer of AU cells.

Three groups of cells were used in the experiment. Group A was used as the control to determine the level of phagocytosis of the phage by the cells. It was inoculated with labeled phage for 60 minutes without DMSO. The 60 minute time period was selected because that was the greatest length of time during the experiment that the cells

to DMSO and phage. Two other groups of cells were used, varying in length of exposure to, and the concentration of, DMSO. Group B was inoculated with labeled phage and exposed to 10% and 20% DMSO for 1-1/2 minutes. This group was used to determine whether the action of DMSO was in the presence of cells was immediate or delayed. Group C was inoculated with labeled phage and also exposed to 10% and 20% DMSO for 60 minutes. The controls for the autoradiograph emulsion consisted of the following: (a) Exposure of a test grid to 10 sec of sunlight and a 100 watt lightbulb, and (b) unlabeled T_3 phage, AU cells, and 20% DMSO.

All cell monolayers were washed three times with Hanks' basal salt solution and the cells were removed from the glass surface with a sterile rubber tipped glass rod. They were immediately fixed in 6.25% glutaraldehyde in Sorenson's buffer solution (25). Dehydration was done by a sequential gradient of ethyl alcohol, followed by two changes of propylene oxide. The cells were then placed in a 1:1 propylene oxide: epon 812 mixture followed by embedding in epon 812 at 60 C for 48 hours.

Ultrathin sections of the hardened cell blocks were cut on a Sorvall MT-2 ultramicrotome at 500 to 700 A. Each

section was placed on a colloidion based copper mesh (100 and 200) grid. The emulsion used was the Ilford L-4 (Ilford Co., England) nuclear emulsion. It has a grain diameter of 1200 A and a sensitivity to H^3 of 132 grains/100 decays (26). Its principle qualities are good autoradiographic resolution (about 0.1μ)(6), low background, high sensitivity, insensitivity to yellow-green light, good reproducibility on monogranular layering, and easy storage.

The loop technique was used to place a thin layer of emulsion on the grids (7). Ten milligrams of Ilford L-4 was melted at 45 C for 15 minutes in 20ml of distilled water in a 250ml beaker. The mixture was constantly stirred, then placed in an ice bath for 3 minutes, and left at room temperature for 20 to 30 minutes. This procedure resulted in a highly viscous emulsion. The grids were placed on circular glass cover slips on aluminum planchets. The planchets were in turn placed on corks sufficiently large to stabilize them. A 4cm. diameter wire loop was dipped into the emulsion and slowly withdrawn. The film formed in the loop gelled rapidly. It was quickly placed over the planchets causing a fine even emulsion to fall over the grids. The grids were placed in a light-free box with dehydrant and allowed to develop 4 weeks in the dark.

The emulsion was developed in Kodak D-19 for 1 minute, rinsed in water for 30 seconds. It was then fixed in Kodafix solution for 1 minute, and rinsed in slowly circulating water for 5 minutes.

After the emulsion was developed, the sections were doubled stained in uranyl acetate (11) for 30 minutes followed by 15 minutes in lead citrate-Reynolds buffer solution (23). The sections were examined for beta tracks with a Philips 100 electron microscope.

RESULTS

The toxicity studies were performed to determine the concentration of DMSO which could be used without causing noticable changes in the cells' growth rate of appearance. The toxicity studies were conducted for a maximum at 60 minutes since, as Amstey has shown (2), the action of DMSO was relatively rapid.

The results of the cellular toxicity studies are shown in Table 1. DMSO in 10% and 15% concentration did not alter the rate of acid production of the cells for periods up to 60 minutes. Cellular appearance was slightly granular while the rate of monolayer formation remained the same as the controls. Cells exposed to 20% DMSO showed no effects for periods of 10 minutes or less. A slight reduction in growth rate was noted if 20% DMSO was left on the cells for 30 and 60 minutes. The cells showed a noticable granularity and their relative rate of monolayer formation was retarded. Exposure to 50% DMSO for 1 minute and 10 minutes markedly disrupted cellular rate of acid production although many of the cells survived and were recovered.

TABLE 1.--Cytotoxic effect of DMSO, in various concentrations, on human epithelial cells.

DMSO Concentration	Time (min)			
	1	10	30	60
100%	R	F	F	F
75%	R	F	F	F
50%	R	R	R	F
20%	N	N	R	R
15%	N	N	N	N
10%	N	N	N	N
5%	N	N	N	N

F=cells became fixed and were unrecoverable.
R=cells were recoverable but metabolic rate was reduced.
N=cells' metabolism was not altered.

The cells were very granular for 3 days thereafter. Exposure for thirty minutes of 50% DMSO caused most of the cells to round up, become highly granular, and only a small ratio of the cells were recovered. Sixty minutes exposure to 50% DMSO resulted in the fixation of the cells to the glass wall. These cells were unrecoverable. Cells exposed to 75% or 100% DMSO for 1 minute were severely altered in their rate of acid production. Cells exposed for 10 minutes or longer to either 75% or 100% DMSO resulted in their irreversible fixation to the glass walls.

DMSO in 10% concentration was used for the plaque reduction studies because there was no detectable

alteration in the cellular rate of acid production over the 60 minute test period.

The plaque reduction studies were performed to determine the following:

a) The level of infectious unit reduction when the phage was placed in the presence of AU cells and 10% DMSO (Table 2, t),

b) The effect 10% DMSO alone would have on the infectious phage titer (Table 2, b),

c) The level of infectious unit reduction when the phage was placed in the presence of AU cells alone (Table 2, c).

The reduction in infectious units of phage was determined by counting the number of plaques on a plate covered with E. coli B. Each plaque represents one infectious unit or plaque forming unit (pfu).

The "input" or stock titer (Table 2,a) was used as a standard and represented 0% reduction of phage (pfu). Control (b) used to determine the effect DMSO has on the phage, shows reduction ranges of 0% to 4.4% over the tests.

Table 2.--The effect of DMSO on the reduction in pfu of phage particles from the suspending medium in the presence of AU cells.

Test	Time	Ave. No. Plaques	Phage Titer	Per Cent Reduction
1.	Input[a]	95	9.5×10^3	0
	Control[b]	92	9.2×10^3	3
	1 minute[t]	77	7.7×10^3	18.5
	2 minutes	66	6.6×10^3	30.5
	5 minutes	65	6.5×10^3	32.0
	60 minutes	57	5.7×10^3	40.0
2.	Input	219	21.9×10^2	0
	Control	215	21.5×10^2	1
	1 minute	181	18.1×10^2	17.4
	3 minutes	141	14.1×10^2	35.6
	5 minutes	116	11.6×10^2	47.0
	30 minutes	101	10.1×10^2	53.9
	60 minutes	98	9.8×10^2	55.3
	Control[c]	205	20.5×10^2	6.4
3.	Input	178	17.8×10^2	0
	Control	215	17.4×10^2	0.3
	1 minute	151	15.1×10^2	15.2
	2 minutes	136	13.6×10^2	23.6
	5 minutes	102	10.2×10^2	42.8
	30 minutes	95	9.5×10^2	46.1
	60 minutes	87	8.7×10^2	51.2
	Control	172	17.2×10^2	3.4
4.	Input	68	6.8×10^3	0
	Control	72	7.2×10^3	-6
	1 minute	51	5.1×10^3	25
	3 minutes	55	5.5×10^3	19.2
	5 minutes	60	6.0×10^3	12
	30 minutes	49	4.9×10^3	28
	60 minutes	43	4.3×10^3	37
	Control	70	7.0×10^3	-2

Table 2 (Continued)

Test	Time	Ave. No. Plaques	Phage Titer	Per Cent Reduction
5.	Input	294	29.4×10^2	0
	Control	288	28.8×10^2	2.1
	1 minute	253	25.3×10^2	13.9
	3 minutes	239	23.9×10^2	18.7
	5 minutes	226	22.6×10^2	23.2
	30 minutes	218	21.8×10^2	25.8
	60 minutes	211	21.1×10^2	28.3
	Control	289	28.9×10^2	1.7
6.	Input	71	7.1×10^3	0
	Control	69	6.9×10^3	2.9
	1 minute	57	5.7×10^3	19.7
	2 minutes	53	5.3×10^3	25.4
	5 minutes	50	5.0×10^3	29.6
	30 minutes	42	4.2×10^3	40.9
	60 minutes	39	3.9×10^3	45.1
7.	Input	70	7.0×10^3	0
	Control	68	6.8×10^3	2.9
	1 minute	55	5.5×10^3	21.4
	2 minutes	53	5.3×10^3	24.3
	5 minutes	50	5.0×10^3	28.6
	30 minutes	44	4.4×10^3	37.2
	60 minutes	40	4.0×10^3	42.9
	Control	70	7.0×10^3	0
8.	Input	74	7.4×10^3	0
	Control	71	7.1×10^3	4
	1 minute	54	5.4×10^3	27
	2 minutes	51	5.1×10^3	31.1
	5 minutes	49	4.8×10^3	35.2
	30 minutes	41	4.1×10^3	44.6
	60 minutes	37	3.7×10^3	50.0

Table 2 (Continued)

Test	Time	Ave. No. Plaques	Phage Titer	Per Cent Reduction
9.	Input	68	6.8×10^3	0
	Control	65	6.5×10^3	4.4
	1 minute	60	6.0×10^3	12.0
	2 minutes	54	5.4×10^3	20.6
	5 minutes	50	5.0×10^3	26.4
	30 minutes	47	4.7×10^3	30.9
	60 minutes	43	4.3×10^3	36.7
10.	Input[a]	65	6.5×10^3	0
	Control[b]	66	6.6×10^3	-1
	1 minute[t]	61	6.1×10^3	6.2
	2 minutes	56	5.6×10^3	13.9
	5 minutes	52	5.2×10^3	20.0
	30 minutes	48	4.8×10^3	26.0
	60 minutes	45	4.5×10^3	30.8
11.	Input[a]	68	6.8×10^3	0
	Control[b]	67	6.7×10^3	1.5
	1 minute[t]	55	5.5×10^3	19.2
	2 minutes	49	4.9×10^3	27.9
	5 minutes	45	4.5×10^3	33.8
	30 minutes	40	4.0×10^3	41.2
	60 minutes	37	3.7×10^3	43.6
	Control[c]	67	6.7×10^3	1.6

Input[a]=T_3 stock phage titer.

Control[b]=T_3 and 10% DMSO for 60 minutes.

Test[t]=T_3 and AU cells and 10% DMSO.

Control[c]=T_3 and AU cells for 60 minutes.

Control (c) used to determine the effect the AU cells alone would have on the phage in the supernatant fluid, shows a reduction range of 0% to 6.4%. The experimental groups (Table 2, t) show a rapid drop in phage titer from the supernatant fluid over the first five minutes of exposure to DMSO. The test shows a reduction of approximately 25% of the phage titer from the suspending medium. After five minutes of exposure the rate of removal of phage from the supernatant fluid appears to reduce itself and hereafter, the action of DMSO was completed as far as reduction of phage was concerned.

The results of the studies are shown in Table 2. Summarizing the tests show a consistent reduction in the phage titer (pfu) as the length of time of exposure to DMSO increases. The "Input" represents the titer of phage in a stock suspension that was used for the test. Control (b) represents the titer of phage (pfu) remaining after 60 minutes exposure to 10% DMSO in LAH. Control (c) represents the titer of phage (pfu) remaining in the suspension medium after 60 minutes of exposure to AU cells without any DMSO. The test periods for exposure of AU cells to 10% DMSO with T_3 phage were 1 minute, 2 or 3 minutes, 5 minutes, 30 minutes, and 60 minutes.

Graph I is a composite of all the results of Table 2. Line A shows the per cent reduction of the initial inoculum in the suspending medium after a given time in the presence of AU cells and 10% DMSO. The range of plaques for the tests is shown in the vertical line from one through 30 minutes. Lines B and C represent the two controls for the test (see b and c above) respectively. Both lines are represented by the broken line on the graph because they are concurrent with one another. There is no significant reduction if pfu under either of these control conditions.

The reduction in pfu when the phage was placed in contact with DMSO and AU cells indicated that the phage may be either (1) entering the cell or (2) attached or associated with the cell membrane. To differentiate which of the two possibilities occurred electron microscope autoradiography was utilized to localize the phage. A H^3-thymidine labeled phage was produced from labeled E coli B and extraneous label was removed by dialysis for 18 hours. Table 3 shows the counts per minute obtained for the 9 (20λ) samples taken during the 18 hours of dialysis. The counts per minute (cpm) began after the first hour at 68 cpm and were gradually reduced to a relatively constant level of

Graph I: Effect of 10% DMSO on Reduction of T_3 Phage from Supernatant Suspension in Presence of AU Cells.

A = T_3 phage exposed to 10% DMSO in the presence of AU cells.

B = T_3 phage exposed to 10% DMSO alone.

C = T_3 phage exposed to AU cells without any DMSO.

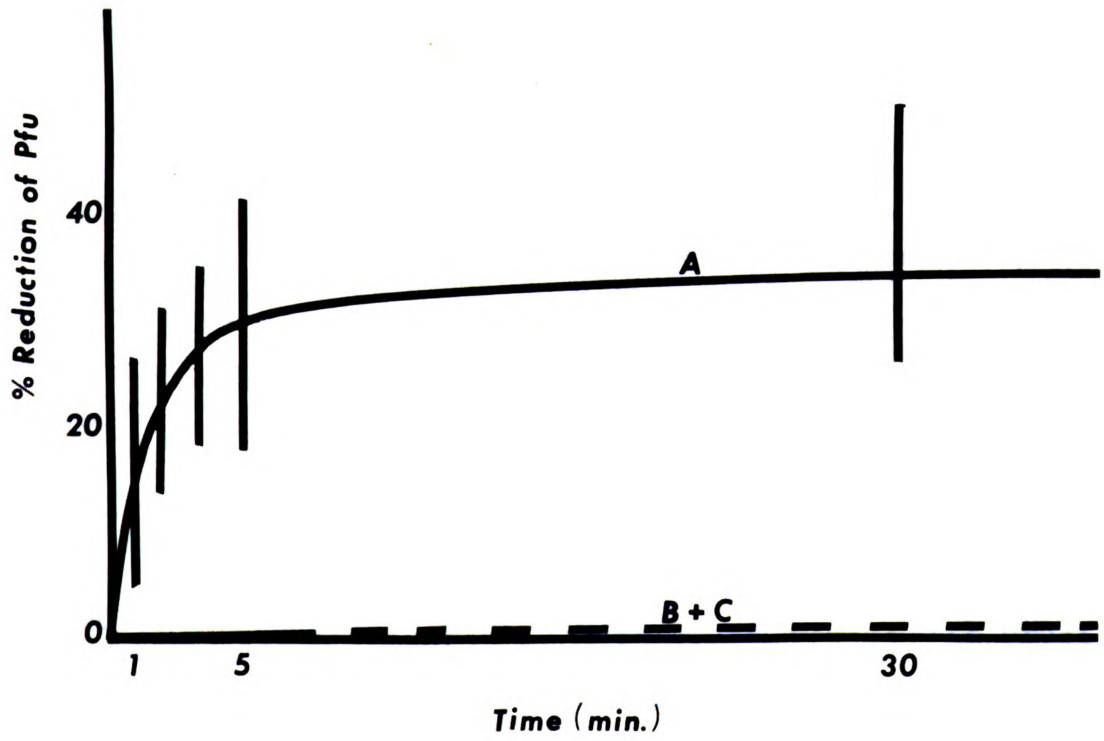

% Reduction of Pfu
40
20
0
A
B + C
1
5
30
Time (min.)

TABLE 3.--Scintillation counts of: dialysis samples (20λ), virus suspension before concentration, and supernatant suspension after concentration.

Sample Number	Time	Cpm	Sample
1	1 hour	68.71	dialysate
2	3 hours	58.60	"
3	4 hours	37.53	"
4	5 hours	33.65	"
5	14 hours	29.28	"
6	15 hours	24.39	"
7	16 hours	26.69	"
8	17 hours	27.00	"
9	18 hours	27.48	"
10	-	5714.00	Virus suspension
11	-	36.88	1st ultracentrifuge supernatant fluid
12	-	43.88	2nd ultracentrifuge supernatant fluid

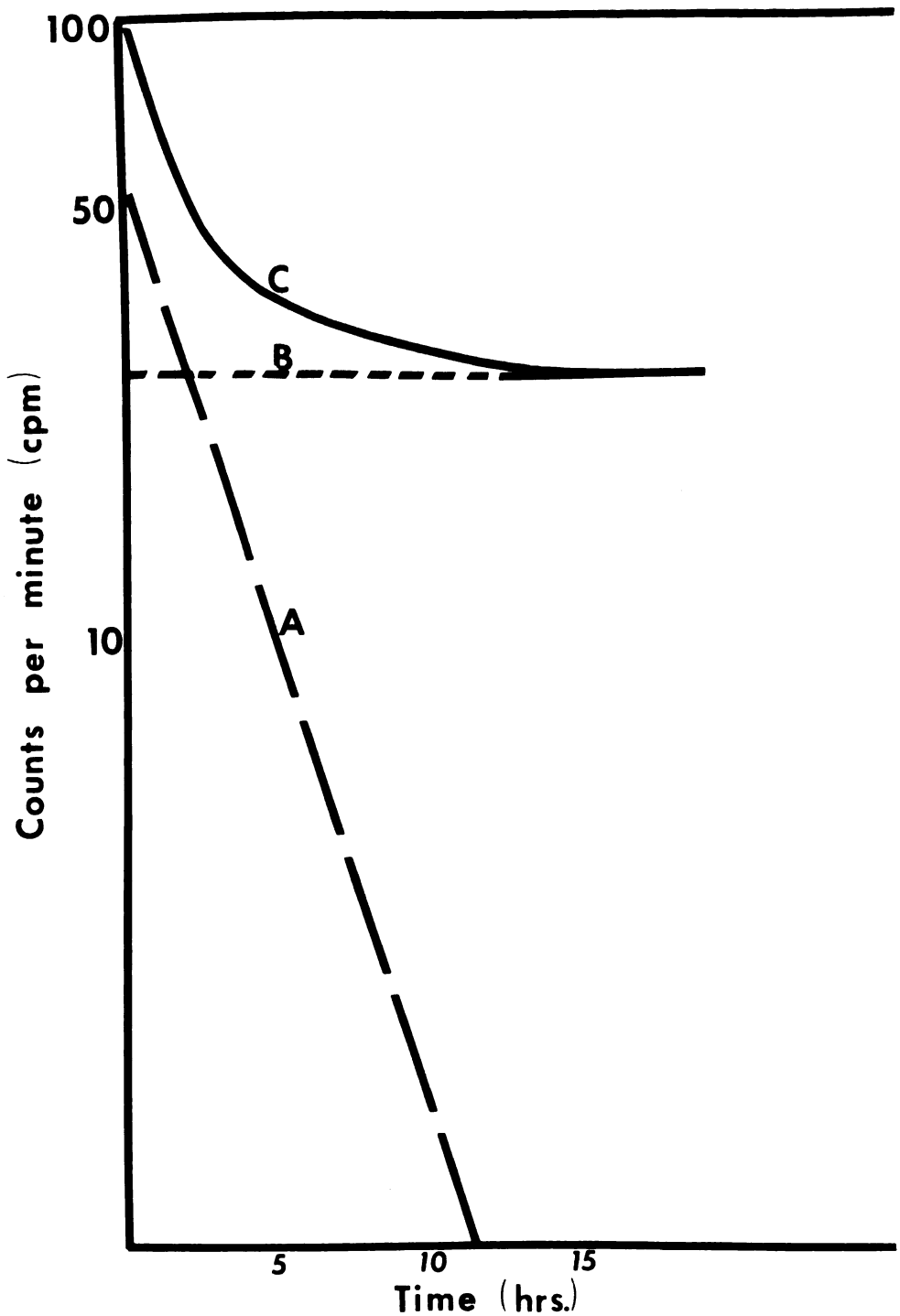
27.00cpm after 18 hours of dialysis. The dialysis procedure was halted at this time because it was felt that the rate of removal of label was not great enough to justify further dialysis. Since the dialyzable label was not identified as to source, it was, for statistical purposes, assumed to be extraneous label not associated with intact phage particles. The rate of removal of the dialyzable label can be seen in Graph II. Line A is the total

Graph II: Dialysis of crude phage lysate to remove dialyzable radiosotope labeled molecules and fragments.

A = total counts per minute (cpm) remaining in suspension.

B = H^3-thymidine remaining in suspension (cpm).

C = labeled particles, dialyzing at a slow consistent rate, remaining in suspension.


100
50
10
C
B
A
Counts per minute (cpm)
5
10
15
Time (hrs.)

reduction of label (counts per minute, cpm) over the eighteen hours of dialysis. Line B shows the removal of this molecule was rapid and efficient because of its small consistent size. Graph II indicates that the H^3 thymidine molecule was removed after 12 hours. Line C shows the removal of a larger molecule which after approximately 18 hours was essentially the only source extraneous label remaining in the suspension. The slow rate at which the label was dialyzed indicates that it was attached to a large molecule which was removed very slowly through the dialyzor. It was necessary to account for the ratio of this extraneous label to labeled phage in suspension when determining the source of beta tracks found on the autoradiographs. Sample ten (Table 3) shows the activity within the dialyzor to be 5,714 cpm. The 50ml suspension, containing both the labeled phage and the extraneous large labeled molecule was concentrated in the ultracentrifuge twice, resulting in a one hundred fold concentration by volume of the suspension. After the first ultracentrifugation the upper 45ml supernatant fluid was counted and discarded (sample 11, Table 3). The supernatant suspension recorded 36.88 cpm for the 20λ sample. The 5 ml concentrated

suspension was resuspended with 5 ml of sterile water and concentrated by ultracentrifugation again. The upper 8ml of supernatant suspension was counted (sample 12, Table 3) and discarded. The discarded supernatant suspension recorded 43.88 cpm. The concentration of the labeled phage resulted in further removal of extraneous sources of label, such as disrupted phage or bacterial DNA, in the discarded supernatant suspensions.

The results of the autoradiograph study are shown in Table 4. Because there was such a rapid reduction in the number of phage particles in the presence of DMSO in the first five minutes of exposure only the test with 90 seconds exposure and the controls were subject to electron microscope examination (Table 2, Graph I). Control group I, consisting of AU cells plus unlabeled T_3 phage plus 20% DMSO for 60 min. showed no beta tracks either inside or outside the cells in a total of 490 cell sections examined. No beta tracks were discernible on any of the sections examined. No beta tracks were discernible on any of the sections examined for this control group. Control Group II, consisting of AU cells plus H^3 labeled T_3 phage for 60 minutes but without DMSO, was found to lack any beta

TABLE 4.--The effect of DMSO on the incorporation of labeled T_3-bacteriophage into AU cells, as shown by counts of beta tracks on the electron microscope autoradiographs.

Test							Total
1. Control I-AU cells & unlabeled T_3&20%DMSO-60min.							
Sect. No.	1	2	3	4	5		5
No. cells	123	47	73	116	129		490
No. tracks	0	0	0	0	0		0
2. Control II-AU cells & H^3-T-60 min.							
Sect. No.	1	2	3	4	5	6	6
No. cells	103	74	126	49	113	73	530
No. tracks	0	0	0	0	0	0	0
3. Experiment III-AU cells&H^3-T_3&10%DMSO-90sec.							
Sect. No.	1	2	3	4			4
No. cells	20	92	73	43			228
No. tracks	1	7	5	3			16
Experiment III-AU cells&H^3-T_3&10%DMSO-90sec.							
Sect. No.	5	6	7	8			4
No. cells	38	47	18	20			123
No. tracks	2	5	2	3			11
Experiment III-AU cells&H^3-T_3&10%DMSO-90sec.							
Sect. No.	9	10	11	12	13		5
No. cells	24	38	36	19	22		140
No. tracks	1	3	2	0	2		8
					Totals		
					Sect. No.		13
					No. cells.		493
					No. tracks		35
4. Experiment IV-AU cells & H^3-T_3&20% DMSO-90sec.							
Sect. No.	1	2	3	4	5	6	
No. cells	12	38	29	73	64	28	
No. tracks	1	1	3	3	5	6	

tracks inside or outside of the cells in 530 cell sections examined. Experimental group III, consisting of AU cells plus H^3 labeled T_3 phage and 10% DMSO for 80 sec. showed 35 beta tracks associated with 493 cells. Experimental group IV, consisting of AU cells plus H^3 labeled T_3 phage plus 20% DMSO showed that there were 15 beta tracks associated with the 244 cell sections examined.

Table 5 is a summary of the data presented in Table 4 and shows the results obtained for each group and the resulting number of beta tracks found associated with each cell that was sectioned. Control group I, lacking

TABLE 5.--Summary of beta tracks per cell found on autoradiographs.

Test	No. of Sections	No. of Tracks	No. of Cells	Beta Tracks per Cell
Control I	5	0	490	0
Control II	6	0	530	0
Experiment III	13	35	493	.071
Experiment IV	6	15	244	.061

labeled phage, shows no beta tracks found on all five of the sections examined. There were no tracks found either inside or outside of the cells. Control group II, lacking DMSO, also showed no beta tracks either inside or outside

of the cells in the six sections examined. Experimental group III with labeled T_3 phage and 10% DMSO and AU cells showed 0.071 beta tracks per cell that was sectioned associated with 493 cells examined. Experimental group IV consisting of the above constituents except 20% DMSO was used instead of the lower concentrates showed 0.061 beta tracks per cell section associated with 244 cells examined.

A statistical evaluation was used to determine if the beta tracks found on the autoradiographs could be attributed to radioisotope that was associated with intact phage particles. The statistical test used was the binomial approximation of numbers (15). The null hypothesis, that the number of beta tracks found on the autoradiographs could all be attributed to extraneous label and not the H^3-thymidine labeled T_3 phage, was established to test the values obtained on the autoradiograph. Rejection of the null hypothesis gives strong evidence for some of the beta tracks coming from the labeled phage. Acceptance or rejection of the null hypothesis was based on the following statistical test: Let B represent the number of cells examined with beta tracks associated with them and C represent the critical number of cells with tracks associated with

them required to reject the null hypothesis. Then, let the 493 cells in experimental group III (Table 1) be the total number of cells examined for this evaluation. The 0.02 level of confidence was selected for the test.

$$P(B<C)=0.02$$

$$E(B)=n.p=493.1/284=1.74$$

$$Var\ (B)=n.p.q=493\ 1/284\ 283/284=1.73$$

$$SD(B)=\sqrt{Var(B)}$$

$$0.98=P(B\geq C)=\Phi\left(\frac{c-0.5-1.74}{1.324}\right)$$

$$0.98=2.06\Phi$$

$$2.06=\left(\frac{c-0.5-1.74}{1.324}\right)$$

$$c=3.24$$

Since the 35 beta tracks found on the autoradiographs of this group (Table 5, Experiment III) exceeds the critical value of 3.24, the null hypothesis was rejected.

The following electron micrographs were prepared to demonstrate the location of the labeled phage particle within the cells. Figure 1 shows a representative cell from control group I. This group lacked any labeled phage and was used to demonstrate the lack of background radiation causing beta tracks. Figure 2 is representative

micrograph from control group II which had labeled phage but lacked DMSO. There were no beta tracks seen in any of the sections from either of these groups. Figure 3 shows a low magnification micrograph of a beta track associated with the cytoplasm of the cell. Figure 4 is a higher magnification micrograph of Figure 3 demonstrating the beta track more clearly. Figure 5 also shows a beta track located well within the cytoplasm of the cell. Figure 6 shows beta tracks located at the cytoplasmic membrane indicating that a labeled particle may be either inside or closely associated on the outside of the membrane. Figure 7 shows a beta track located in the nucleus of a cell and Figure 8 is a higher magnification micrograph of Figure 7 which clearly demonstrates the beta track within the nucleus. Figure 9 also shows a beta track located within the nucleus of the cell. Figures 10 and 11 are controls for the nuclear emulsion. Figure 10 is a micrograph of the emulsion that was exposed for 10 seconds to sunlight while Figure 11 is a micrograph of the emulsion that was exposed to a flash of a 100 watt light bulb.

Figure 1. A human epithelial cell from experimental group I, exposed to 10% DMSO and unlabeled T_3 bacteriophage. No beta tracks were located in any of the sections in this group (n) nucleus, (c) cytoplasm. Magnification 26,200.

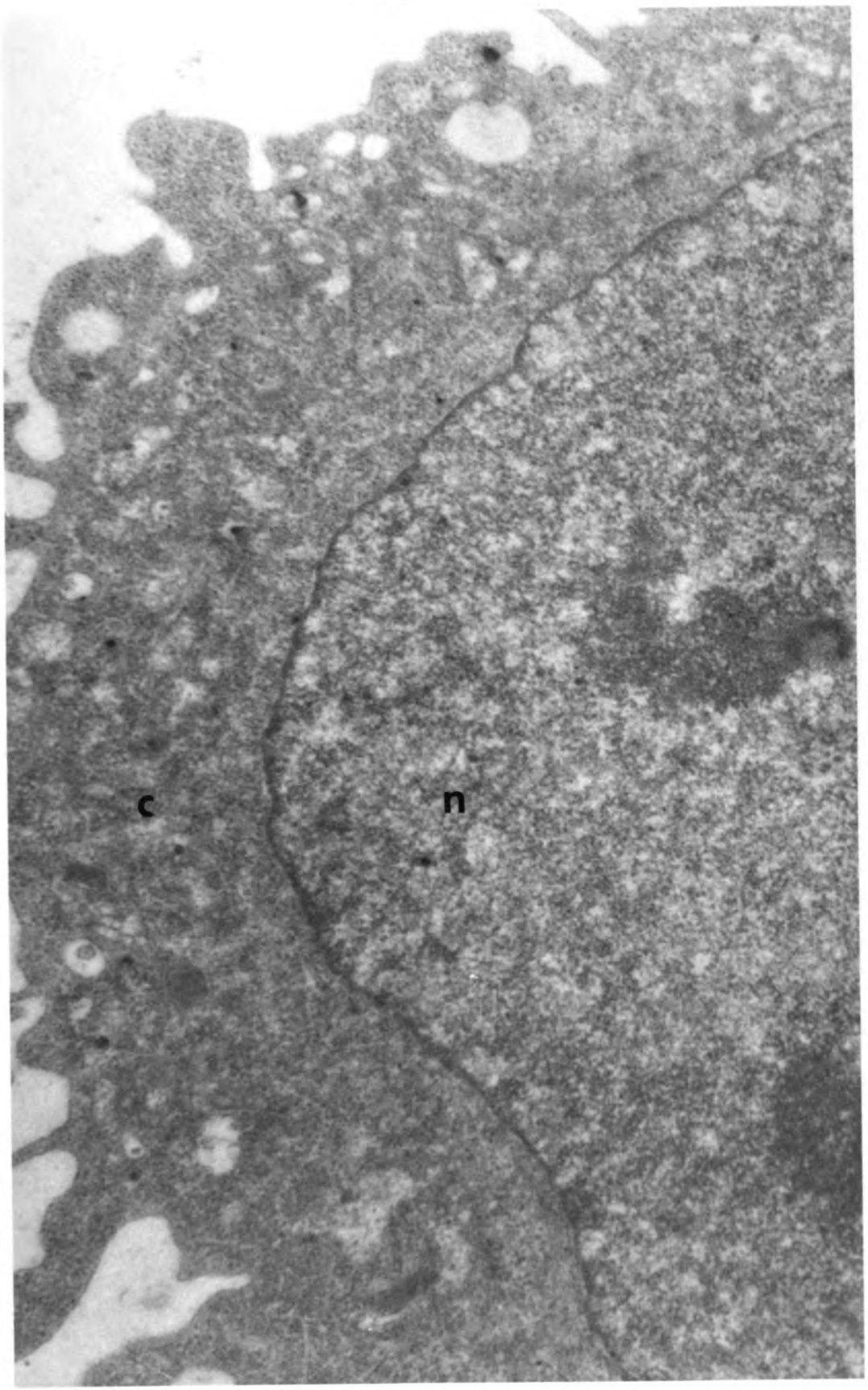

c
n

Figure 2. A human epithelial cell from experimental group II, exposed to H^3-labeled T_3 bacteriophage but without any DMSO. No beta tracks were found in any of these sections indicating either the cells did not phagocytize any of the labeled phage or the level of phagocytosis was too low to detect in the number of cell sections that were examined. (c) cytoplasm, (n) nucleus. Magnification 26,200.

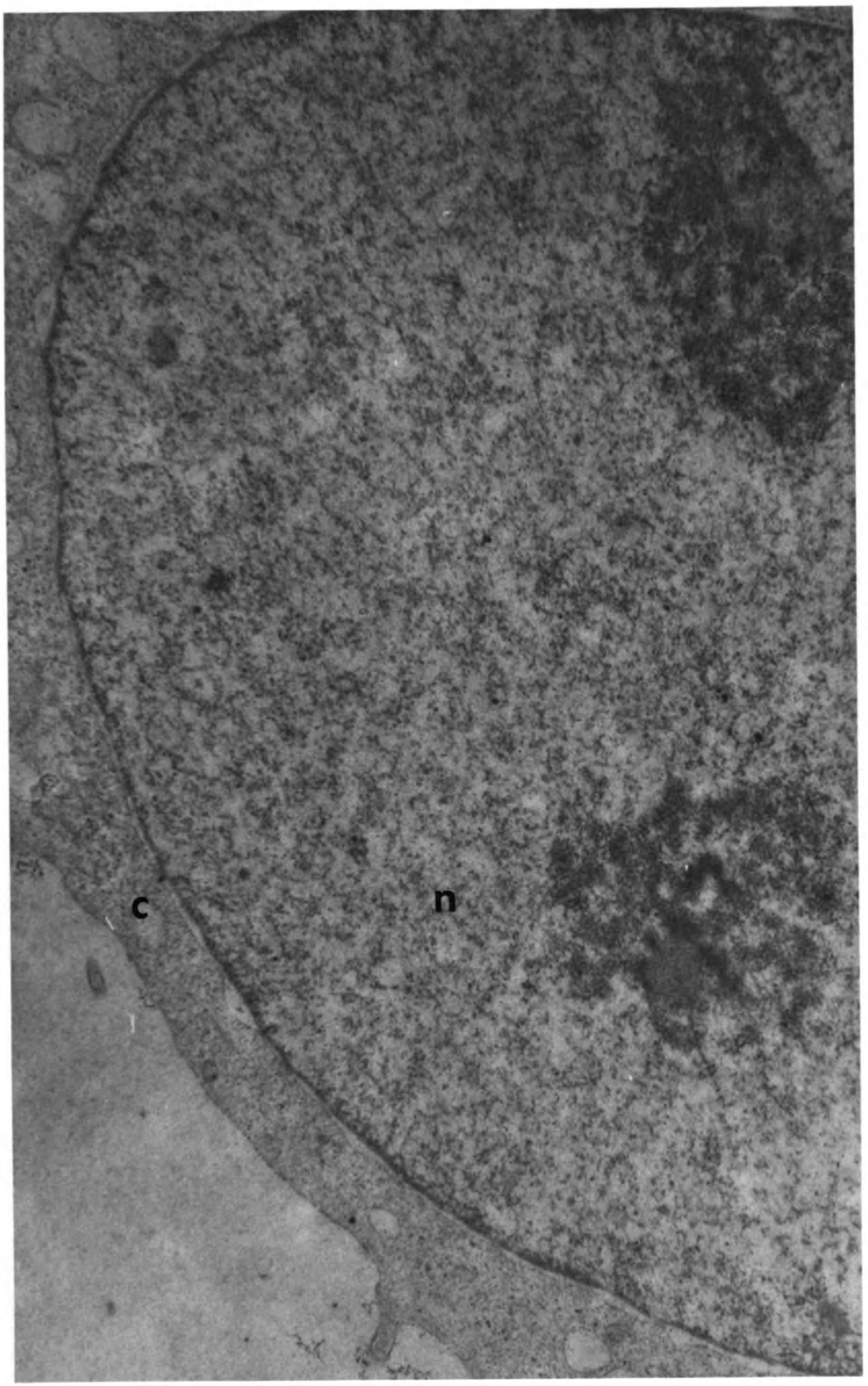

c
n

Figure 3. A beta track (arrow) located over the cytoplasm indicating the location of a labeled phage. This epithelial cell is from the experimental group III, consisting of AU cells, labeled phage, and 10% DMSO. (c) cytoplasm, (n) nucleus. Magnification 26,200.

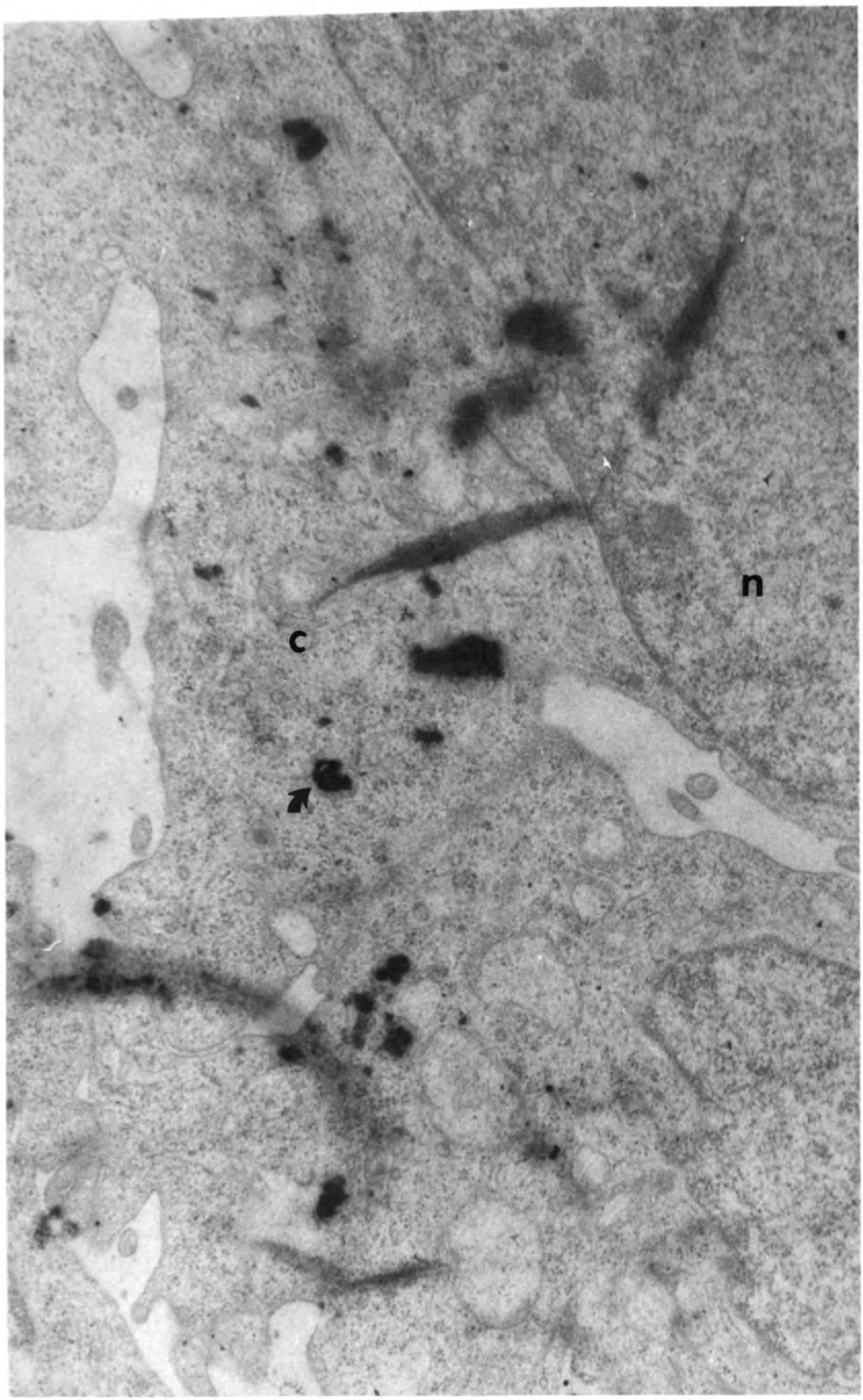

n
c

Figure 4. A higher magnification of the previous (Fig. 3) micrograph. The beta track (arrow) is located within the cytoplasmic membrane. (cm) cytoplasmic membrane, (c) cytoplasm. Magnification 59,300.

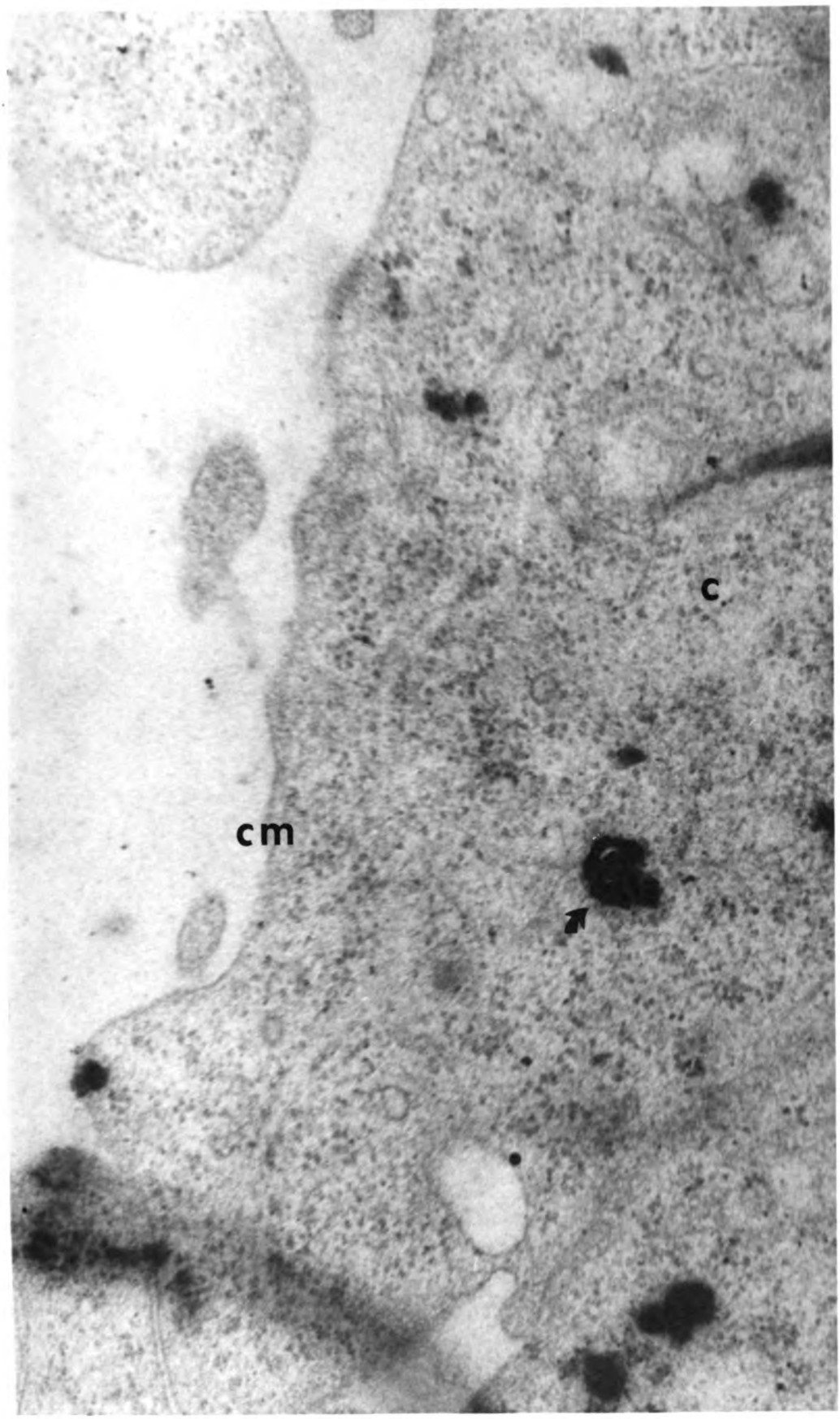

c
cm

Figure 5. This beta track (arrow) is located within the cytoplasm. The cell was exposed to H^3 labeled T_3 phage and 10% DMSO for 90 seconds. (cm) cytoplasmic membrane, (c) cytoplasm. Magnification 59,300.

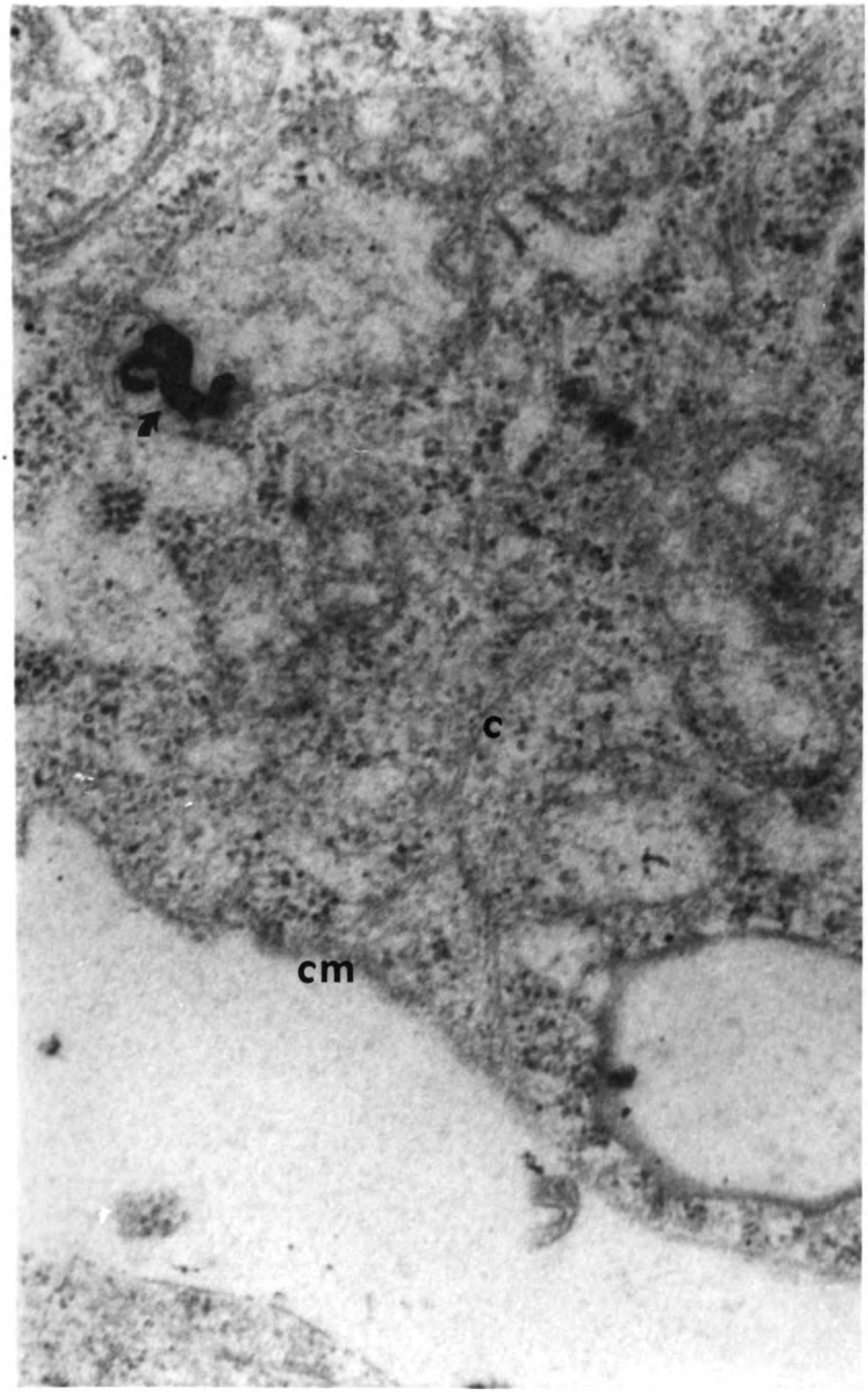

c
cm

Figure 6. This cell has two beta tracks (arrow) associated with the cell membrane. The cell was exposed to H^3 labeled T_3 phage and 20% DMSO. The location of the tracks is too close to the membrane to clearly determine whether the track is inside or associated on the membrane. (cm) cytoplasmic membrane, (c) cytoplasm, (n) nucleus. Magnification 26,200.

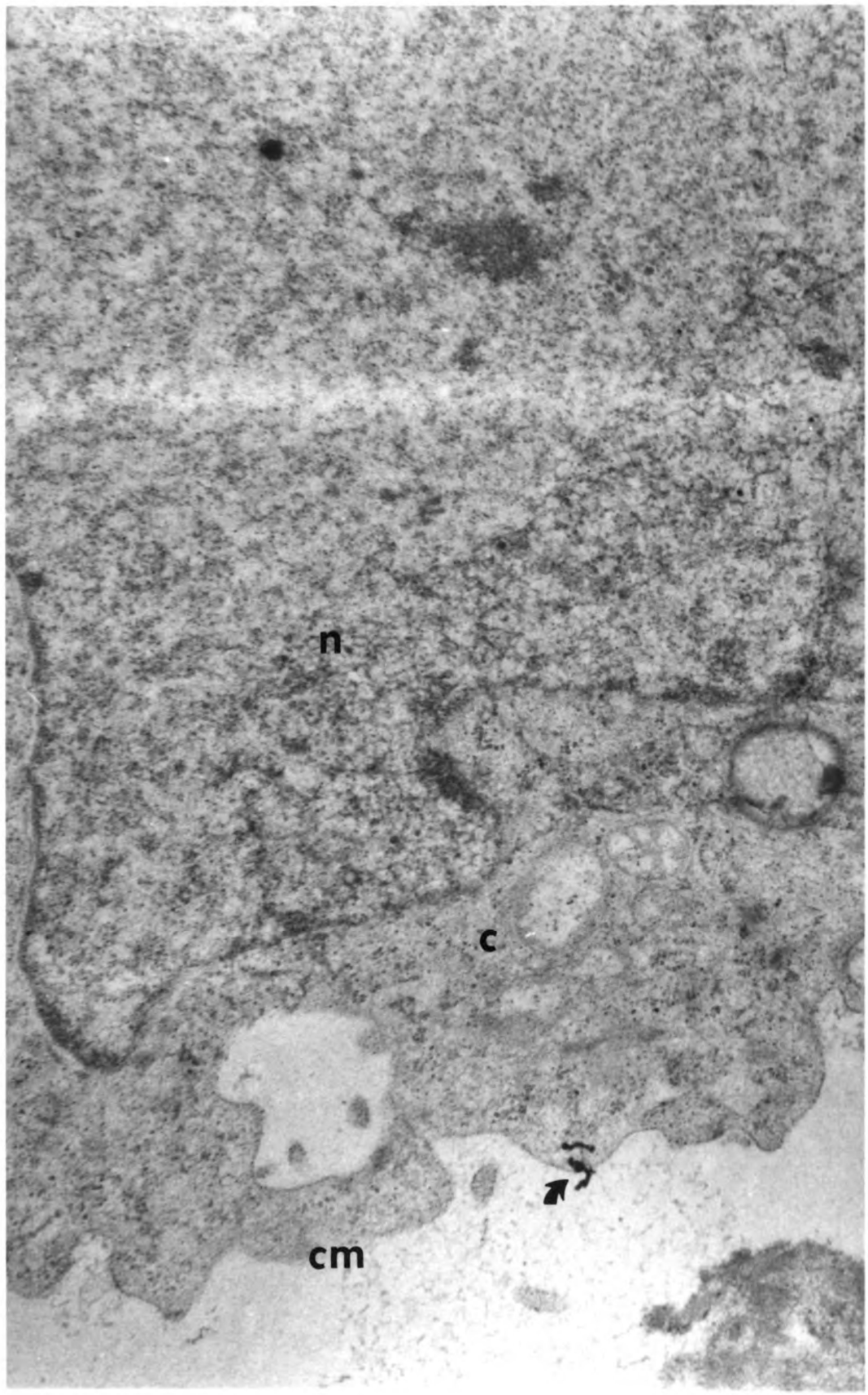

n
c
cm

Figure 7. A beta track (arrow) is located associated with the cell cytoplasm. The cell was exposed to 20% DMSO and H^3-labeled T_3 phage. (c) cytoplasm, (n) nucleus, Magnification 26,200.

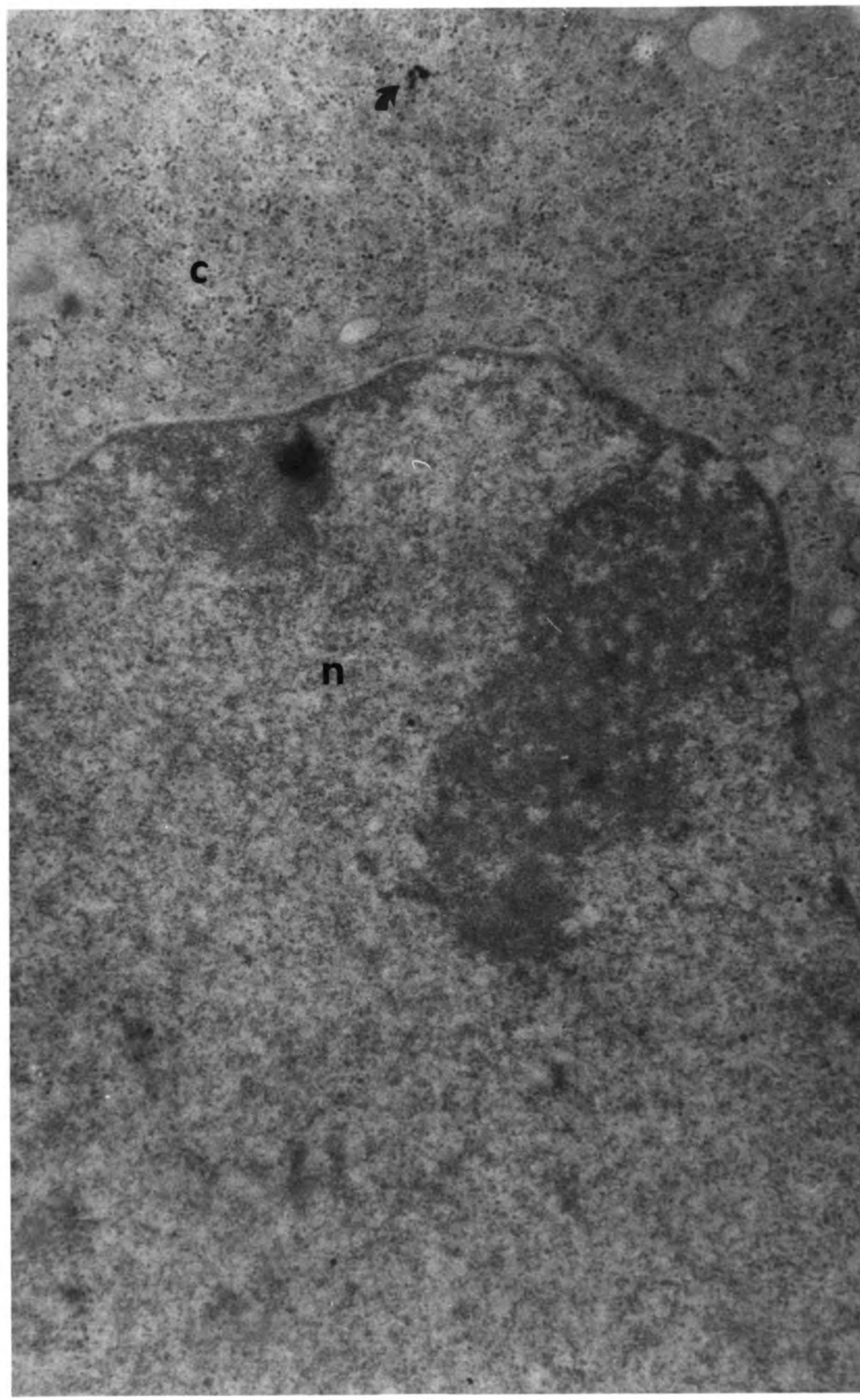

c
n

Figure 8. A beta track (arrow) located over the nucleus of a cell exposed to 10% DMSO and H^3 labeled T_3 phage. The location of this track indicated that the labeled particle was transported to the cells nucleus. (n) nucleus, (c) cytoplasm. Magnification 26,200.

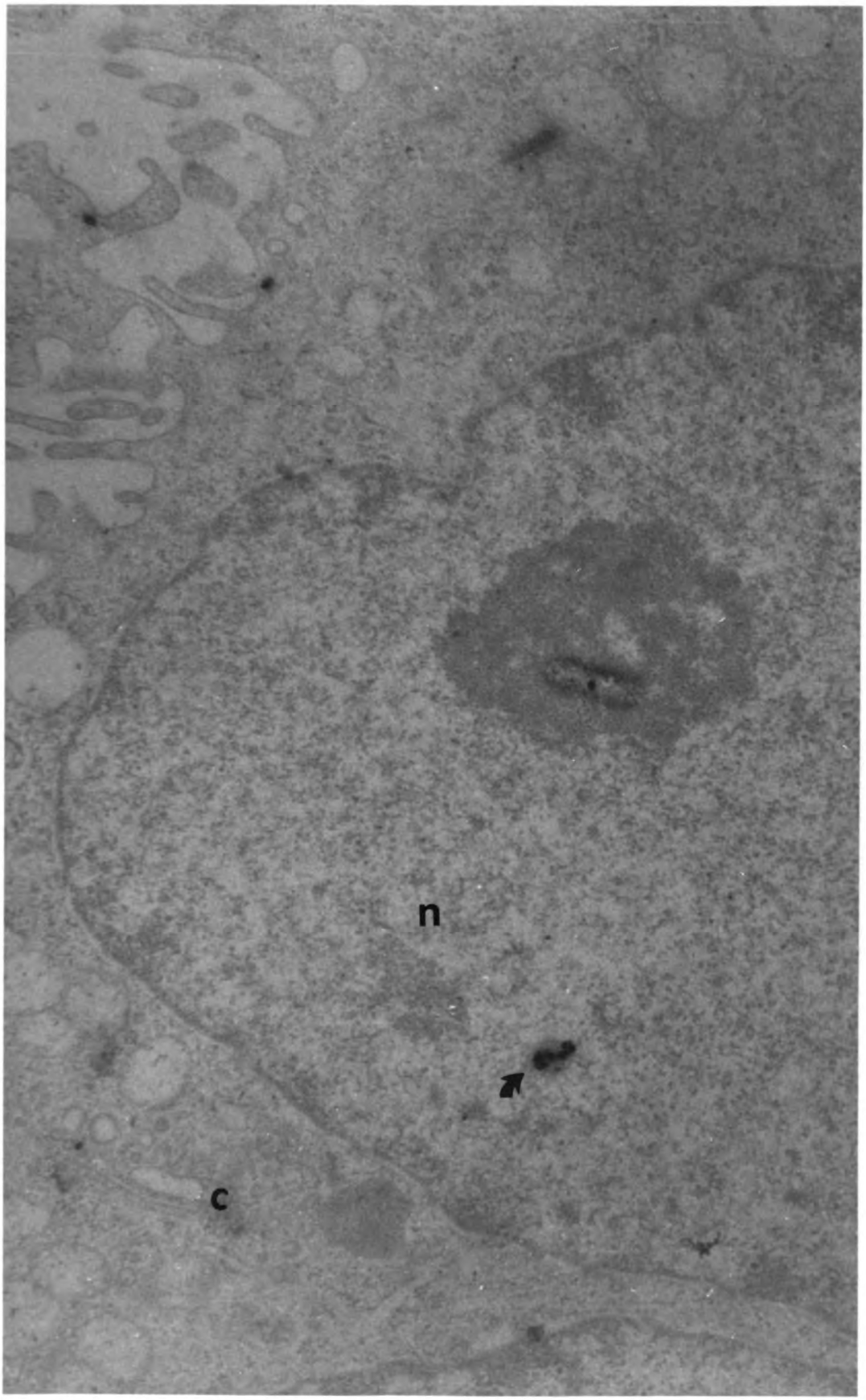

n
c

Figure 9. A higher magnification of the previous (Fig. 8) micrograph. The beta track can be clearly located well within the nucleus of the cell. (c) cytoplasm, (n) nucleus. Magnification 59,300.

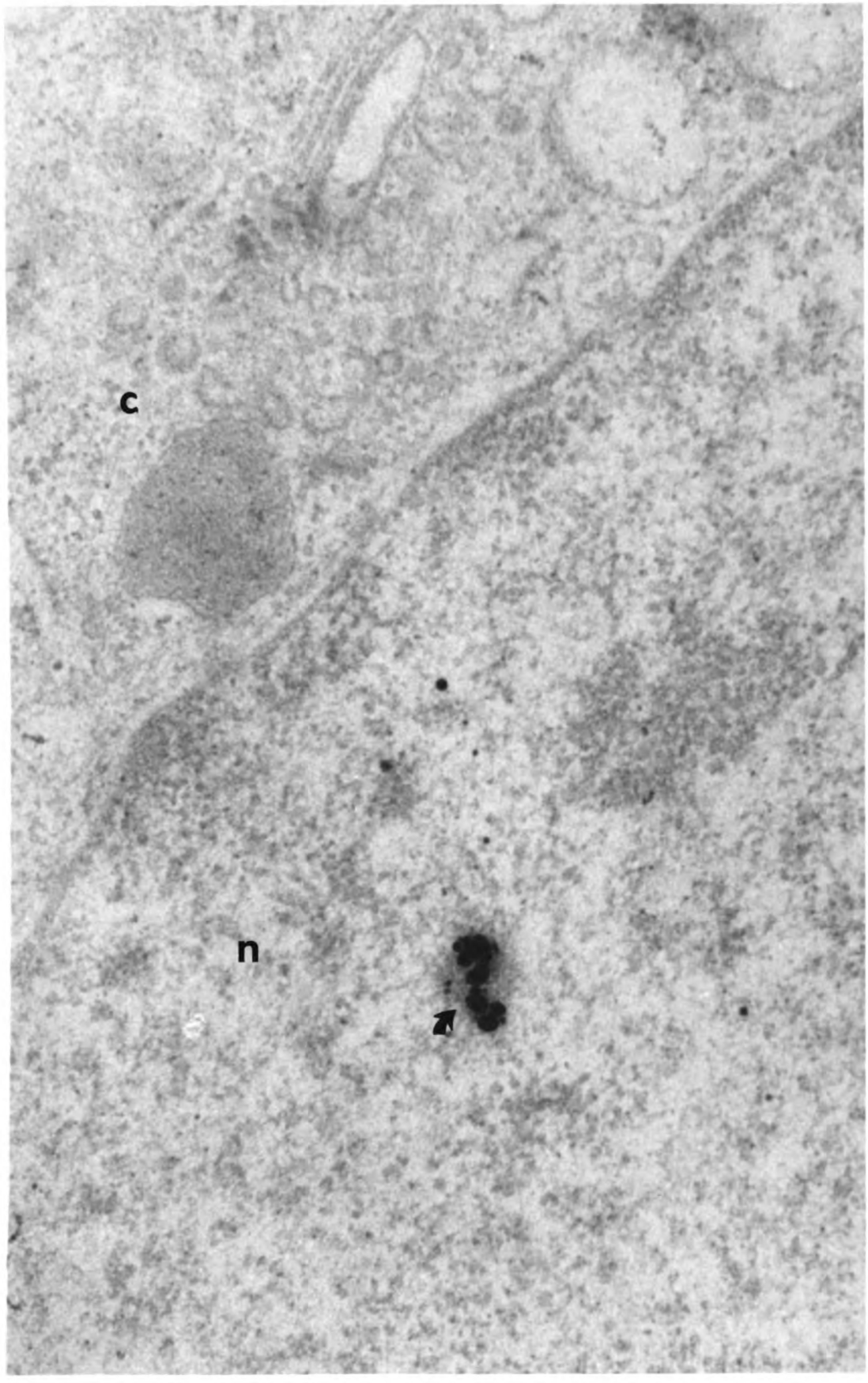

c
n

Figure 10. A beta track (arrow) is located over the nucleolus within the cell's nucleus. The track is a small one very close to the nuclear membrane. The cell was exposed to 10% DMSO and H^3 labeled T_3 phage. (nu) nucleolus, (c) cytoplasm, (nm) nuclear membrane. Magnification 59,300.

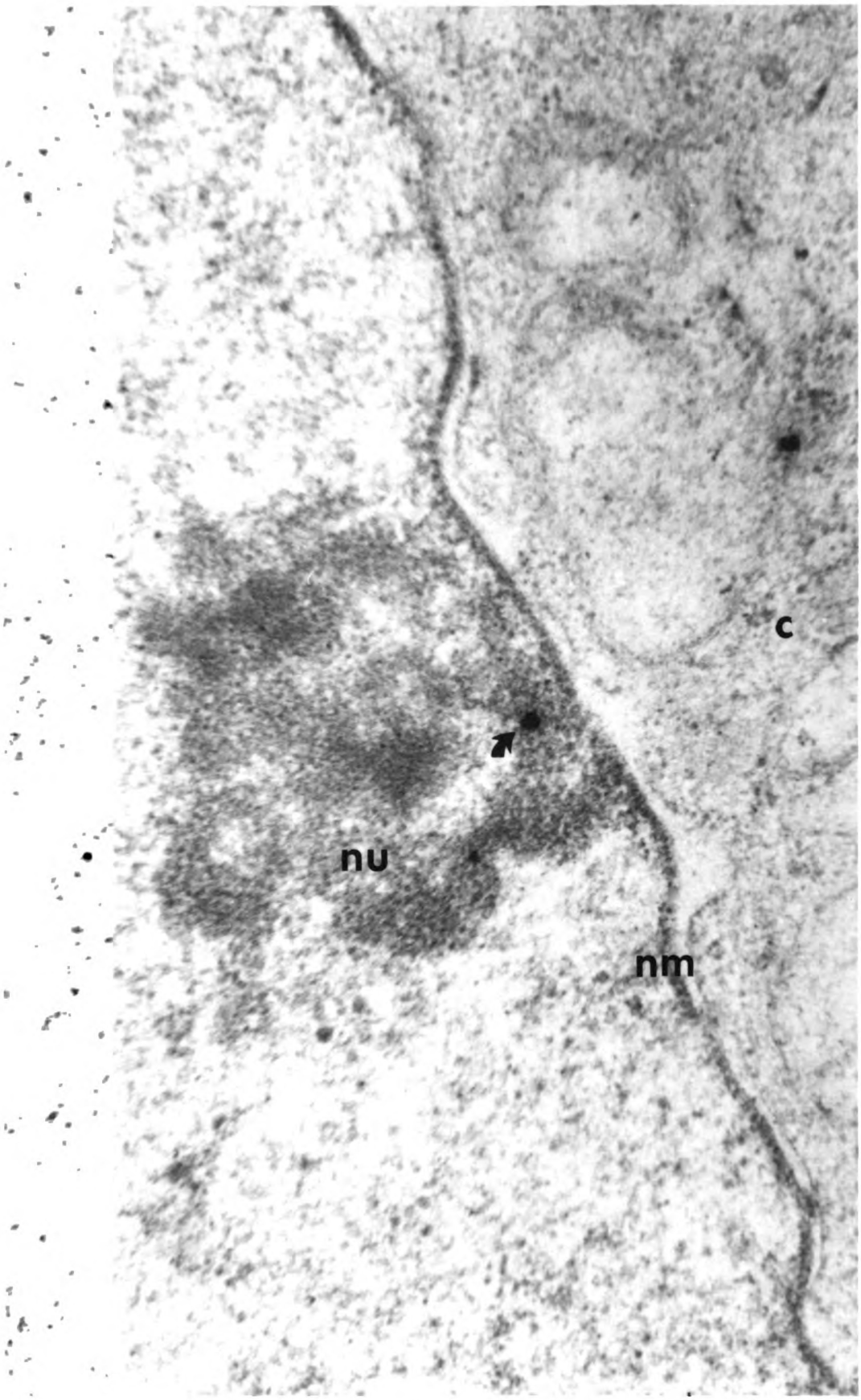

c
nu
nm

Figure 11. This micrograph demonstrates beta tracks made in the nuclear emulsion. The source of radiation was a 10 sec. exposure to sunlight. Magnification 59,300.

Figure 12. Beta tracks formed in nuclear emulsion after exposure to a flash of light from a 100 watt overhead lightbulb. The extensive tracks are readily photographed on the grids. Magnification 59,300.

DISCUSSION

The cytotoxicity studies showed that DMSO in low concentrations (less than 20%) had little effect on the immediate metabolism of appearance of the AU cells. Because we wanted to use DMSO in a concentration which would cause the least damage or alteration in the cells' appearance and metabolism and yet mediate the passage of virus into the cell, the 10% and 20% concentration of DMSO was used throughout the other tests. Concentration of 20% to 50% DMSO results in severe alterations in the relative rate of acid production compared to control cells. The treated cells appear rounded and heavily granular. Treatment of the cells with 75% or greater concentration of DMSO causes the cell to become fixed to the glass wall. These results showed that DMSO severely altered the metabolism of the epithelial cells when used in concentrations of more than 20%. The action appears to be readily reversible upon removal of the DMSO.

The plaque reduction tests showed that there was no significant reduction in T_3 phage pfu over the 60 minute

test period when exposed to 10% DMSO by itself. The percent reduction of phage was slightly less when placed in LAH plus 10% DMSO than when placed in the presence of AU cells alone. The difference in results obtained from these two control groups was about the same as the variations in results that were obtained from test to test within each group itself. This indicates that DMSO in 10% concentration has no effect on the T_3 phage and that the AU cells alone do not incorporate any of the phage to a detectable level.

Graph I showed that most of the reduction of the phage in the presence of AU cells and 10% DMSO had occurred within the first six minutes of exposure to DMSO. After this period of exposure to 10% DMSO the rate of phage reduction appeared to parallel the rate of reduction of phage which occurred under the control conditions. This indicates that during the first 6 minutes there was a significant and rapid removal of the infectious phage from the medium in the presence of 10% DMSO and AU cells. This removal of phage was not due to action of the cells alone as there was only a slight reduction in the phage titer in the controls when the phage was placed in the presence of

the cells without DMSO (Graph II, Line C). This indicated that 10% DMSO may have caused the AU cells to incorporate a significant amount of the phage from the suspension.

Electron microscope autoradiographs of H^3 labeled T_3 phage were prepared to explain the reduction in pfu from the suspending medium that occurred in the presence of DMSO. The phage was labeled with H^3 thymidine that had previously been incorporated into the nucleic acid of the host. Since the bacteria and phage were grown and labeled on solid medium agar plates, the quantity of extraneous label that was not incorporated in the phage nucleic acid but present in the crude lysate would be low. The labeled thymidine molecule is small and, therefore, rapidly dialyzable. Other sources of label in the crude lysate could be disrupted bacterial DNA or ruptured phage DNA components. Graph II indicated that a small molecule of labeled material was removed within 12 hours by dialysis. It was presumed, because of its rapid rate of removal, that this small molecule was H^3-thymidine that was not incorporated into the phage DNA. The best explanation for the presence of the low level of radioisotope remaining in the dialyzor after dialysis for 18 hours was the presence of a larger

component of labeled DNA. The unknown source of label remaining in suspension had to be accounted for as a possible source which could produce beta tracks on the autoradiographs. Since this unknown labeled material was dialyzable while the phage particle was not, a comparison of the dialyzable radioactive counts to total counts within the dialyzing suspension would give an indication of the relative amount of the two components. This ratio of counts was 1 count to 284 counts. This indicated that the unknown source of label composed 1/284 of the total label within the phage suspension. This ratio does not take into account the proportion of the unknown label that was removed by discarding the supernatant fluid after two cycles of concentration by ultracentrifugation. This procedure removed much of the unknown label but its source was not determined because the counts indicated that it was in an extremely small quantity. The low level of extraneous label had to be accounted for in statistically determining if the beta tracks found on the autoradiographs were caused by labeled phage or extraneous sources of label. The calculations for the statistical evaluation made of experimental group III were based on the ratio of the unknown source of label to

to total label present in the dialyzor after 18 hours of dialysis. This is an overestimate of the amount of unknown label present because it does not take into account the quantity of label removed in the supernatant suspension after ultracentrifugation. Since the evaluation was based on the dialysis results, causing an overestimate in the quantity of extraneous label, the probability of accepting the null hypothesis, that the tracks were due to extraneous label and not the labeled phage, was increased.

In Table 4, control group I did not show any background tracks due to outside sources of radiation. Control group II showed no beta tracks on the autoradiographs when the labeled phage was placed in the presence of the AU cells without any DMSO. The lack of any beta tracks in this control group indicates that the rate of phagocytosis, if any, was so extremely low that it wasn't detectable and therefore no beta tracks were found inside the cells and that in washing the cells all of the labeled phage was removed resulting in no beta tracks present outside the cells on the autoradiographs.

Since there was no background source of radiation causing beta tracks, the only source of radiation other

than the labeled phage which could cause beta tracks on the autoradiographs was the extraneous dialyzable label present in the phage suspension. The statistical evaluation conducted on experimental group III, taking into account an overestimated source of extraneous label, resulting in the rejection of the null hypothesis, gives strong evidence that most of the tracks were caused by labeled T_3 phage. In the statistical evaluation C represents the critical value upon which acceptance or rejection of the null hypothesis is based. C has a value of 3.24 meaning that 4 or less beta tracks found associated with 493 cells could all be explained as being caused by the extraneous source of label in the phage suspension. Since 35 beta tracks were found associated with 493 cells, the null hypothesis was rejected.

Figures 1 and 2 represent cells taken from control groups I and II respectively. Both show no beta tracks and are representative of the type of cells that were selected for examination on the autoradiographs. Only cells with large centered nuclei were selected insuring as best as possible that only cells sectioned through their center were counted. By following this procedure, it was felt

that the possibility of mislocating the labeled particle would be reduced to a minimum. Figures 3, 5, 6, and 7 show beta tracks located in the cytoplasm of the cells. Figure 4 is a higher magnification micrograph of Figure 3 demonstrating the beta track more clearly. In Figure 6, the beta tracks are located very close to the cell membrane and are difficult to determine if the labeled particle is inside the cell or associated with the membrane. Figures 7 and 9 demonstrate beta tracks located defintiely within the nucleus of the cells. Figure 8 is a higher magnification micrograph of Figure 7 demonstrating unequivocably the location of the labeled particle. Figures 10 and 11 represent controls for the nuclear emulsion. The controls demonstrate that the emulsion was sensitive to a source of radiation and that a relatively even monolayer of emulsion was layered over the sections.

The location of a statistically significant number of beta tracks associated with the cells gives clear evidence that DMSO was a penetrant carrier capable of mediating the T_3 bacteriophage into a foreign host. In examining the autoradiographs in the electron microscope, no beta tracks were detectable in the sections lacking DMSO

whereas many beta tracks were readily detectable in the sections exposed to 10% DMSO.

Normal virus-host interaction requires a period of time for chemical attachment before penetration of the membrane by the viral particle can occur. In this study, the speed (90 seconds) in which the labeled phage was found within the cells eliminates normal chemical transport of the phage. The speed of action indicates that the DMSO may have a direct interaction with the cell membrane. This interaction resulted in the rapid incorporation of the T_3 phage into the AU cells. This mediating capacity may make DMSO useful in incorporating other virus particles into host cells not normally attacked due to a lack of available virus-cell absorption sites.

The action of DMSO itself is, at present, unknown. It is the subject of intensive study for its solvent properties, cell membrane effects, cryoprotective characteristics, and its carrier penetrant capacity. It is a controversial compound and will require extensive research to determine its ultimate action and its possible side effects, especially its effect on the permeability of the cell membrane.

SUMMARY

Cytotoxic studies determined that concentrations of 20% or less of DMSO appeared to have little or no immediate effect on the AU epithelial cells. Any effect that it did have was readily reversible. Concentrations between 20% and 50% resulted in increasing severe alterations in the cells' appearance and metabolism. Concentrations greater than 50% caused severe damage and often fixation to the cells. Plaque reduction studies in the presence of 10% DMSO showed a reduction in the number of phage particles in the supernatant fluid when placed in the presence of AU cells. The reduction occurred rapidly, within six minutes of exposure, and thereafter no significant activity by the DMSO could be determined. Electron microscope autoradiographs of H^3-thymidine labeled T_3 bacteriophage demonstrated the location of the particles within the nucleus and cytoplasm of the cells.

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