CONFIDENTIAL

THE UNIVERSITY OF MICHIGAN

SCHOOL OF DENTISTRY

Progress Report

Period: July 1, 1962, to March 1, 1963

ACTION OF DETERGENTS AND FLAVOR AGENTS ON LIVING TISSUE

J. K. Avery
S. S. Han
P. J. Martin
T. C. Neumeier

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OFFICE OF RESEARCH ADMINISTRATION ANN ARBOR

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PART I

TISSUE CULTURE STUDIES

RESEARCH PLAN FOR TISSUE CULTURE STUDIES ON DETERGENTS AND FLAVOR AGENTS

I. VIABILITY STUDIES, GROSS

A. Gross and low-power microscopic observations of the effect of dentrifrice agents on monolayer cultures in flasks and culture tubes.

B. Concentrations of 2%, 1%, .6%, .4%, .2%, .1%, .06%, .04%, and .02% used.

II. VIABILITY STUDIES, MICRO

A. Immediate effect of low concentrations on cell viability.

B. Time effect of low concentrations on cell viability.

1. To gain information concerning differences in detergent or flavor agent action after prolonged contact with cells.

2. The viability of the cells will be based on the uptake or exclusion of vital dyes.

a. Dye uptake tests—methylene blue and tetrazolium stains.

b. Dye exclusion tests—trypan blue, and erythrosin B.

III. GROWTH AND PROLIFERATION STUDIES

A. Very low concentrations of flavor agents or detergents used in combination with a seven-day monolayer of epithelial and/or connective tissue cells.

B. Cells counted at various time intervals to study relative effect of very low concentrations of detergents or flavor agents on the growth and proliferation of the cells.

IV. TOXICITY STUDIES

A. To determine normal cellular characteristics and any changes in cell size, shape, opacity, alteration of nucleus or nucleolus by low concentrations of detergent and/or flavor agents.

B. To determine normal cellular structure and any changes in the cell in relation to "specific" or "nonspecific" effects of detergents and/or flavor agents.

1. Histochemical studies

2. Time-lapse cinematography

3. Electron microscopy

V. METABOLISM STUDIES

A. Glucose requirements of the cells and rate of lactic acid formation with the various detergents or flavor agents.

B. DNA and RNA levels determined; alteration in metabolism ascertained.

VI. ORAL BACTERIA STUDIES

A. The concentrations of the various detergents or flavor agents necessary to affect various oral bacteria.

B. The effect of the time that various detergents or flavor agents are in contact with various oral bacteria.

INTRODUCTION

This report is a summary of the progress on the research contract entitled "Action of Detergents and Flavor Agents on Living Tissue" during the period July 1, 1962, to March 1, 1963.

During this period study has been concentrated in the area of microscopic analysis of cell viability after contact with low concentrations of the antiseptics Thiomersal, Cetylpyridinium chloride, and Hyamine 1622, and the detergent Tween 60. Relative effects of these reagents, using a method for analysis of the lethal dose for 50% of the cells, comprises Part II of the research plan.

Growth and proliferation studies of HEp_2 cells affected by these same reagents were done during this period. Viability studies were included here also in order to gain more accurate data than could be obtained from cell counts alone because viable and nonviable, nonlysed cells cannot accurately be differentiated by cell counting methods. In the present studies, cell counts coupled with dye exclusion tests were run to determine more accurately cell growth and viability. This is Part III of the research plan.

Toxicity studies also were carried out during this period. Both histochemical studies and electron microscope studies are included in this report. No time-lapse studies are reported at this time as both the camera and the time-lapse equipment have been undergoing repairs and modification. Some data have been collected but not sufficient to report at this time. It will be included in a future report. The histochemical studies reported are in concern with the reagents Thiomersal, Cetylpyridinium chloride, Hyamine 1622, and Tween 60 at several concentrations. The May-Grünwald-Giemsa staining procedure was employed to differentiate DNA proteins and RNA proteins and the McManus Sudan Black B stain to demonstrate the presence and location of lipids. The electron microscope study includes ultrastructural changes of the HEp_2 cell after introduction of 0.0075% sodium lauryl sulfate into the cell suspension. This is Part IV of the research plan.

During this period Oral Bacteria Studies were continued in which the bactericidal and bacteriostatic effects of Thiomersal, Cetylpyridinium chloride, Hyamine 1622, and Tween 60 were evaluated using Lactobacillus acidophilus. These studies were a repeat of those described in the July, 1962, report and the findings are similar. This is Part VI of the research plan.

II. VIABILITY STUDIES, MICRO

A. PURPOSE

This study was initiated in order to determine the relative effects of certain reagents on HEp_2 cells (human epithelial cells) over a relatively short period of time and at low concentrations. The chemical substances used in the investigation were Thiomersal, Cetylpyridinium chloride, and Hyamine 1622 (antiseptics), and Tween 60 (a detergent).

B. PROCEDURE

During the middle of the log phase the HEp_2 cells were harvested from a suspension culture. Using the growth medium, $Eagles_{75}$-$tryptose\ phosphate_{15}$-$calf\ serum_{10}$, the cells were diluted to a concentration of 100,000 cells per milliliter. One-tenth ml of reagent solution and 0.01 ml of 0.4% erythrosin B staining agent were added to 0.9 ml of the cell suspension five minutes before commencing the count of viable cells. The concentrations of reagent used were 0.0001%, 0.00025%, 0.0005%, 0.00075%, 0.001%, 0.0025%, 0.005%, 0.0075%, 0.01%, and 0.025%. Controls were run for each concentration of each reagent.

Over a period of 25 minutes the viable and nonviable cells were counted at 5-minute intervals with a hemacytometer. Using the Reed-Muench method* the lethal dose for 50% of the cells (LD_{50}) was determined.

C. RESULTS

At the various low concentrations of the reagents used, Cetylpyridinium chloride appeared to be the most toxic. After 5 minutes the LD_{50} occurred at a concentration of 0.0074%. After 25 minutes the LD_{50} occurred at a concentration of 0.0043%.

Hyamine 1622 was the least toxic, producing no LD_{50} at 5 minutes. At 10 minutes a concentration of 0.0224% produced an LD_{50} and at 25 minutes the concentration required for an LD_{50} was 0.0216%.

Tween 60 showed an LD_{50} at a concentration of 0.0197% after 5 minutes and 0.0144% after 25 minutes, a very slight decrease. Time apparently is not a factor in the toxicity of Tween 60 or Hyamine 1622.

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*Reed, L. J., and Muench, H., Am. J. Hyg. 27:493, 1938.

Thiomersal, however, showed a relatively great increase in toxicity as the time interval increased. At 5 minutes the LD_{50} occurred at a concentration of 0.0234% but after 25 minutes only 0.0008% was required to produce an LD_{50}.

Controls which were run concomitantly showed a viability of from 83% to 95%.

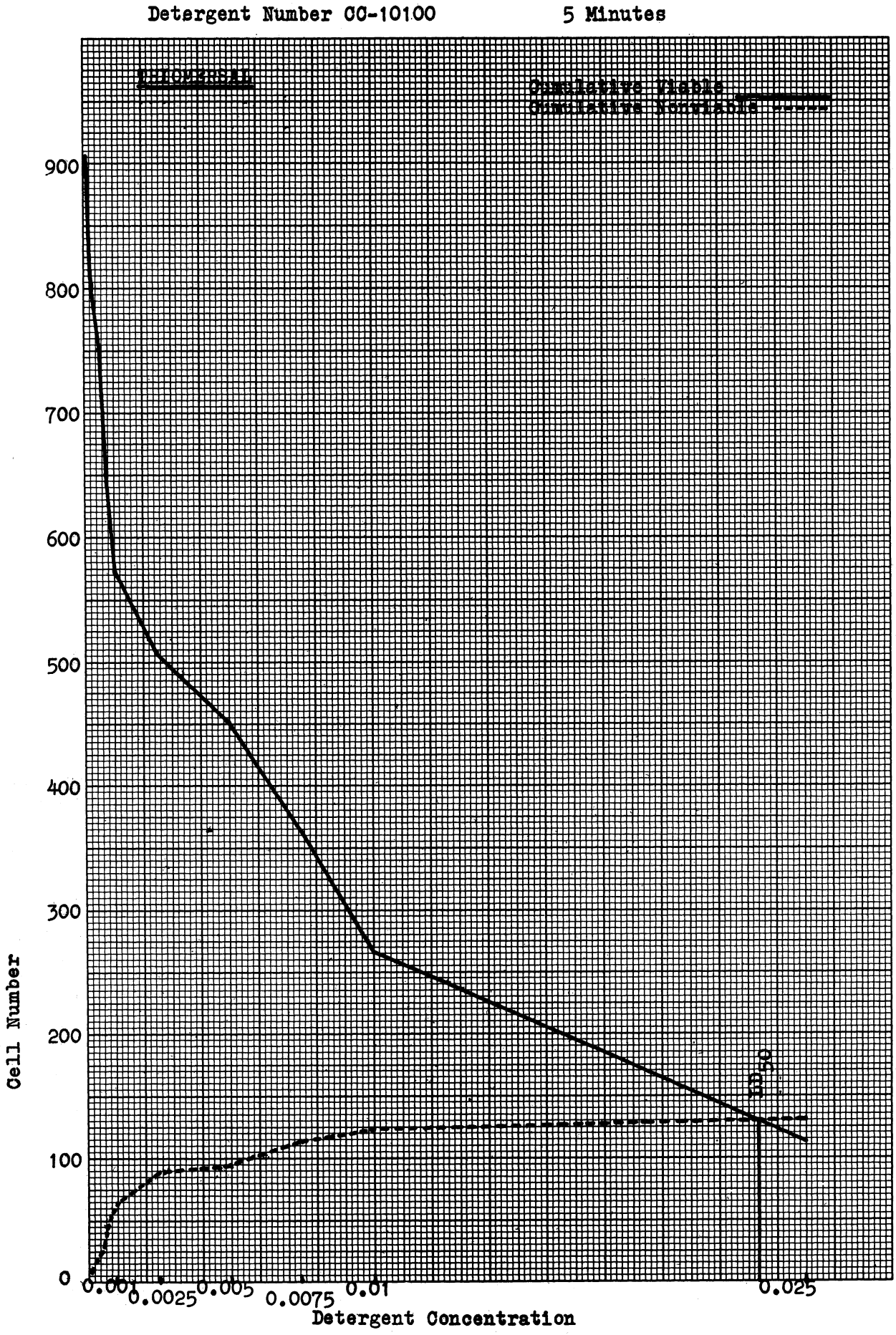
See tables and graphs following.

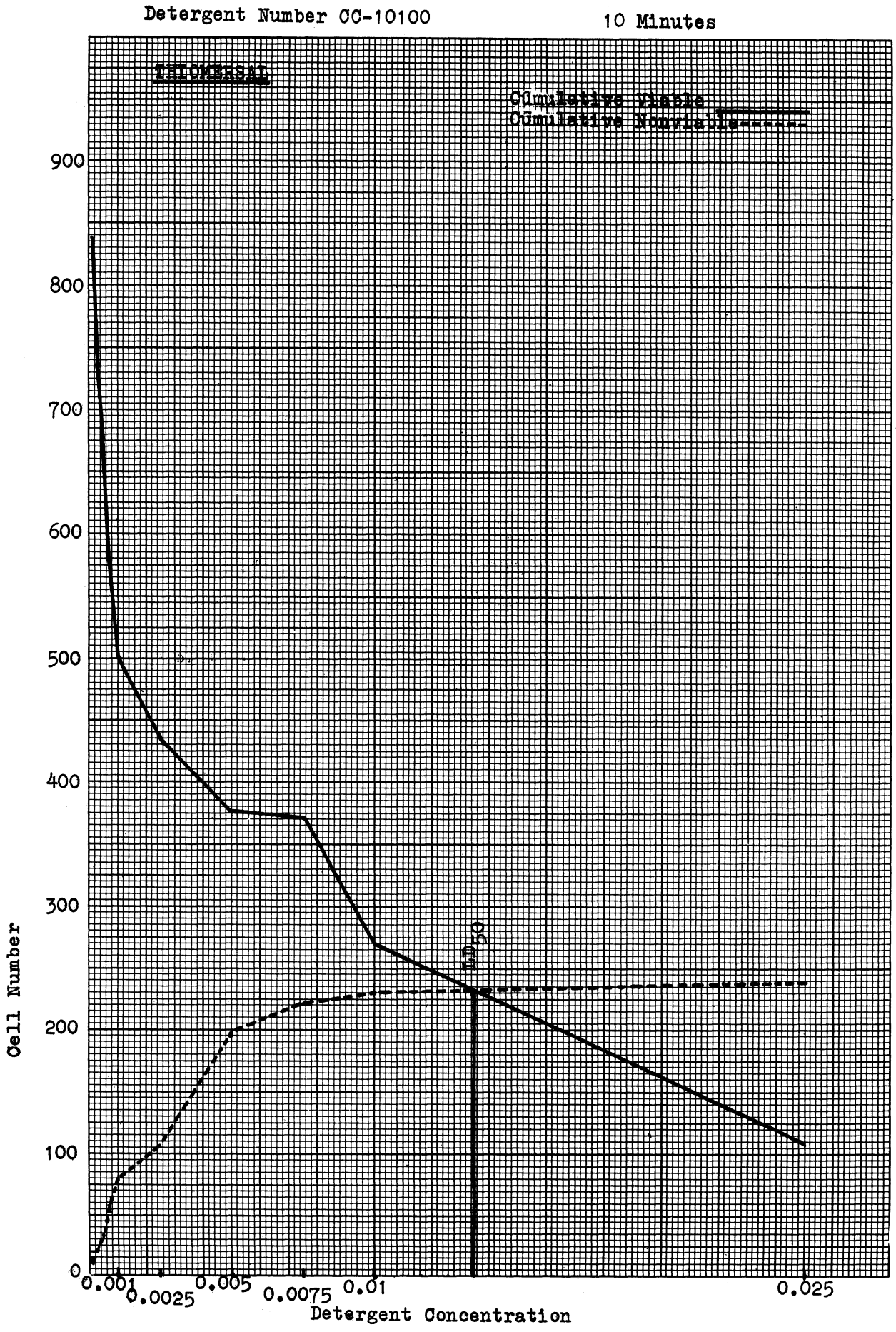
TABLE I

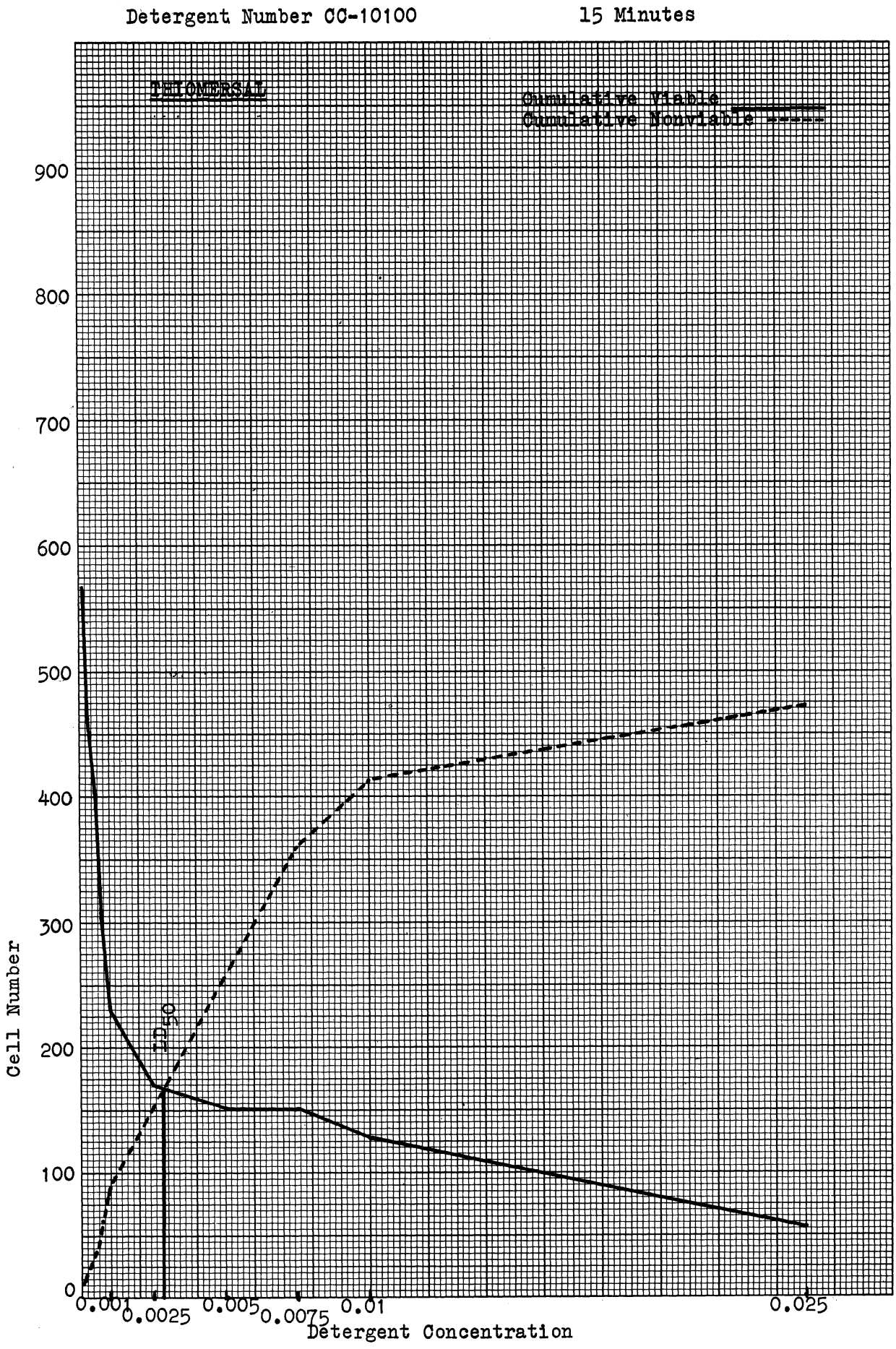
LD_{50}* OF REAGENTS AT VARIOUS TIME INTERVALS

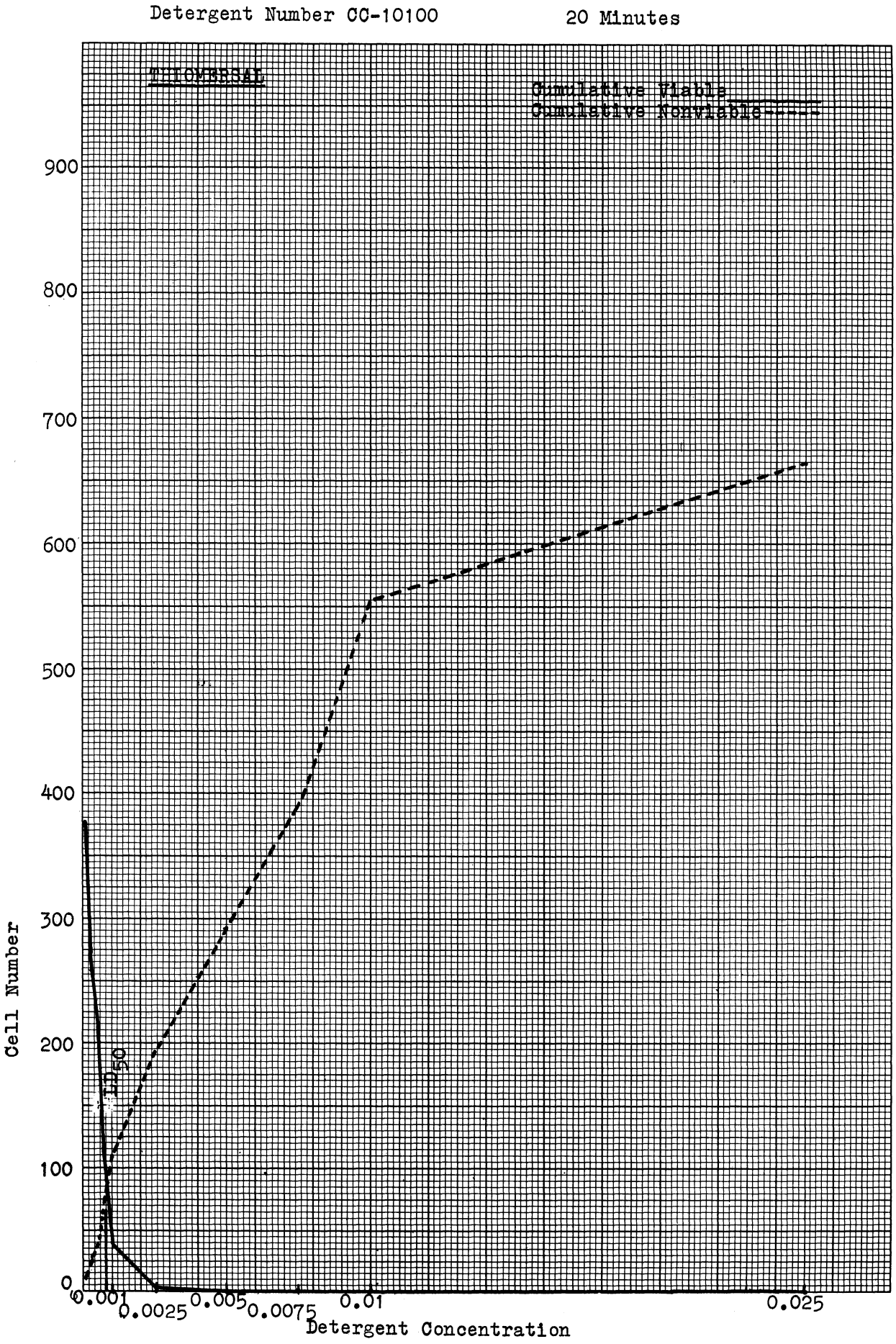
Reagent	Minutes				
	5	10	15	20	25
Thiomersal	0.0234	0.0135	0.0028	0.0008	0.0008
Cetylpyridinium Chloride	0.0074	0.0072	0.0072	0.0066	0.0043
Hyamine 1622	--	0.0224	0.0208	0.0206	0.0216
Tween 60	0.0197	0.0166	0.0163	0.0164	0.0144

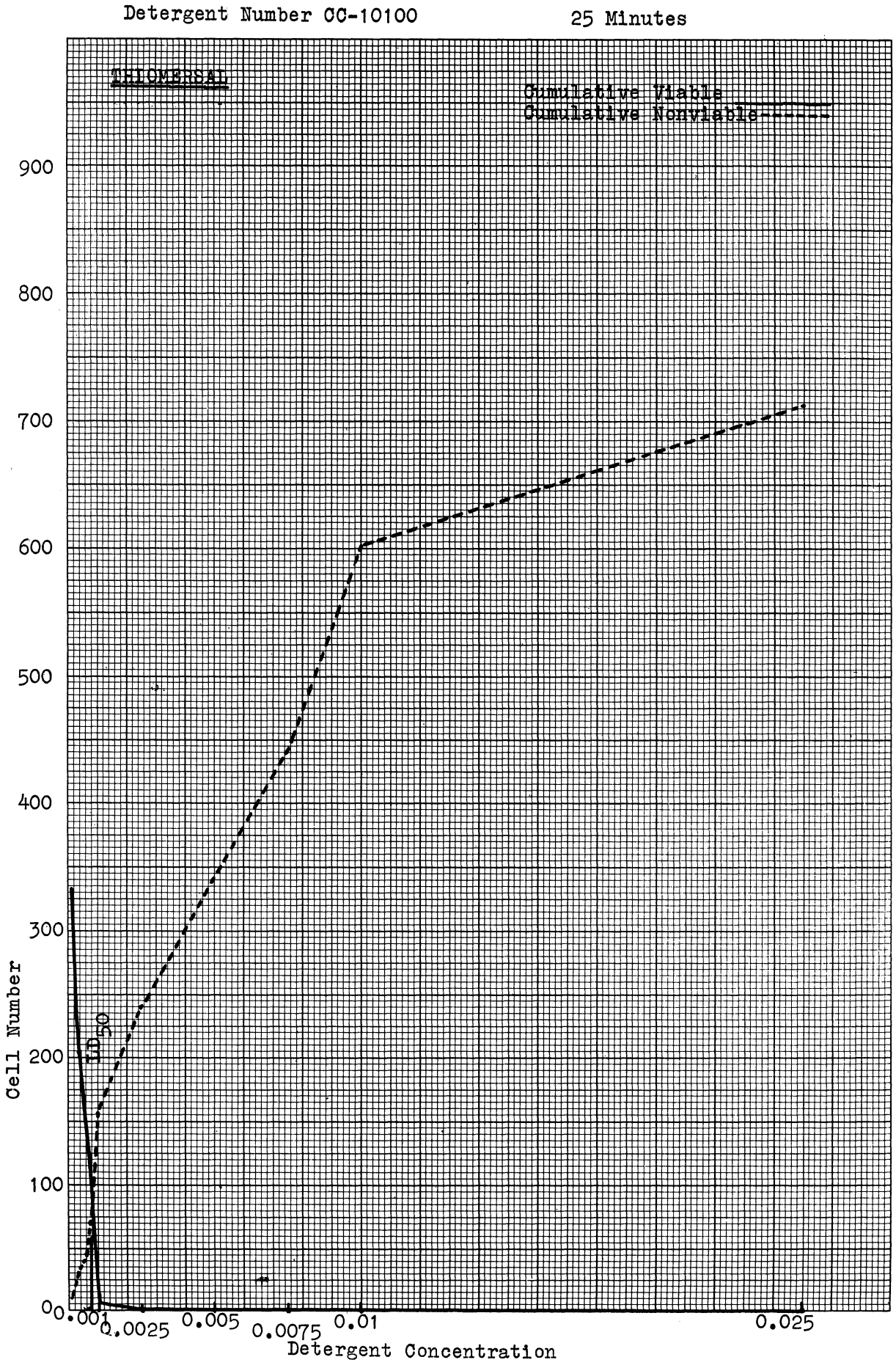
*Expressed in percent.


Detergent Number CC-10100
5 Minutes
TRICHOSEAL
Cumulative Viable
Cumulative Nonviable
900
800
700
600
500
400
300
200
100
0
Cell Number
0.001
0.0025
0.005
0.0075
0.01
0.025
Detergent Concentration
LD50

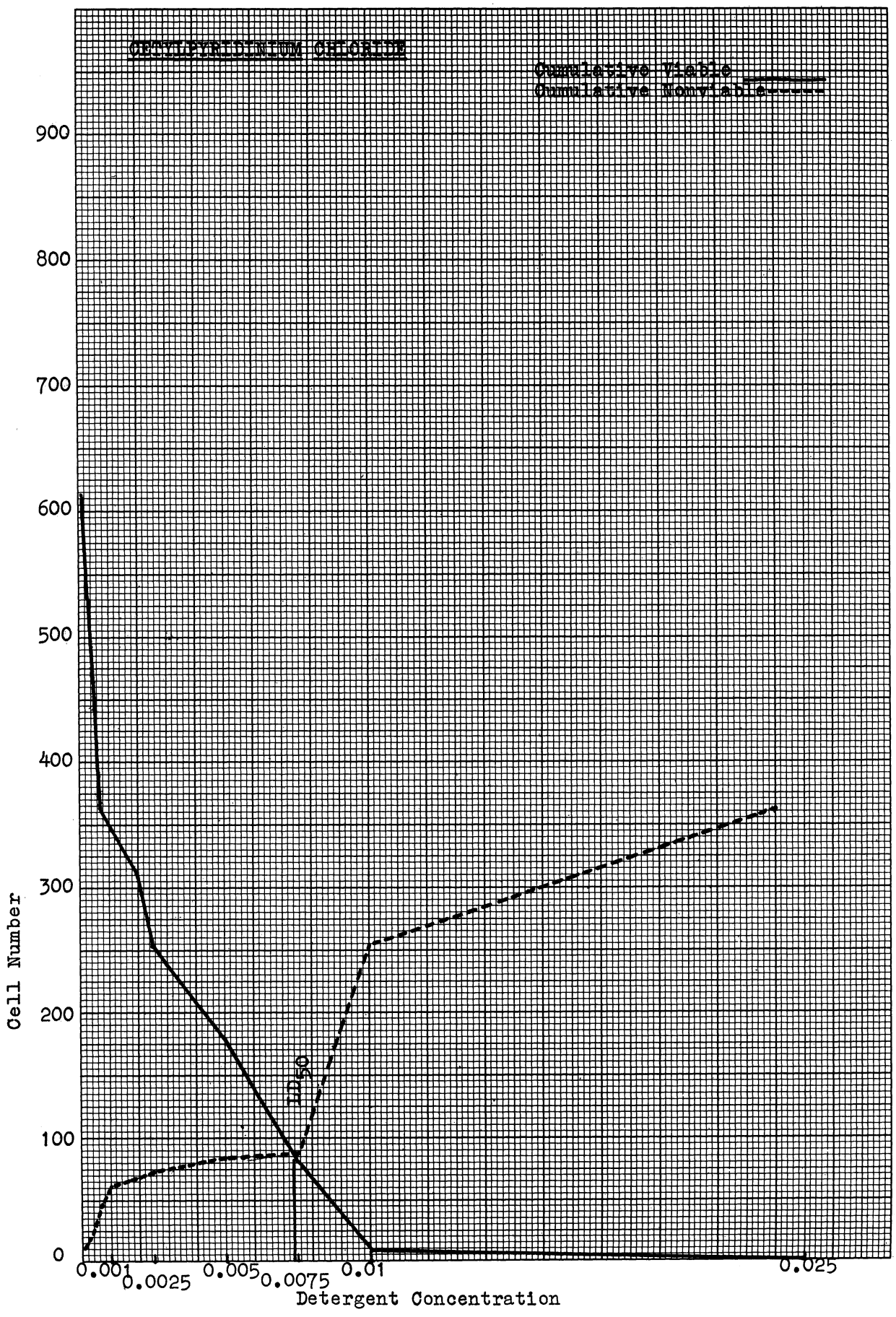

Detergent Number CC-10100
10 Minutes
TRITON X-100
Cumulative Viable
Cumulative Nonviable
900
800
700
600
500
400
300
200
100
0
Cell Number
LD50
0.001
0.0025
0.005
0.0075
0.01
0.025
Detergent Concentration

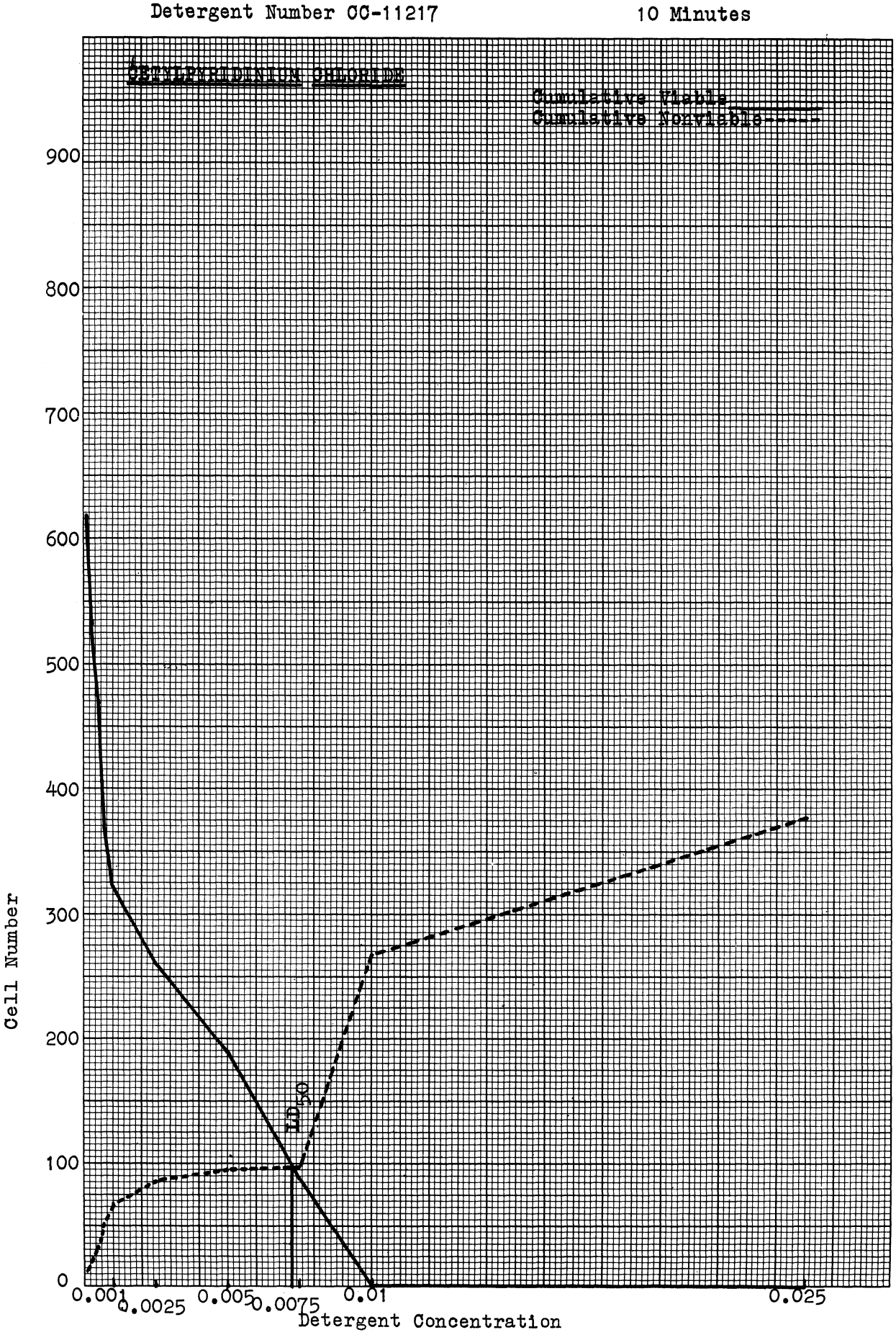

Detergent Number CC-10100
15 Minutes
THIOMERSAL
Cumulative Viable
Cumulative Nonviable
900
800
700
600
500
400
300
200
100
0
Cell Number
LC50
0.001
0.0025
0.005
0.0075
0.01
0.025
Detergent Concentration

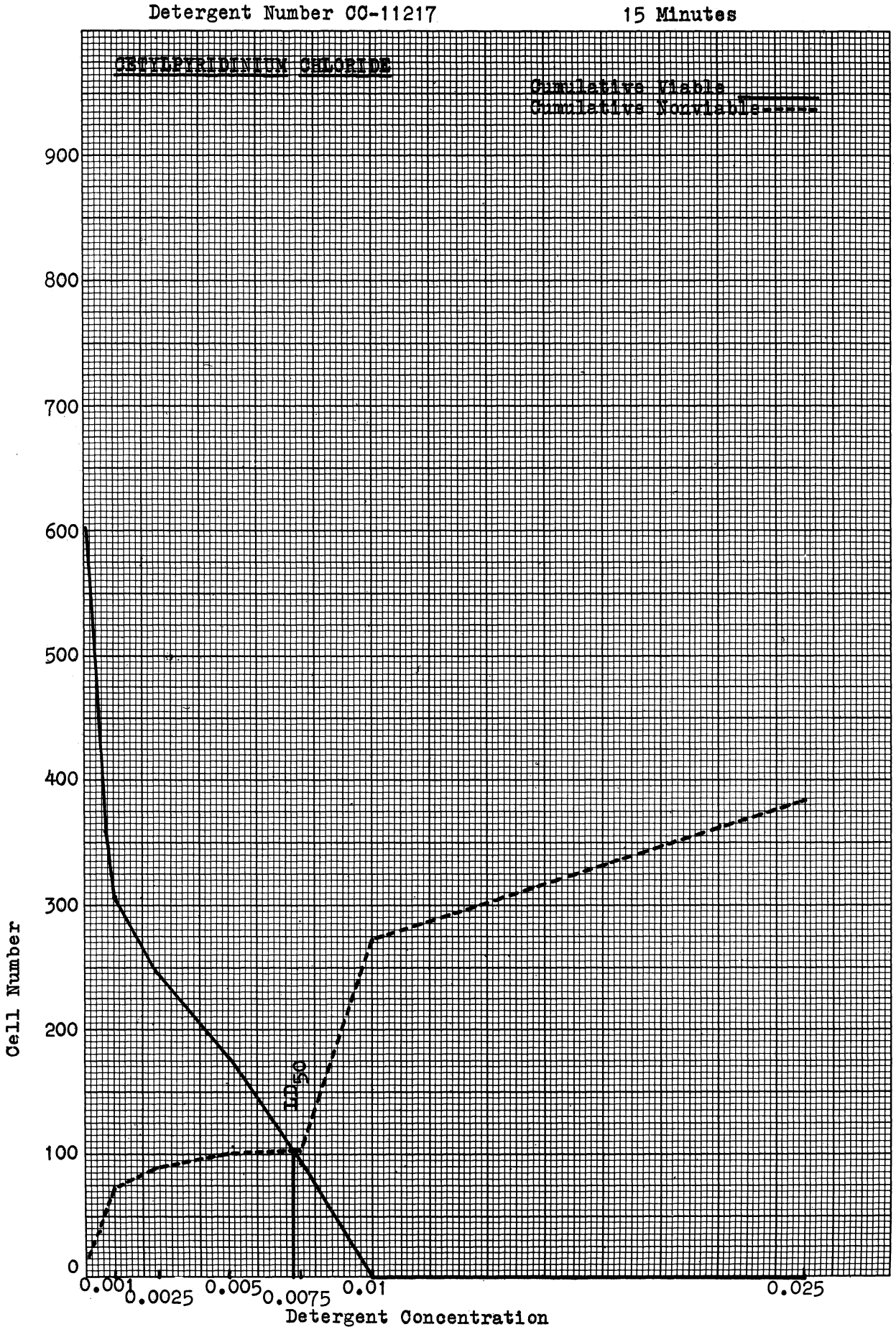

Detergent Number CC-10100
20 Minutes
TRICYRESAL
Cumulative Viable
Cumulative Nonviable
900
800
700
600
500
400
300
200
100
0
Cell Number
LD50
0.001
0.0025
0.005
0.0075
0.01
0.025
Detergent Concentration

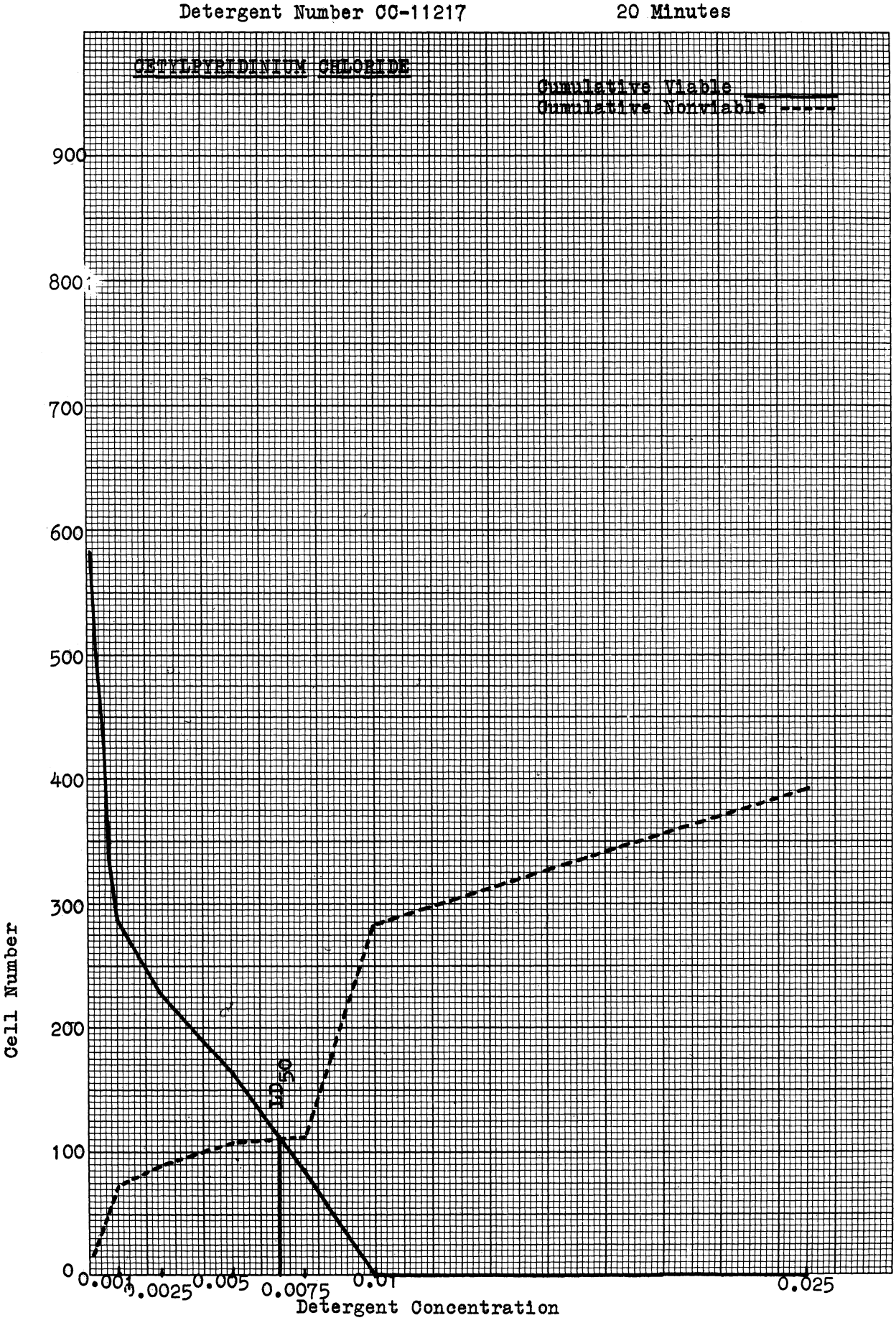

Detergent Number CC-10100
25 Minutes
THIOMERSAL
Cumulative Viable
Cumulative Nonviable
900
800
700
600
500
400
300
200
100
0
Cell Number
LD50
0.001
0.0025
0.005
0.0075
0.01
0.025
Detergent Concentration

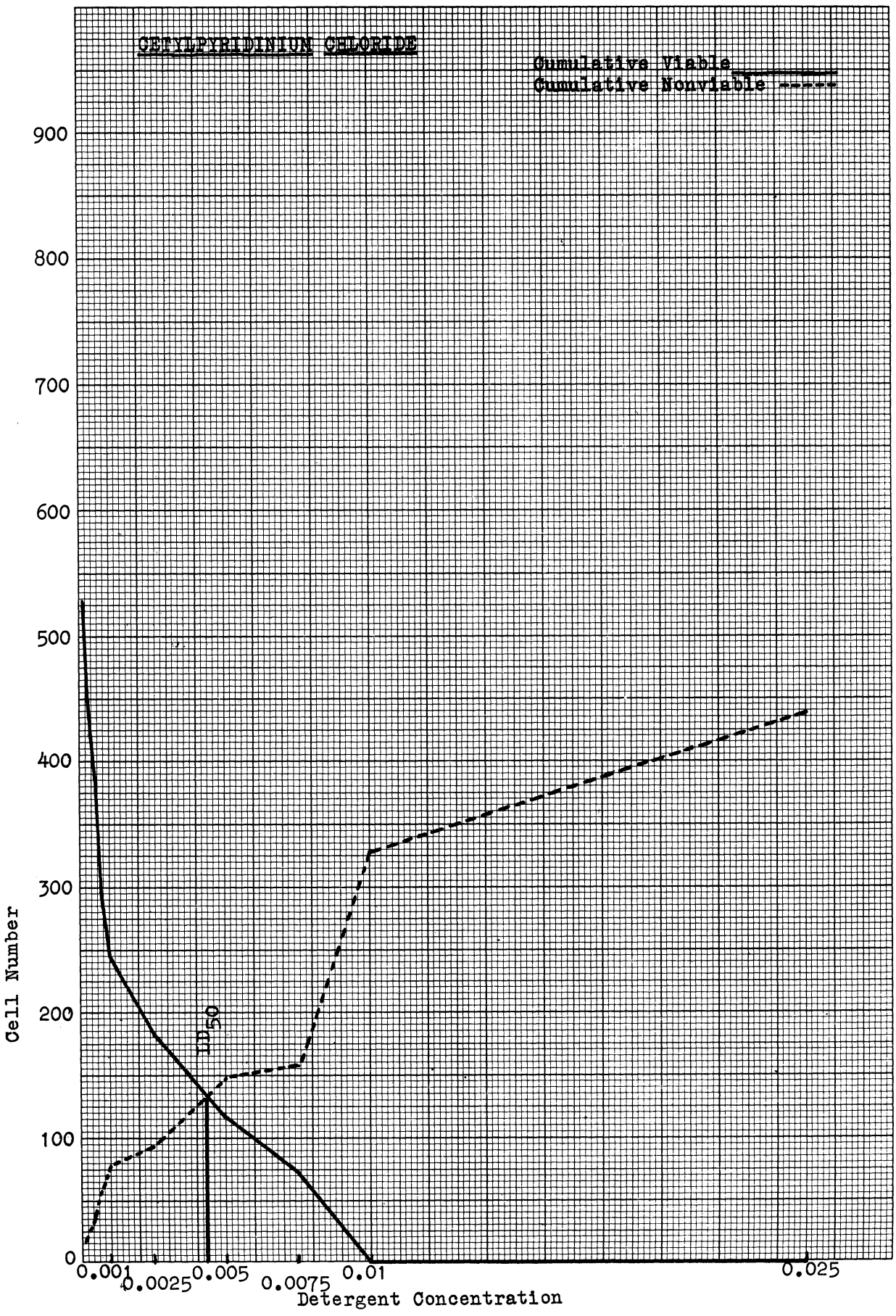
Detergent Number CC-11217 5 Minutes

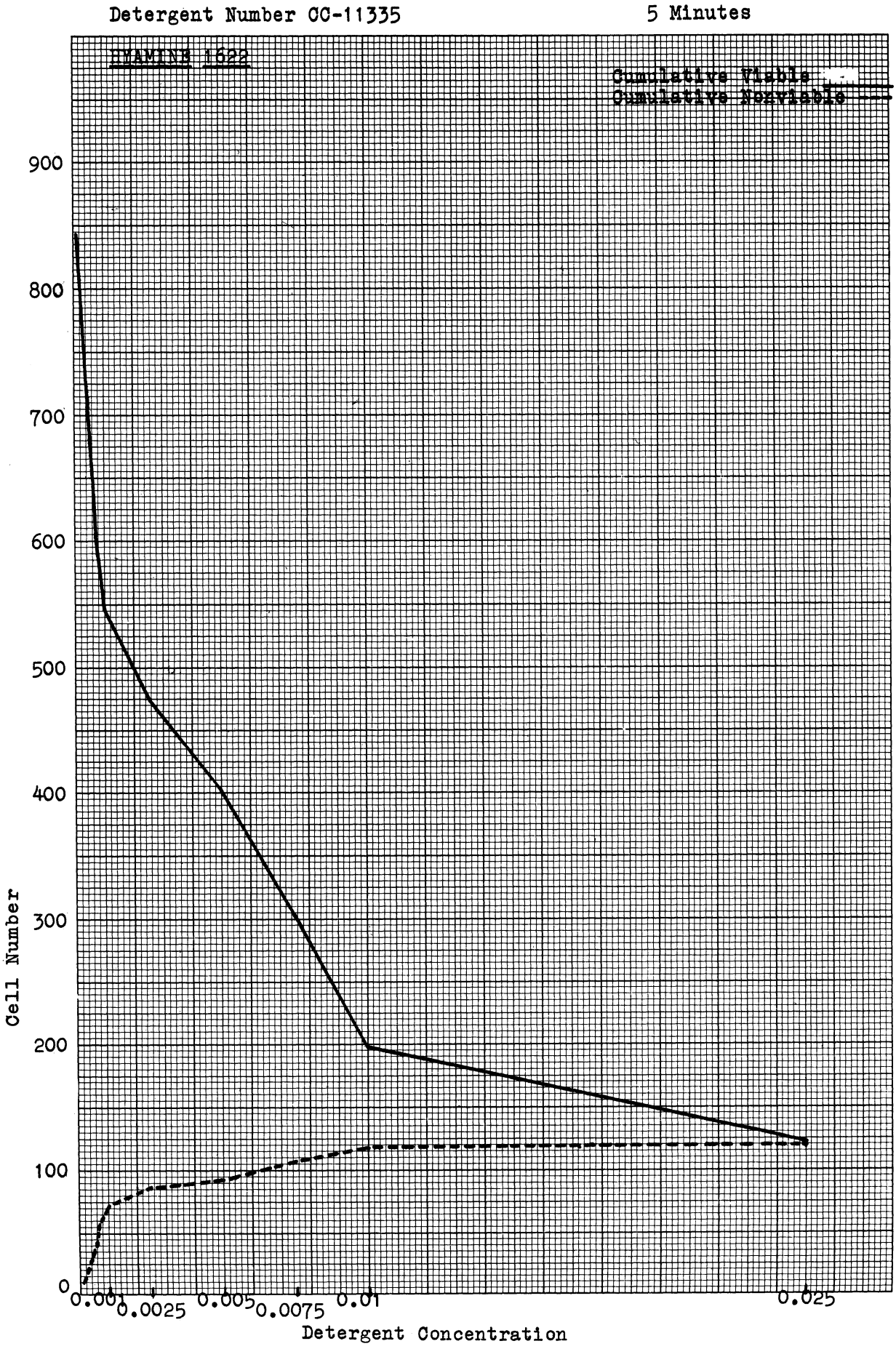



Detergent Number CC-11217
10 Minutes
CETYLPYRIDINIUM CHLORIDE
Cumulative Viable
Cumulative Nonviable
900
800
700
600
500
400
300
200
100
0
Cell Number
ED50
0.001
0.0025
0.005
0.0075
0.01
0.025
Detergent Concentration

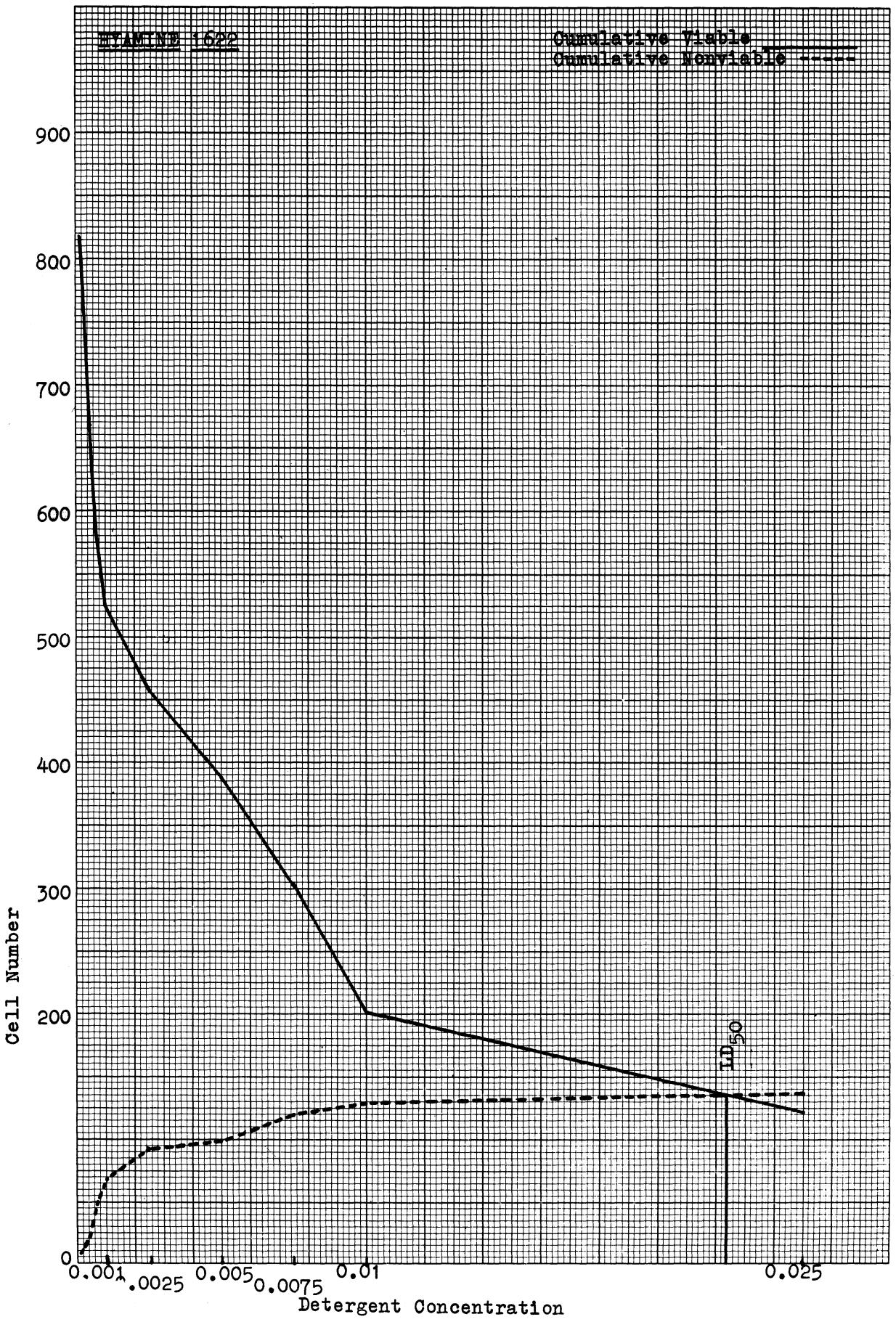

Detergent Number CC-11217
15 Minutes
CETYLPYRIDINIUM CHLORIDE
Cumulative Viable
Cumulative Nonviable
900
800
700
600
500
400
300
200
100
0
Cell Number
ED50
0.001
0.0025
0.005
0.0075
0.01
0.025
Detergent Concentration

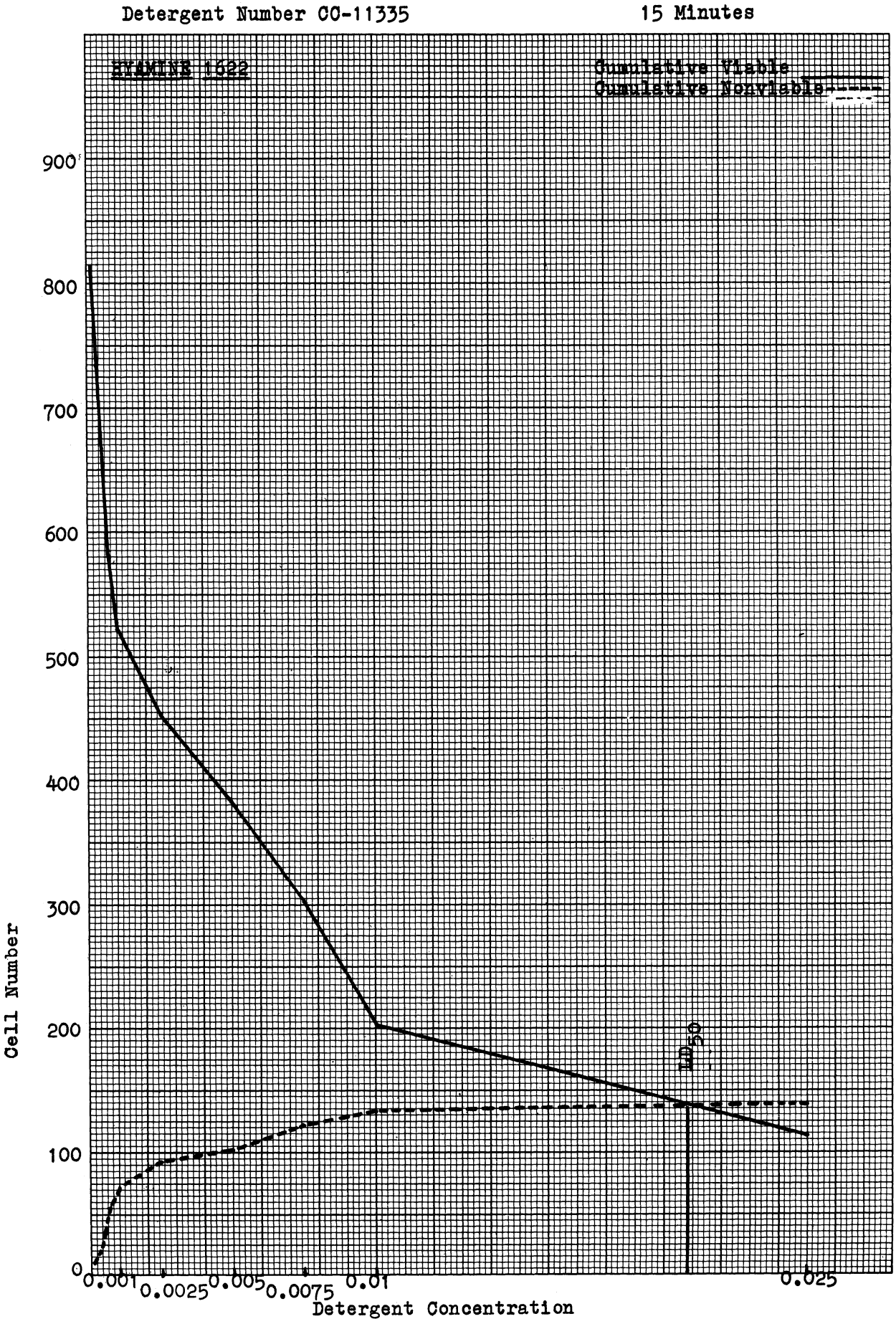

Detergent Number CC-11217
20 Minutes
CETYLPYRIDINIUM CHLORIDE
Cumulative Viable
Cumulative Nonviable
900
800
700
600
500
400
300
200
100
0
Cell Number
LD50
0.001
0.0025
0.005
0.0075
0.01
0.025
Detergent Concentration

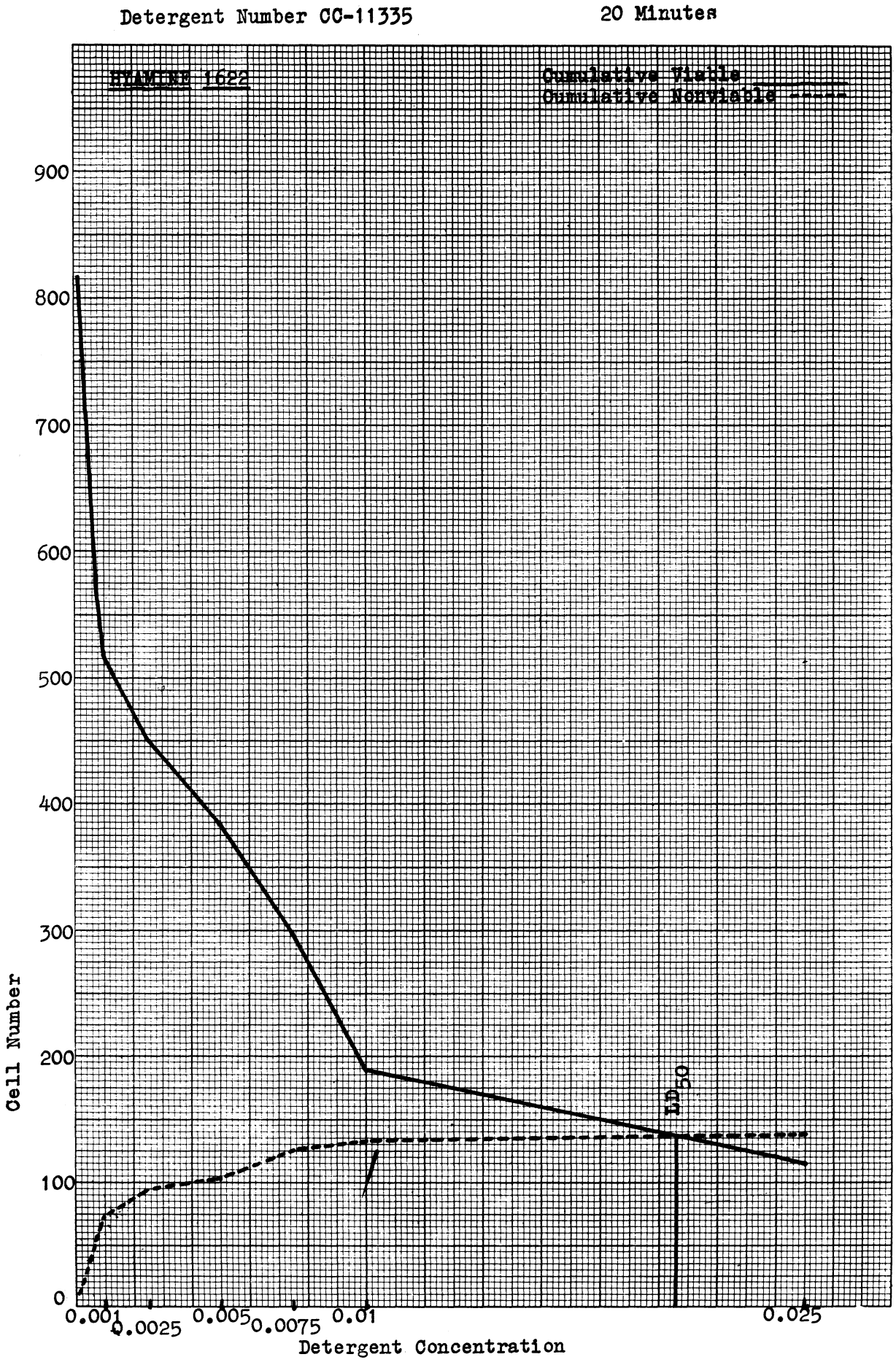

Detergent Number CC-11217
25 Minutes
CETYLPYRIDINIUM CHLORIDE
Cumulative Viable
Cumulative Nonviable
900
800
700
600
500
400
300
200
100
0
Cell Number
LD50
0.001
0.0025
0.005
0.0075
0.01
0.025
Detergent Concentration

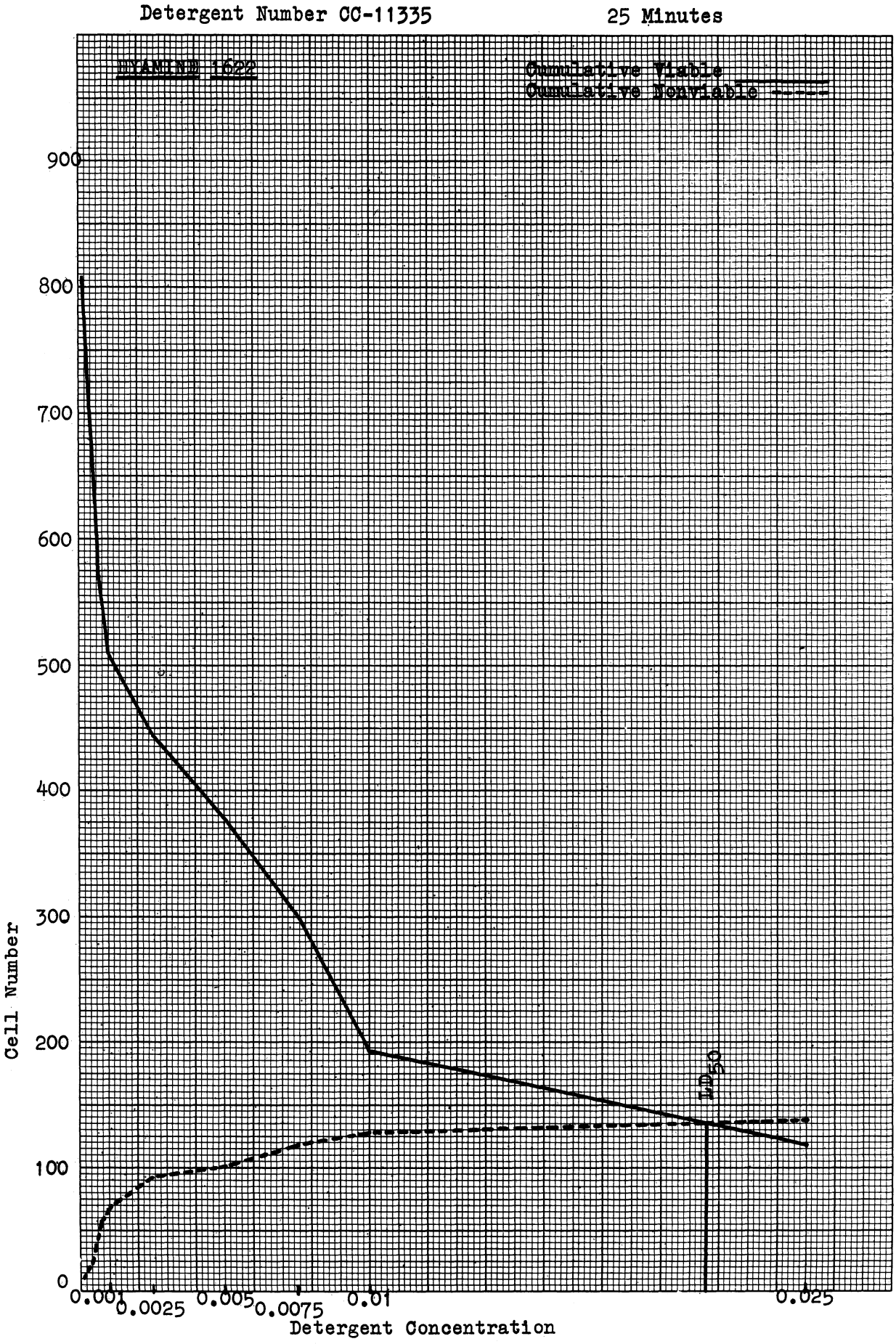

Detergent Number CC-11335
5 Minutes
EXAMINE 1622
Cumulative Viable
Cumulative Nonviable
900
800
700
600
500
400
300
200
100
0
Cell Number
0.001
0.0025
0.005
0.0075
0.01
0.025
Detergent Concentration

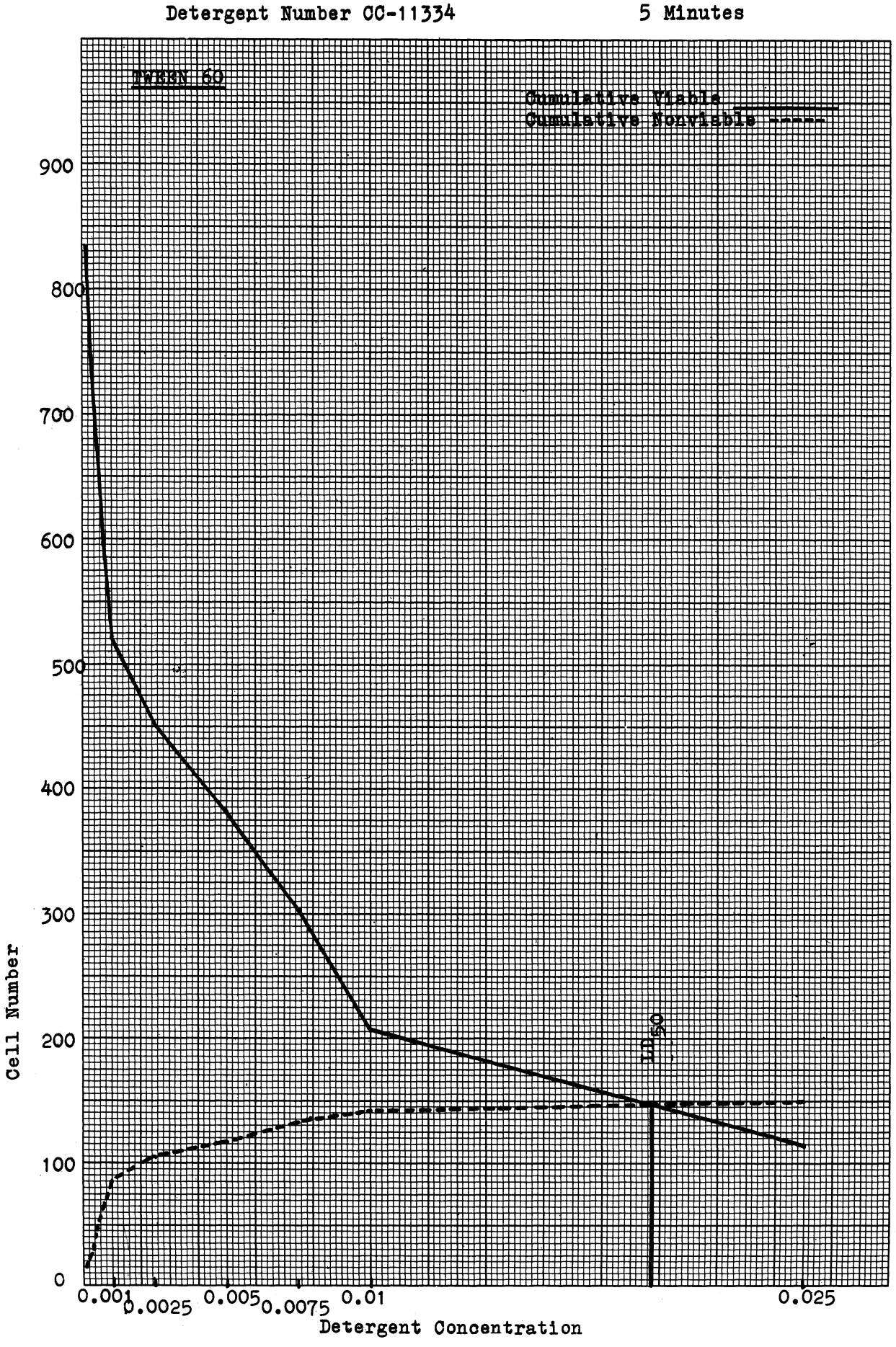
Detergent Number CC-11335 10 Minutes

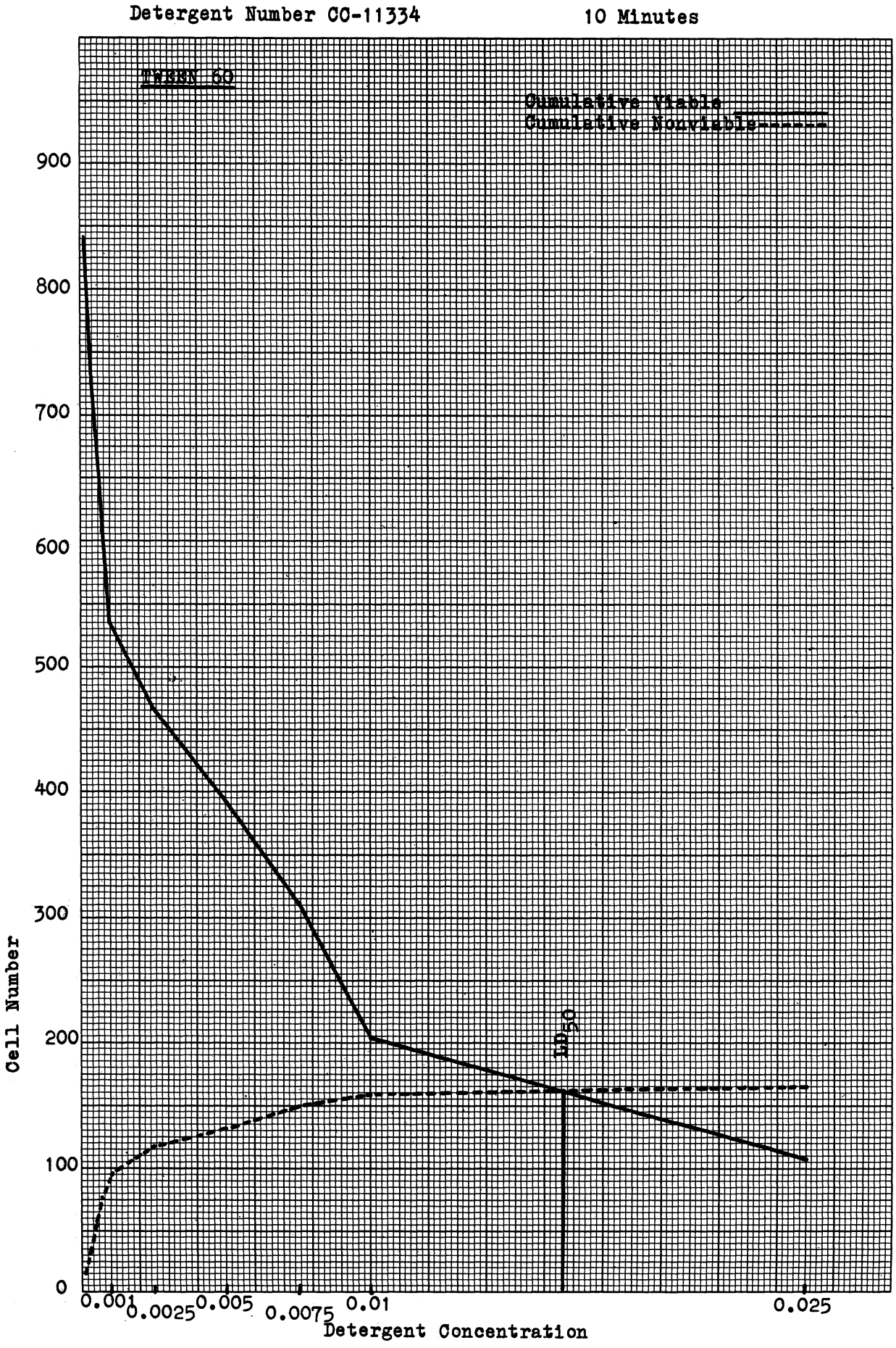


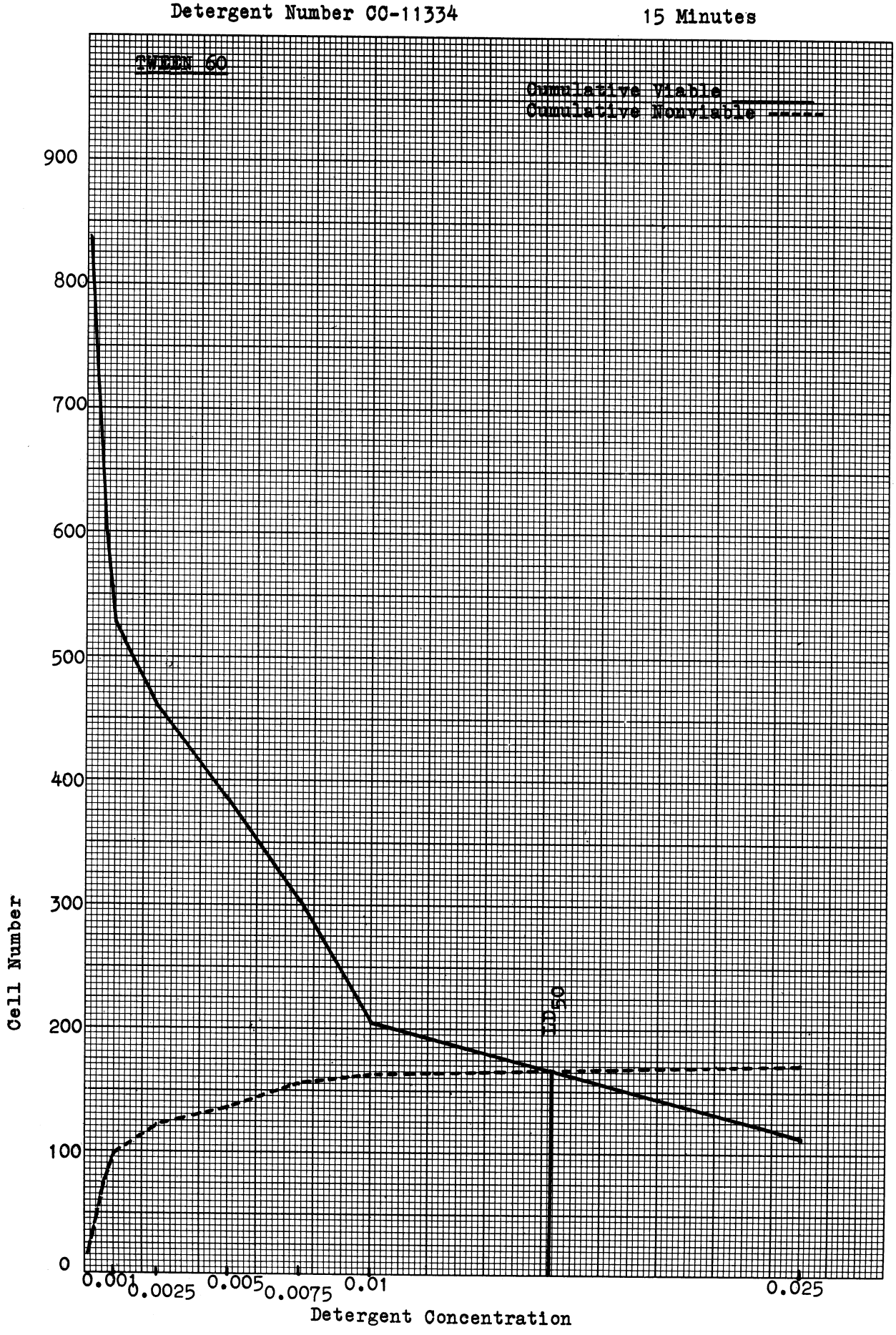

Detergent Number CO-11335
15 Minutes
HYAMINE 1622
Cumulative Viable
Cumulative Nonviable
900
800
700
600
500
400
300
200
100
0
Cell Number
0.001
0.0025
0.005
0.0075
0.01
0.025
Detergent Concentration
LD50

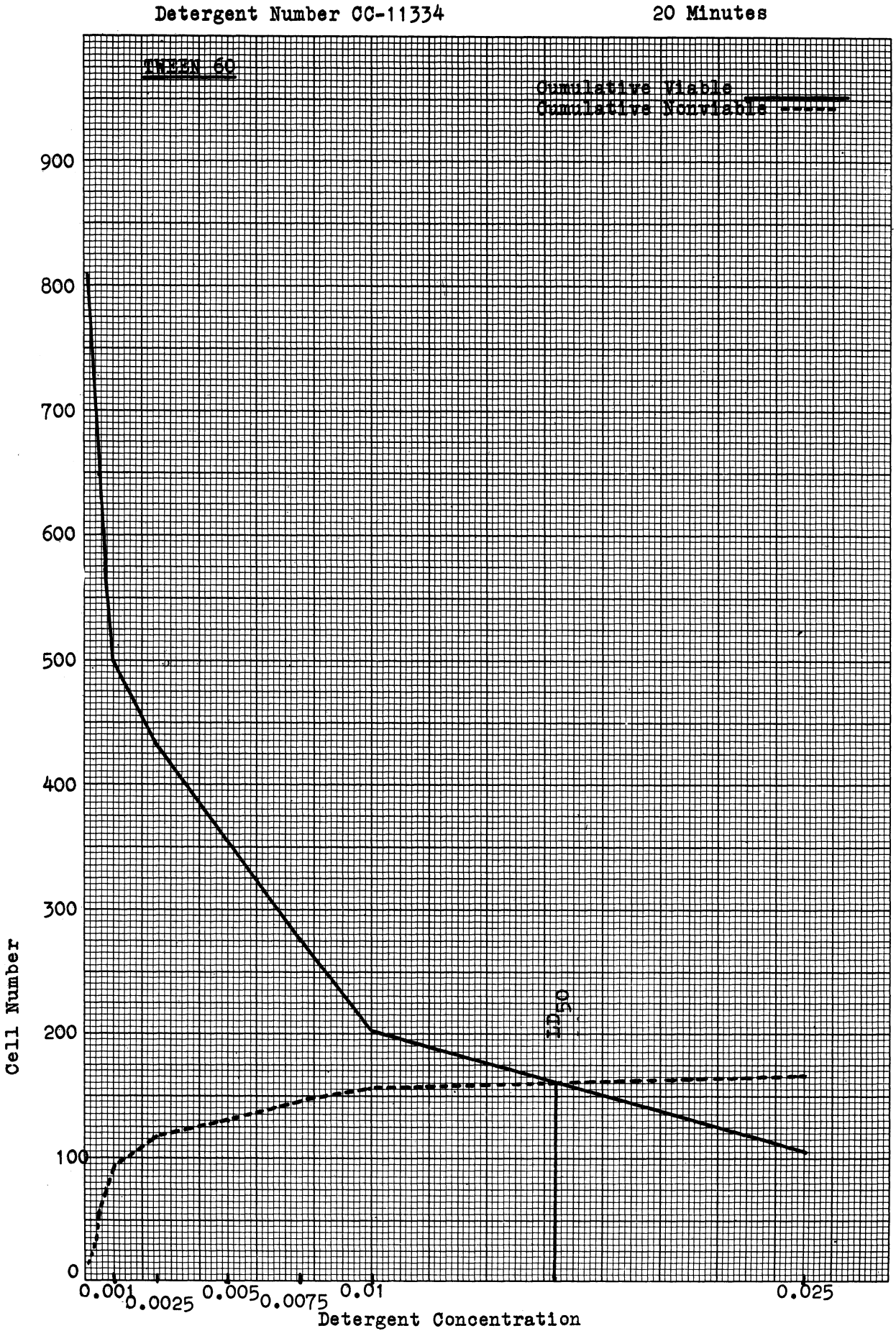

Detergent Number CC-11335
20 Minutes
STAMINE 1622
Cumulative Viable
Cumulative Nonviable
900
800
700
600
500
400
300
200
100
0
Cell Number
0.001
0.0025
0.005
0.0075
0.01
0.025
Detergent Concentration
LD50


Detergent Number CC-11335
25 Minutes
EXAMINE 1622
Cumulative Viable
Cumulative Nonviable
900
800
700
600
500
400
300
200
100
0
Cell Number
LD50
0.001
0.0025
0.005
0.0075
0.01
0.025
Detergent Concentration


Detergent Number CC-11334
5 Minutes
TWEEN 60
Cumulative Viable
Cumulative Nonviable
900
800
700
600
500
400
300
200
100
0
Cell Number
0.001
0.0025
0.005
0.0075
0.01
0.025
Detergent Concentration


Detergent Number CO-11334
10 Minutes
TWEEN 60
Cumulative Viable
Cumulative Nonviable
900
800
700
600
500
400
300
200
100
0
Cell Number
LD50
0.001
0.0025
0.005
0.0075
0.01
0.025
Detergent Concentration


Detergent Number CO-11334
15 Minutes
TWEEN 60
Cumulative Viable
Cumulative Nonviable
900
800
700
600
500
400
300
200
100
0
Cell Number
0.001
0.0025
0.005
0.0075
0.01
0.025
Detergent Concentration
LD50


Detergent Number CC-11334
20 Minutes
TWEEN 60
Cumulative Viable
Cumulative Nonviable
900
800
700
600
500
400
300
200
100
0
Cell Number
EC50
0.001
0.0025
0.005
0.0075
0.01
0.025
Detergent Concentration

Detergent Number CC-11334 25 Minutes

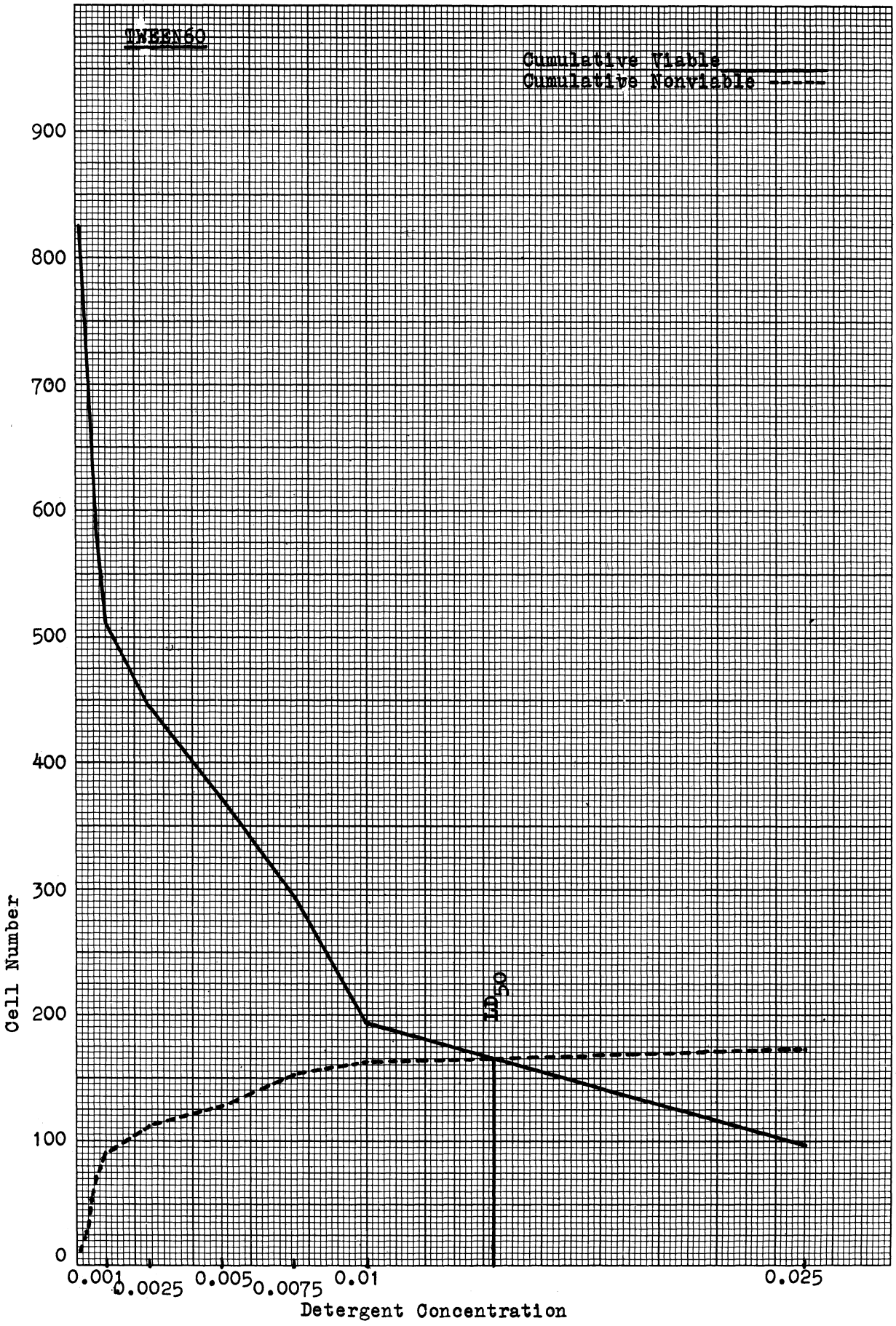


TABLE II

VIABILITY OF CELLS AT VARIOUS TIME INTERVALS

Thiomersal

Minutes	Conc., %	Nonviable	Viable	Total	% Viable	Cumulative	
						Nonviable	Viable
5	.0001	8	102	110	93	8	906
	.00025	10	53	63	84	18	804
	.0005	7	100	107	93	25	751
	.00075	21	77	98	79	46	651
	.001	19	66	85	78	65	574
	.0025	24	57	81	70	89	508
	.005	5	88	93	94	94	451
	.0075	19	97	116	84	113	363
	.01	9	152	161	94	122	266
	.025	9	114	123	93	131	114
10	.001	10	102	112	91	10	838
	.00025	14	60	74	81	24	736
	.0005	9	98	107	92	33	676
	.00075	25	77	102	75	58	578
	.001	20	66	86	77	78	501
	.0025	28	59	87	68	106	435
	.005	89	6	95	6	195	376
	.0075	26	100	126	79	221	370
	.01	9	162	171	95	230	270
	.025	8	108	116	93	238	108
15	.0001	11	103	114	90	11	567
	.00025	15	60	75	80	26	464
	.0005	10	97	107	91	36	404
	.00075	26	76	102	75	62	307
	.001	27	61	88	69	89	231
	.0025	65	18	83	22	154	170
	.005	102	0	102	0	256	152
	.0075	104	24	128	19	360	152
	.01	53	70	123	57	413	128
	.025	59	58	117	50	472	58

TABLE II (Continued)

Thiomersal

Minutes	Conc., %	Nonviable	Viable	Total	% Viable	Cumulative	
						Nonviable	Viable
20	.0001	10	102	112	91	10	376
	.00025	18	61	79	77	28	274
	.0005	10	98	108	91	38	213
	.00075	27	75	102	74	65	115
	.001	45	37	82	45	110	40
	.0025	82	3	85	3	192	3
	.005	102	0	102	0	294	0
	.0075	99	0	99	0	393	0
	.01	161	0	161	0	554	0
	.025	111	0	111	0	665	0
25	.0001	10	102	112	91	10	333
	.00025	20	61	81	75	30	231
	.0005	10	97	107	91	40	170
	.00075	31	68	99	69	71	73
	.001	85	4	89	4	156	5
	.0025	84	1	85	1	240	1
	.005	102	0	102	0	342	0
	.0075	100	0	100	0	442	0
	.01	160	0	160	0	602	0
	.025	111	0	111	0	713	0

TABLE II (Continued)

Cetylpyridinium Chloride

Minutes	Conc., %	Nonviable	Viable	Total	% Viable	Cumulative	
						Nonviable	Viable
5	.0001	11	83	94	88	11	614
	.00025	8	67	75	89	19	531
	.0005	11	100	111	90	30	464
	.00075	16	52	68	76	46	364
	.001	16	58	74	78	62	312
	.0025	11	75	86	87	73	254
	.005	11	97	108	90	84	179
	.0075	3	76	79	96	87	82
	.01	167	5	172	2	254	6
	.025	109	1	110	1	363	1
10	.0001	12	85	97	88	12	619
	.00025	11	64	75	85	23	534
	.0005	10	99	109	91	33	470
	.00075	18	50	68	74	51	371
	.001	17	60	77	78	68	321
	.0025	17	72	89	81	85	261
	.005	10	100	110	91	95	189
	.0075	2	88	90	98	97	89
	.01	170	1	171	1	267	1
	.025	110	0	110	0	377	0
15	.0001	16	80	96	83	16	601
	.00025	11	63	74	85	27	521
	.0005	11	99	110	90	38	458
	.00075	17	53	70	76	55	359
	.001	19	61	80	76	74	306
	.0025	15	69	84	82	89	245
	.005	11	83	94	88	100	176
	.0075	3	93	96	97	103	93
	.01	171	0	171	0	274	0
	.025	110	0	110	0	384	0

TABLE II (Continued)

Cetylpyridinium Chloride

Minutes	Conc., %	Nonviable	Viable	Total	% Viable	Cumulative	
						Nonviable	Viable
20	.0001	16	80	96	83	16	581
	.00025	10	63	73	86	26	501
	.0005	12	97	109	89	38	438
	.00075	17	53	70	76	55	341
	.001	18	60	78	77	73	288
	.0025	16	65	81	80	89	228
	.005	17	78	95	82	106	163
	.0075	5	85	90	94	111	85
	.01	171	0	171	0	282	0
	.025	110	0	110	0	392	0
25	.0001	16	80	96	83	16	529
	.00025	11	63	74	85	27	449
	.0005	13	91	104	88	40	386
	.00075	19	50	69	72	59	295
	.001	19	62	81	76	78	245
	.0025	16	66	82	80	94	183
	.005	53	43	96	45	147	117
	.0075	11	74	85	87	158	74
	.01	171	0	171	0	329	0
	.025	110	0	110	0	439	0

TABLE II (Continued)

Hyamine 1622

Minutes	Conc., %	Nonviable	Viable	Total	% Viable	Cumulative	
						Nonviable	Viable
5	.0001	10	77	87	88	10	841
	.00025	11	75	86	87	21	764
	.0005	16	86	102	84	37	689
	.00075	21	58	79	73	58	603
	.001	13	69	82	84	71	545
	.0025	15	74	89	83	86	476
	.005	6	99	105	94	92	402
	.0075	14	104	118	88	106	303
	.01	11	78	89	88	117	199
	.025	1	121	122	99	118	121
10	.0001	9	80	89	90	9	818
	.00025	8	69	77	90	17	738
	.0005	15	83	98	95	32	669
	.00075	18	61	79	77	50	586
	.001	17	66	83	79	67	525
	.0025	25	72	97	74	92	459
	.005	7	87	94	92	99	387
	.0075	22	99	121	82	121	300
	.01	8	78	86	91	129	201
	.025	7	123	130	95	136	123
15	.0001	10	80	90	89	10	815
	.00025	10	66	76	87	20	735
	.0005	17	88	105	84	37	669
	.00075	19	60	79	76	56	581
	.001	16	67	83	81	72	521
	.0025	21	70	91	77	93	454
	.005	8	82	90	91	101	384
	.0075	20	99	119	83	121	302
	.01	12	89	101	88	133	203
	.025	5	114	119	96	138	114

TABLE II (Continued)

Hyamine 1622

Minutes	Conc., %	Nonviable	Viable	Total	% Viable	Cumulative	
						Nonviable	Viable
20	.0001	10	80	90	89	10	816
	.00025	11	69	80	86	21	736
	.0005	17	89	106	84	38	667
	.00075	19	60	79	76	57	578
	.001	15	66	81	81	72	518
	.0025	22	69	91	76	94	452
	.005	8	85	93	91	102	383
	.0075	23	110	133	83	125	298
	.01	8	73	81	90	133	188
	.025	6	115	121	95	139	115
25	.0001	10	80	90	89	10	808
	.00025	10	68	78	87	20	728
	.0005	18	89	107	83	38	660
	.00075	19	61	90	68	57	571
	.001	13	63	76	83	70	510
	.0025	23	68	91	75	93	447
	.005	7	76	83	92	100	379
	.0075	17	110	127	87	117	303
	.01	11	76	87	87	128	193
	.025	8	117	125	94	136	117

TABLE II (Continued)

Tween 60

Minutes	Conc., %	Nonviable	Viable	Total	% Viable	Cumulative	
						Nonviable	Viable
5	.0001	15	86	101	85	15	835
	.00025	11	76	87	87	26	749
	.0005	24	80	104	77	50	673
	.00075	17	73	90	81	67	593
	.001	18	69	87	79	85	520
	.0025	20	70	90	78	105	451
	.005	12	78	90	87	117	381
	.0075	17	96	113	85	134	303
	.01	7	93	100	93	141	207
	.025	9	114	123	93	150	114
10	.0001	15	86	101	85	15	842
	.00025	17	70	87	80	32	756
	.0005	27	78	105	74	59	686
	.00075	17	73	90	81	76	608
	.001	19	69	88	78	95	535
	.0025	22	73	95	77	117	466
	.005	14	80	94	85	131	393
	.0075	19	109	128	85	150	313
	.01	7	96	103	93	157	204
	.025	8	108	116	93	165	108
15	.0001	16	83	99	84	16	838
	.00025	16	78	94	83	32	755
	0005	27	78	105	74	59	677
	.00075	18	71	89	80	77	599
	.001	21	68	89	76	98	528
	.0025	23	75	98	76	121	460
	.005	14	81	95	85	135	385
	.0075	20	99	119	83	155	304
	.01	6	94	100	94	161	205
	.025	9	111	120	92	170	111

TABLE II (Concluded)

Tween 60

Minutes	Conc., %	Nonviable	Viable	Total	% Viable	Cumulative	
						Nonviable	Viable
20	.0001	14	81	95	85	14	809
	.00025	16	79	95	83	30	728
	.0005	28	77	105	73	58	649
	.00075	17	72	89	81	75	572
	.001	19	69	88	78	94	500
	.0025	23	76	99	77	117	431
	.005	13	80	93	86	130	355
	.0075	15	71	86	83	145	275
	.01	10	98	108	91	155	204
	.025	10	106	116	91	165	106
25	.0001	12	82	94	87	12	825
	.00025	15	79	94	84	27	743
	.0005	27	79	106	74	54	664
	.00075	18	71	89	80	72	585
	.001	18	68	86	79	90	514
	.0025	22	75	97	77	112	446
	.005	15	77	92	84	127	371
	.0075	25	101	126	80	152	294
	.01	11	97	108	90	163	193
	.025	9	96	105	91	172	96

III. GROWTH AND PROLIFERATION STUDIES

A. PURPOSE

The purpose of the study during this period of investigation was to determine and compare the effects of low concentrations of Thiomersal, Cetylpyridinium chloride, and Hyamine 1622 (antiseptics), and Tween 60 (a detergent) on the growth and viability of human epithelial cells. In the past, growth of HEp_2 cells has been determined by counting on a Coulter counter the total number of cells. Since viable and nonviable, nonlysed cells cannot be accurately differentiated by this method, stains indicating viability were included in the present study because little or no cytolysis was expected with the low concentrations of reagents used. Also, this method of figuring percent of viable cells is a more accurate indication of reagent toxicity than counts of the total number of remaining cells in a growing culture.

B. PROCEDURE

Fifty-milliliter suspension cultures of HEp_2 cells were planted at concentrations ranging from 1.2×10^5 to 4.5×10^5 cells per ml and allowed to enter early log phase of growth. The growth medium was similar to that used previously—$Eagles_{75}$, $tryptose\ phosphate_{15}$, and $calf\ serum_{10}$, with 0.1% methyl cellulose and 0.1% sodium citrate added. Log phase was entered at intervals ranging from 21.5 to 45.5 hours when the cell count ranged from 2.2×10^5 to 8×10^5 and at that time one ml of one of the antiseptic and detergent solutions was introduced. The concentrations of the solutions used were 0.001%, 0.00075%, 0.0005%, and 0.0001%. All of these concentrations were used with the cultures of lower cell count and in addition the 0.001% solutions were used with cultures of the highest cell count in order to demonstrate any varied effects due to a different density of the cell population. The cultures were placed on a rotary shaker for constant agitation.

Every 24 hours two one-ml samples were withdrawn from the cultures for counting and viability determinations. The total cell count was obtained by use of the Coulter counter. The percentage of viable cells was determined by staining with 0.4% erythrosin B and counting the stained (nonviable) and unstained (viable) cells with a hemacytometer.

The inclusion of the viability determination demonstrates more clearly the toxic effects of the antiseptic and detergent solutions. In previous studies the inhibition of growth was difficult to differentiate from death of the cells when lysis of the dead cells did not occur. Also, any error introduced by fractionation of the cells, a possibility that exists because frac-

tionation may increase the total cell count as determined by the Coulter counter, is eliminated.

C. RESULTS

1. Effects of 0.001% Solutions

At this concentration a large variance was made in the initial cell population density. Examinations were made using an initial inoculum of 1.2×10^5 cells per ml and 4.5×10^5 cells per ml. The solutions were added when the cell count reached approximately 2.2×10^5 cells per ml and 8×10^5 cells per ml respectively.

A definite variation in toxic reaction was noticed between the different cell population densities. For example at the higher level the population appears to be unaffected by Tween 60. At the lower level there is a reduction in growth rate and the plateau phase occurs at the same time as in the control but at a lower cell number. At the lower cell population Cetylpyridinium chloride seems to be only slightly more toxic than Tween 60 when the total cell count is considered. However, at a higher cell population Cetylpyridinium chloride is the most toxic of the four chemicals tested. This could be attributed to an increase in total cell lysis by Cetylpyridinium chloride in a higher cell population as compared to that by other chemicals.

On the basis of viable cell counts, the cells in the series of tests at the lower cell population have been affected to such an extent that Thiomersal, Cetylpyridinium chloride, and Hyamine 1622 caused almost immediate death and the viable cell count was so low that any practical comparison of these three antiseptics was impossible. Tween 60 showed only a slight effect upon the viability. At the higher population levels there is a more distinct variation in viability. Cetylpyridinium chloride is the most toxic, Thiomersal is slightly more toxic than Hyamine 1622, and Tween 60 is almost identical to the control.

An interesting finding that was observed at both high and low population levels is that Thiomersal produced an immediate reduction in the number of viable cells but with Cetylpyridinium chloride and Hyamine 1622 the reduction did not occur for at least 24 hours. However, Thiomersal did not prove to be ultimately the most toxic.

2. Effects of 0.00075% Solutions

This concentration level seems to divide the four compounds into two groups. Tween 60 and Hyamine 1622 had a slight effect on the cells while Cetylpyridinium chloride and Thiomersal showed absolute toxicity. The former group seems only to

inhibit mitosis without any toxic effect on the cell proper.

3. Effects of 0.0005% Solutions

The results at this concentration are similar to those at 0.00075%. The decrease in viable cells again points out the toxic action of Thiomersal and Cetylpyridinium chloride. One difference between the two concentrations, however, is that the time required before reduction in the number of viable cells by Thiomersal and Cetylpyridinium chloride has been prolonged. At 0.00075% the reaction time varies from zero to 24 hours. At the concentration of 0.0005% this lag is increased to an interval ranging from 65 to 89 hours.

4. Effects of 0.0001% Solutions

At this concentration Thiomersal, in addition to Tween 60 and Hyamine 1622, appears to be almost nontoxic. With Hyamine 1622 the growth rate is even greater than in the control. Thiomersal did show a toxic reaction after approximately 76 hours. Cetylpyridinium chloride caused a reduced rate of growth of the cells 26 hours after its introduction but the percentage of viable cells did not begin to decrease until after 47 hours.

D. SUMMARY

Tween 60 has been shown to be the least toxic throughout the concentration range examined. Hyamine 1622 is the second least toxic over all but is less toxic than Tween 60 at the lowest concentration tested. Thiomersal rates third in degree of nontoxicity and Cetylpyridinium chloride is the most toxic.

A decrease in the degree of toxicity of the three antiseptic reagents in a more dense cell population has been shown. This may be due to a number of factors. Possibly a more rapidly growing culture shows more resistance to the toxicity of these reagents or perhaps the increased amount of protein inactivates the antiseptics. It is also possible that a given amount of these reagents can react only with an optimum number of cells. If that be the case then it would explain why a dense and continuously growing cell population would appear to be less affected than a more sparse cell population.

As has been reported previously, the concentration of the reagents is a definite factor in the degree of toxicity. The present study has shown also that the time required before a decrease in the number of viable cells becomes evident is dependent upon the concentration of the reagents. A reduction in concentration will increase the length of time preceding a reaction.

These studies showed that a relatively small difference in concentration

of the three antiseptics produced a great difference in toxicity. At 0.001%, 0.00075%, and 0.0005% they were quite toxic but at 0.0001% they were, with the possible exception of Cetylpyridinium chloride, nontoxic.

In previous reports on higher concentrations viability tests were not necessary because total lysis of the cells usually occurred immediately after introduction of the reagent. However, at the lower concentrations tested in the present study significant cytolysis was not expected and it was essential to determine the viability of the cells. While the total cell count often showed little change the number of viable cells dropped sharply.

TABLE III

GROWTH AND VIABILITY

0.001%—High Cell Population

Hours Cultured	Hours in Reagent	Hours of Interval	Control			Thiomersal			Cetylpyridinium Chloride		
			Total No. of Cells x 10^5/ml	% Viable	No. of Viable Cells x 10^5/ml	Total No. of Cells x 10^5/ml	% Viable	No. of Viable Cells x 10^5/ml	Total No. of Cells x 10^5/ml	% Viable	No. of Viable Cells x 10^5/ml
0	0	0	4.531	94	4.26	4.567	94	4.29	4.532	94	4.26
26.5	0	26.5	8.520	97	8.26	7.028	97	6.82	8.111	97	7.87
48	21.5	21.5	12.938	94	12.16	8.259	87	7.18	9.380	86	8.07
72	45.5	24	15.507	95	14.73	8.123	30	2.44	7.004	5	0.35
96	69.5	24	17.855	92	16.42	7.312	5	0.37	6.045	3	0.18
119	92.5	23	15.203	80	12.16	7.200	0	0	5.861	0	0
144	117.5	25	14.757	37	5.47	7.833	0	0	5.193	0	0
			Tween 60			Hyamine 1622					
0	0	0	4.537	94	4.26	4.521	94	4.25			
26.5	0	26.5	8.100	92	7.45	8.003	97	7.76			
48	21.5	21.5	11.933	96	11.45	8.860	68	6.02			
72	45.5	24	14.655	96	14.07	8.287	30	2.48			
96	69.5	24	16.299	89	14.50	7.764	5	0.38			
119	92.5	23	14.375	67	9.63	7.406	0	0			
144	117.5	25	13.710	43	5.90	7.381	0	0			

TABLE III (Continued)

0.001%—Low Cell Population

Hours Cultured	Hours in Reagent	Hours of Interval	Control			Thiomersal			Cetylpyridinium Chloride		
			Total No. of Cells x 10^5/ml	% Viable	No. of Viable Cells x 10^5/ml	Total No. of Cells x 10^5/ml	% Viable	No. of Viable Cells x 10^5/ml	Total No. of Cells x 10^5/ml	% Viable	No. of Viable Cells x 10^5/ml
0	0	0	1.230	90	1.10	1.230	90	1.10	1.230	90	1.10
24	0	24	1.680	75	1.26	1.790	71	1.27	1.650	81	1.38
43.5	0	19.5	2.489	92	2.29	2.387	90	2.15	2.265	95	2.16
62	18.5	18.5	3.163	98	3.10	2.793	55	1.57	2.919	90	2.64
79.5	36	17.5	3.798	90	3.42	3.066	40	1.22	4.016	25	1.00
102	58.5	22.5	6.703	98	6.57	3.110	15	0.46	3.392	10	0.39
124	80.5	22	8.404	99	8.32	2.664	3	0.08	3.452	0.05	0.17
144	100.5	20	8.673	87	7.55	2.636	0	0	2.982	0	0
168	124.5	24	9.183	70	6.43	2.674	0	0	3.026	0	0
188.5	145	20.5	9.361	70	6.55	1.163	0	0	2.990	0	0
212.5	169	24	9.467	60	5.67	--	--	--	--	--	--
236	192.5	23.5	7.121	40	2.84	--	--	--	--	--	--
			Tween 60			Hyamine 1622					
0	0	0	1.260	90	1.13	1.230	90	1.10			
24	0	24	1.640	80	1.31	1.690	83	1.40			
43.5	0	19.5	2.350	85	1.99	2.504	99	2.48			
62	18.5	18.5	2.810	90	2.52	2.979	93	2.77			
79.5	36	17.5	3.277	85	2.78	2.582	12	0.31			
102	58.5	22.5	4.123	90	3.71	2.154	1	0.02			
124	80.5	22	4.414	85	3.75	1.496	0	0			
144	100.5	20	4.594	65	2.98	1.008	0	0			
168	124.5	24	4.553	55	2.51	1.142	0	0			
188.5	145	20.5	3.978	30	1.19	0.780	0	0			
212.5	169	24	3.847	25	0.96	--	--	--			
236	192.5	23.5	3.592	15	0.54	--	--	--			

TABLE III (Continued)

0.00075%

Hours Cultured	Hours in Reagent	Hours of Interval	Control			Thiomersal			Cetylpyridinium Chloride		
			Total No. of Cells x 10^5/ml	% Viable	No. of Viable Cells x 10^5/ml	Total No. of Cells x 10^5/ml	% Viable	No. of Viable Cells x 10^5/ml	Total No. of Cells x 10^5/ml	% Viable	No. of Viable Cells x 10^5/ml
0	0	0	2.100	90	1.89	2.100	90	1.89	2.100	90	1.89
21.5	0	21.5	2.756	95	2.61	2.565	95	2.43	2.701	95	2.56
45.5	24	24	5.279	95	5.02	4.500	90	4.05	3.633	60	2.18
70	48.5	24.5	7.571	97	7.34	5.832	50	2.92	3.706	30	1.11
95	73.5	25	9.940	92	9.14	5.103	37	1.89	3.535	23	0.82
129	107.5	34	12.105	90	10.90	4.229	15	0.63	3.459	20	0.69
143.5	122	14.5	11.755	85	9.99	3.384	5	0.16	3.319	10	0.33
167	145.5	23.5	13.595	87	11.83	3.197	0	0	3.886	5	0.19
191	169.5	24	14.151	84	11.89	--	--	--	--	--	--
214	192.5	23	12.188	82	9.99	--	--	--	--	--	--
			Tween 60			Hyamine 1622					
0	0	0	2.100	90	1.89	2.100	90	1.89			
21.5	0	21.5	2.839	95	2.69	2.820	95	2.67			
45.5	24	24	4.912	95	4.66	4.691	95	4.45			
70	48.5	24.5	6.068	95	5.75	5.814	97	5.63			
95	73.5	25	8.735	96	8.38	7.528	95	7.15			
129	107.5	34	11.332	95	10.76	10.439	95	9.91			
143.5	122	14.5	10.955	90	9.85	9.699	90	8.72			
167	145.5	23.5	11.381	92	10.47	10.257	60	6.15			
191	169.5	24	10.934	81	8.87	9.989	47	4.69			
214	192.5	23	10.850	74	8.02	10.413	41	4.26			

TABLE III (Continued)

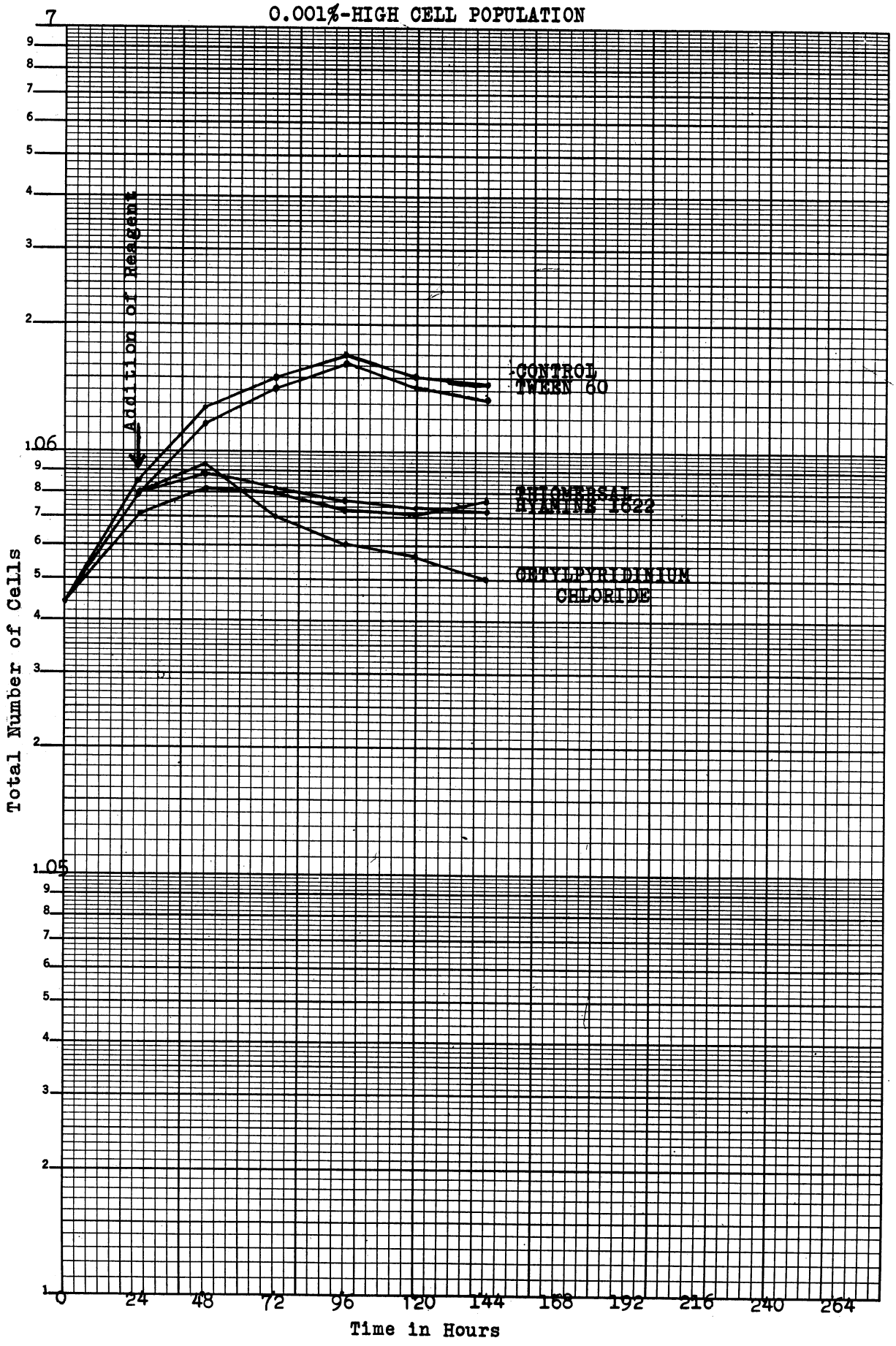
0.0005%

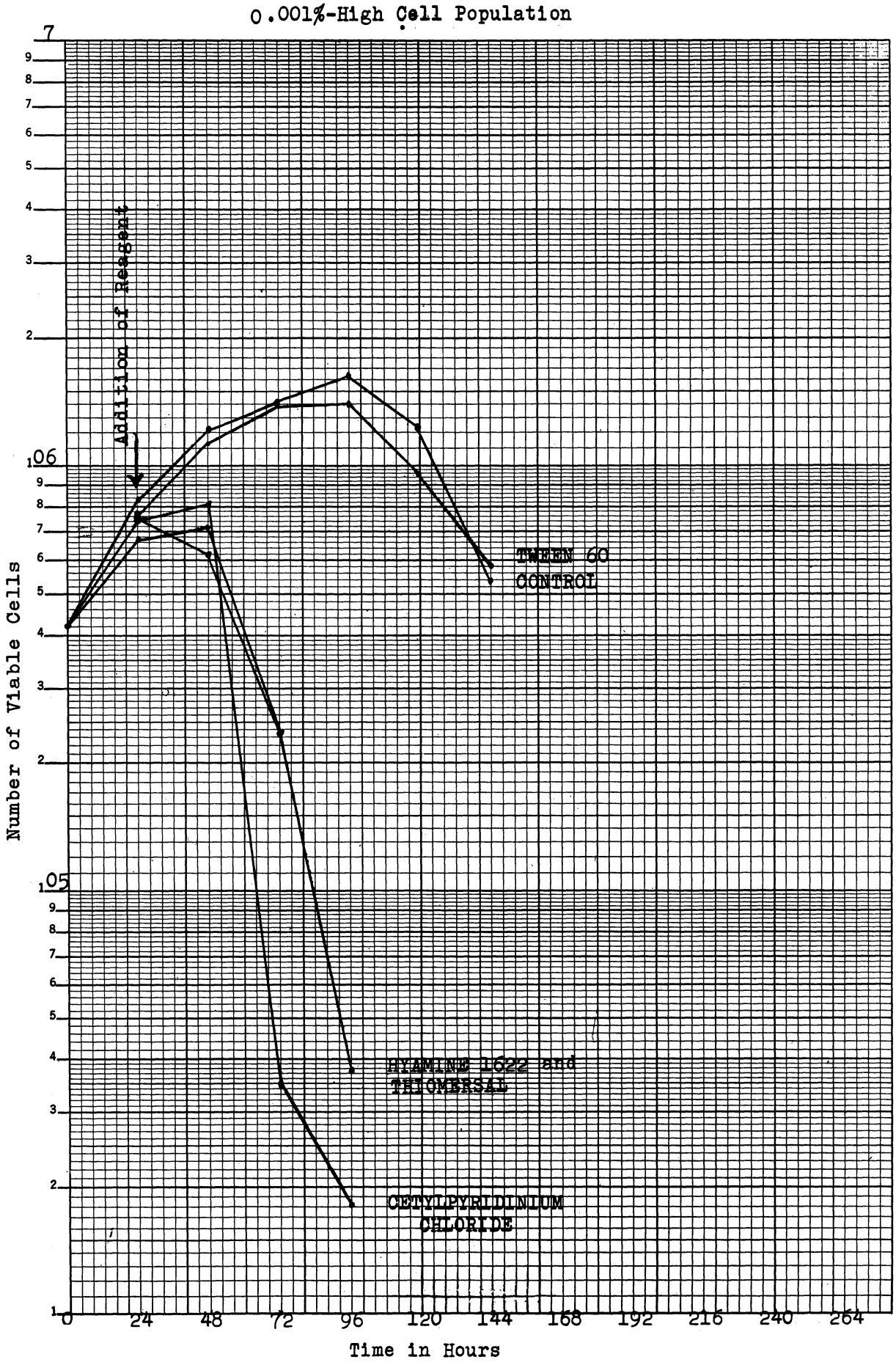
Hours Cultured	Hours in Reagent	Hours of Interval	Control			Thiomersal			Cetylpyridinium Chloride		
			Total No. of Cells x 10^5/ml	% Viable	No. of Viable Cells x 10^5/ml	Total No. of Cells x 10^5/ml	% Viable	No. of Viable Cells x 10^5/ml	Total No. of Cells x 10^5/ml	% Viable	No. of Viable Cells x 10^5/ml
0	0	0	1.317	95	1.25	1.576	95	1.49	1.490	95	1.41
20	0	20	1.897	97	1.84	1.963	99	1.94	1.817	95	1.73
28	0	8	2.352	97	2.30	2.040	99	2.02	2.179	95	2.07
49	21	21	4.136	97	4.01	3.810	100	3.81	2.998	80	2.40
71	43	22	5.473	100	5.47	4.015	99	3.97	3.449	80	2.07
93.5	65.5	22.5	7.578	100	7.57	5.309	97	5.15	3.289	83	2.73
117	89	23.5	9.758	95	9.30	5.775	50	4.89	3.611	33	1.19
141	113	24	9.625	95	9.14	4.976	30	1.49	3.414	1	0.03
165.5	137.5	24.5	11.866	95	11.27	4.574	10	0.45	3.247	0	0
188.5	160.5	23	10.877	90	9.77	4.431	7	0.31	2.946	0	0
212.5	184.5	24	9.412	85	8.00	--	--	--	--	--	--
			Tween 60			Hyamine 1622					
0	0	0	1.598	95	1.52	1.600	95	1.52			
20	0	20	1.960	95	1.89	1.812	97	1.77			
28	0	8	2.350	95	2.21	2.134	97	2.03			
49	21	21	4.072	100	4.07	3.618	97	3.51			
71	43	22	5.268	97	5.00	4.913	97	4.75			
93.5	65.5	22.5	6.667	95	6.33	6.763	95	6.42			
117	89	23.5	8.564	95	8.13	7.342	85	6.22			
141	113	24	8.795	95	8.25	7.757	80	6.20			
165.5	137.5	24.5	8.982	90	8.09	9.042	90	8.13			
188.5	160.5	23	8.459	78	6.60	9.829	75	7.37			
212.5	184.5	24	--	--	--	9.613	60	5.77			

TABLE III (Concluded)

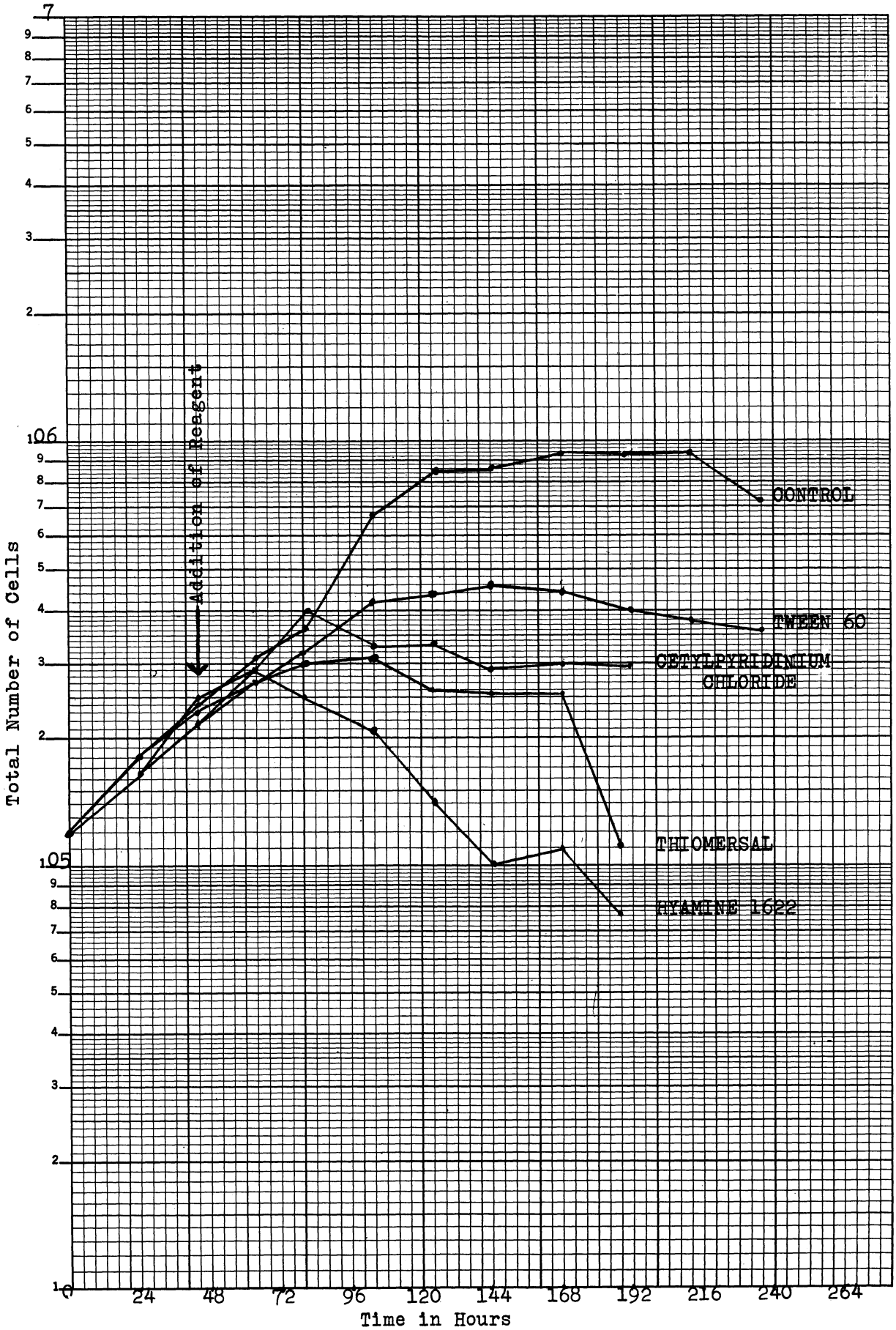
0.0001%

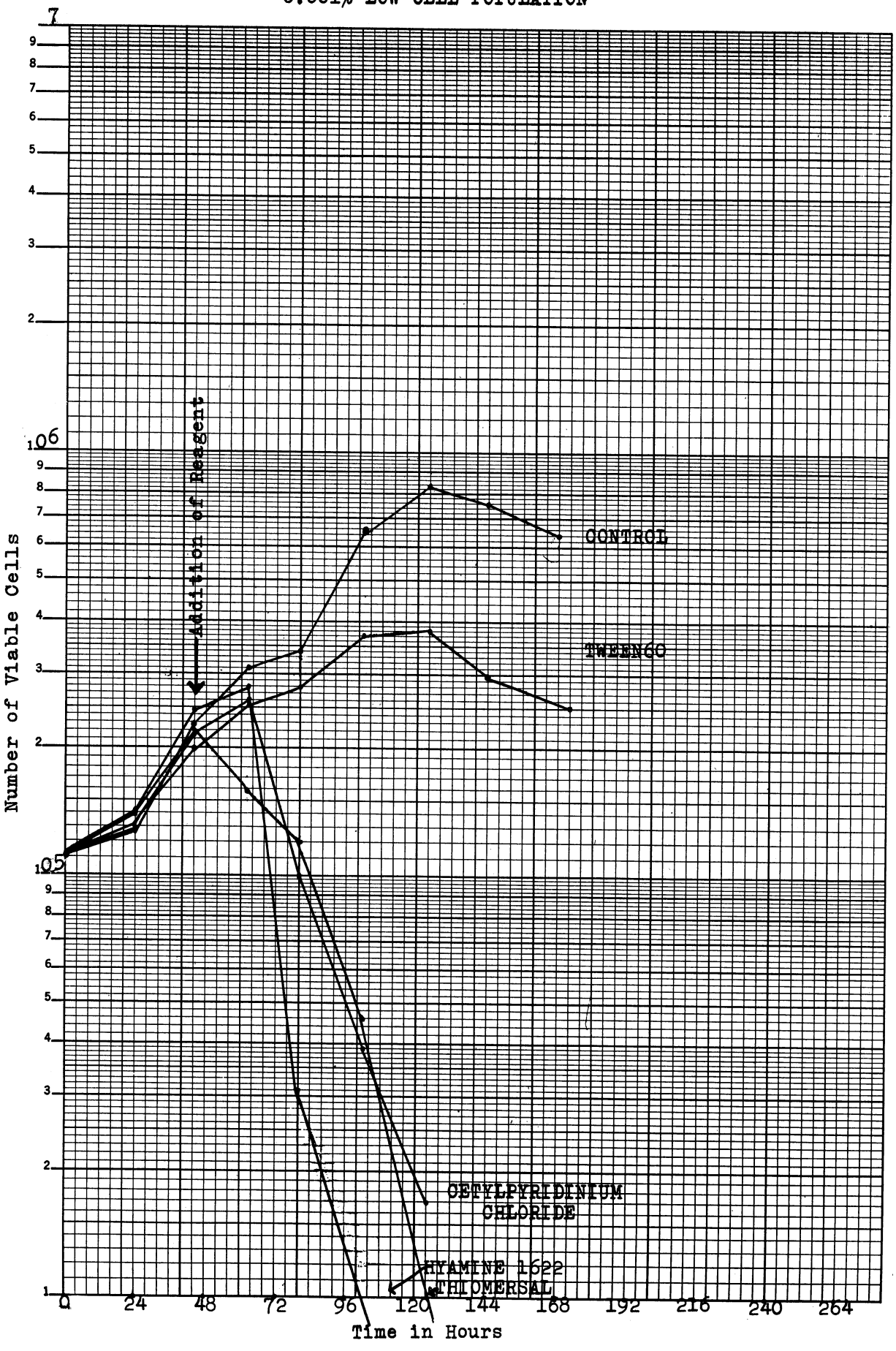
Hours Cultured	Hours in Reagent	Hours of Interval	Control			Thiomersal			Cetylpyridinium Chloride		
			Total No. of Cells x 10^5/ml	% Viable	No. of Viable Cells x 10^5/ml	Total No. of Cells x 10^5/ml	% Viable	No. of Viable Cells x 10^5/ml	Total No. of Cells x 10^5/ml	% Viable	No. of Viable Cells x 10^5/ml
0	0	0	1.819	95	1.72	1.669	95	1.58	1.696	95	1.61
21.5	0	21.5	2.203	80	1.76	2.073	80	1.66	2.121	85	1.80
45.5	0	24	3.615	92	3.32	3.532	95	3.35	3.510	95	3.33
71.5	26	26	5.764	99	5.70	6.757	99	6.69	5.683	99	5.62
92.5	47	21	7.744	95	7.35	7.602	99	7.52	6.158	99	6.09
121.5	76	29	10.267	95	9.75	9.540	95	9.06	7.636	90	6.87
143.5	98	22	11.583	90	10.42	9.322	90	8.39	8.132	35	2.85
			Tween 60			Hyamine 1622					
0	0	0	1.591	95	1.51	1.681	95	1.60			
21.5	0	21.5	2.065	85	1.76	2.112	85	1.79			
45.5	0	24	3.612	97	3.50	3.706	97	3.60			
71.5	26	26	5.585	98	5.47	6.660	99	6.60			
92.5	47	21	7.800	99	7.72	8.878	95	8.44			
121.5	76	29	9.688	95	9.20	10.920	95	10.37			
143.5	98	22	10.592	90	9.53	12.229	90	11.00			

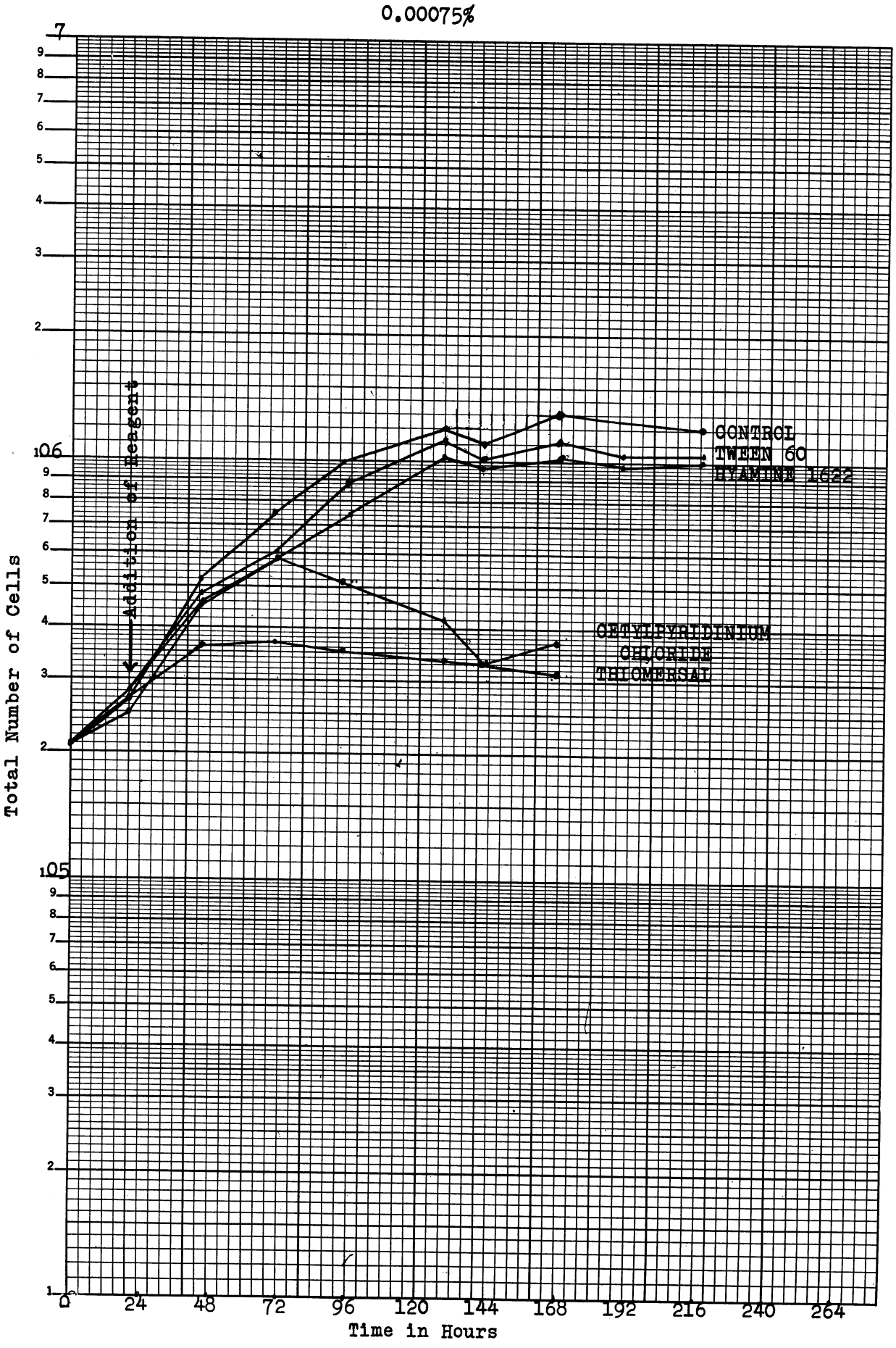

0.001%-HIGH CELL POPULATION
Addition of Reagent
CONTROL
TWEEN 60
THIOMERSAL
MERTHIOLATE
CETYLPYRIDINIUM
CHLORIDE
Total Number of Cells
Time in Hours
0
24
48
72
96
120
144
168
192
216
240
264

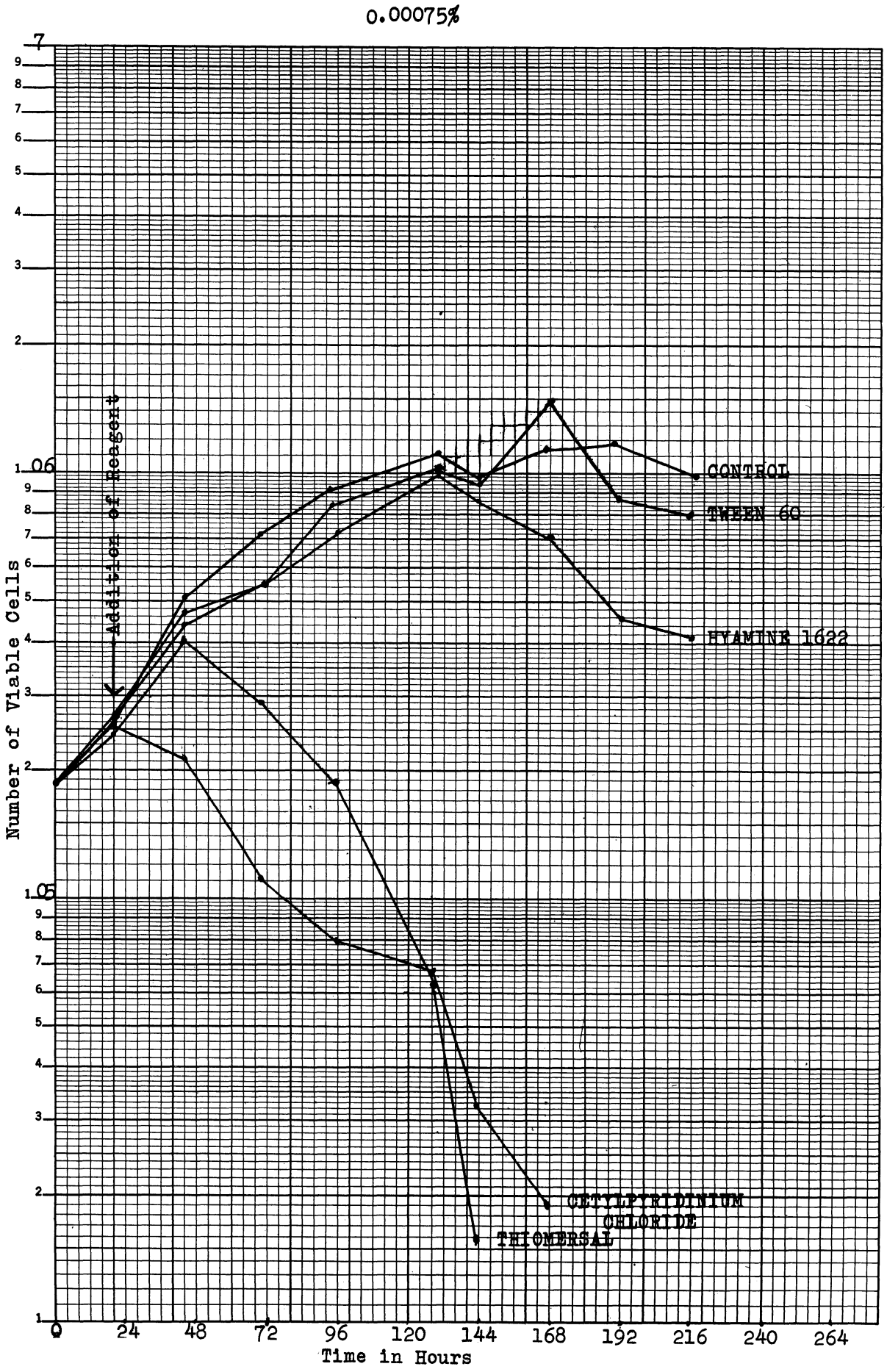

0.001%-High Cell Population
Addition of Reagent
Number of Viable Cells
Time in Hours
TWEEN 60 CONTROL
HYAMINE 1622 and THIOMERSAL
CETYLPYRIDINIUM CHLORIDE
0
24
48
72
96
120
144
168
192
216
240
264

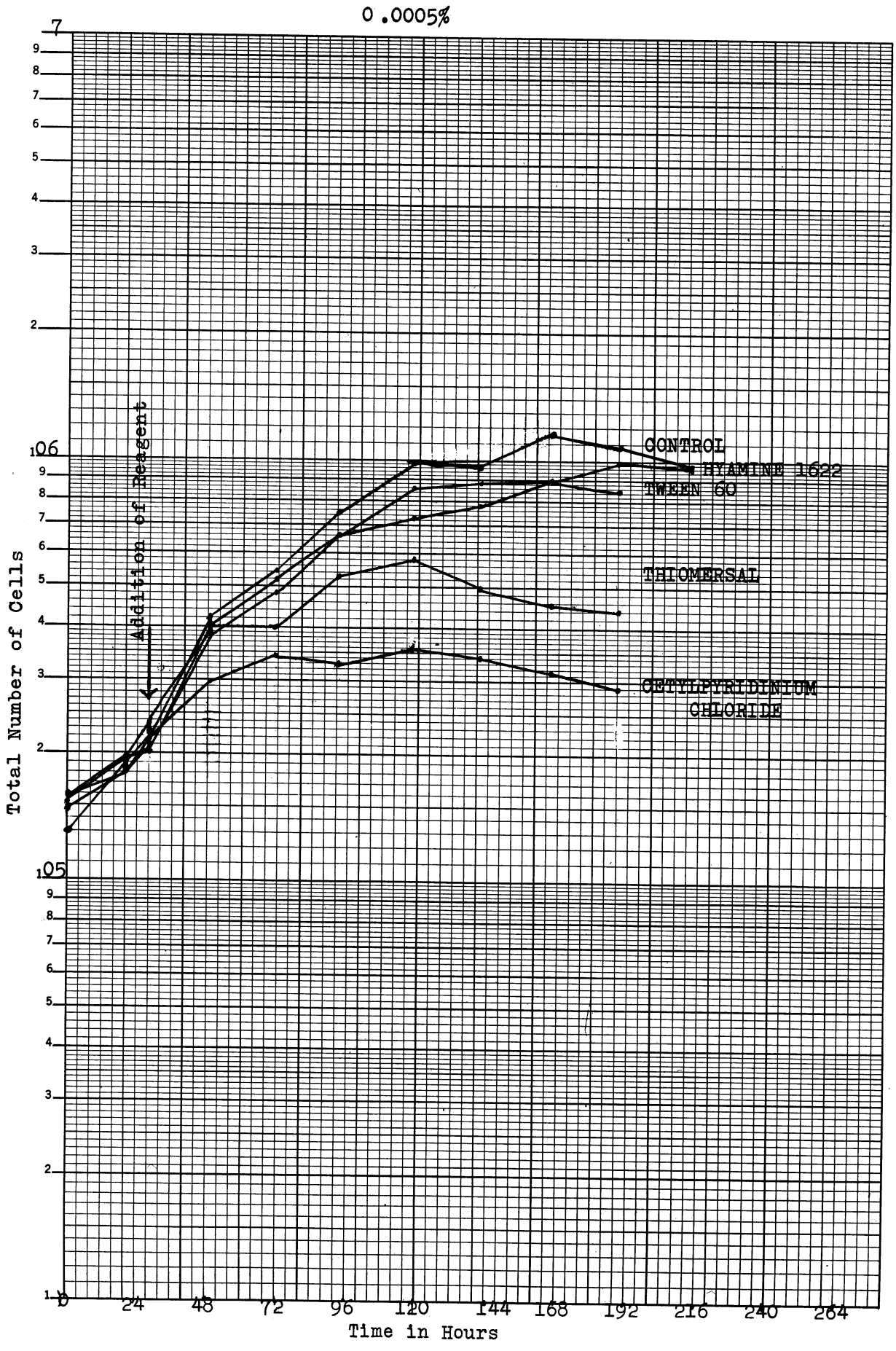
0.001%-LOW CELL POPULATION

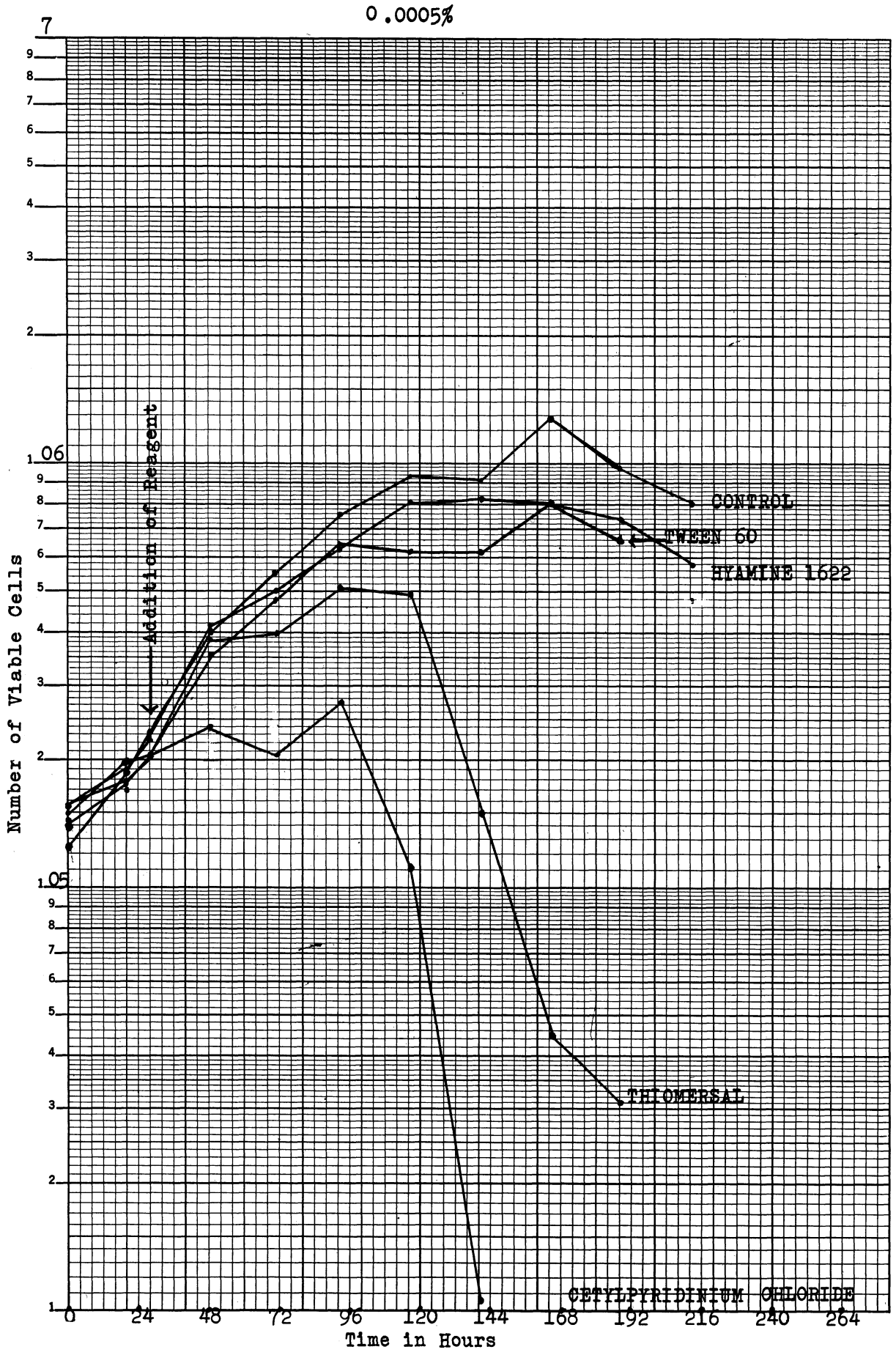



0.001%-LOW CELL POPULATION
Number of Viable Cells
Addition of Reagent
CONTROL
TWEEN60
CETYLPYRIDINIUM CHLORIDE
HYAMINE 1622
THIOMERSAL
Time in Hours
0
24
48
72
96
120
144
168
192
216
240
264


0.00075%
Addition of reagent
CONTROL
TWEEN 60
EYAMINE 1622
CETYLPYRIDINIUM CHLORIDE
THIOMERSAL
Total Number of Cells
Time in Hours
0
24
48
72
96
120
144
168
192
216
240
264


0.00075%
Number of Viable Cells
Addition of Reagent
CONTROL
TWEEN 60
HYAMINE 1622
CETYLPYRIDINIUM CHLORIDE
THIOMERSAL
Time in Hours
0
24
48
72
96
120
144
168
192
216
240
264


0.0005%
Addition of Reagent
CONTROL
HYAMINE 1622
TWEEN 60
THIOMERSAL
CETYLPYRIDINIUM CHLORIDE
Total Number of Cells
Time in Hours
0
24
48
72
96
120
144
168
192
216
240
264


0.0005%
7
9
8
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6
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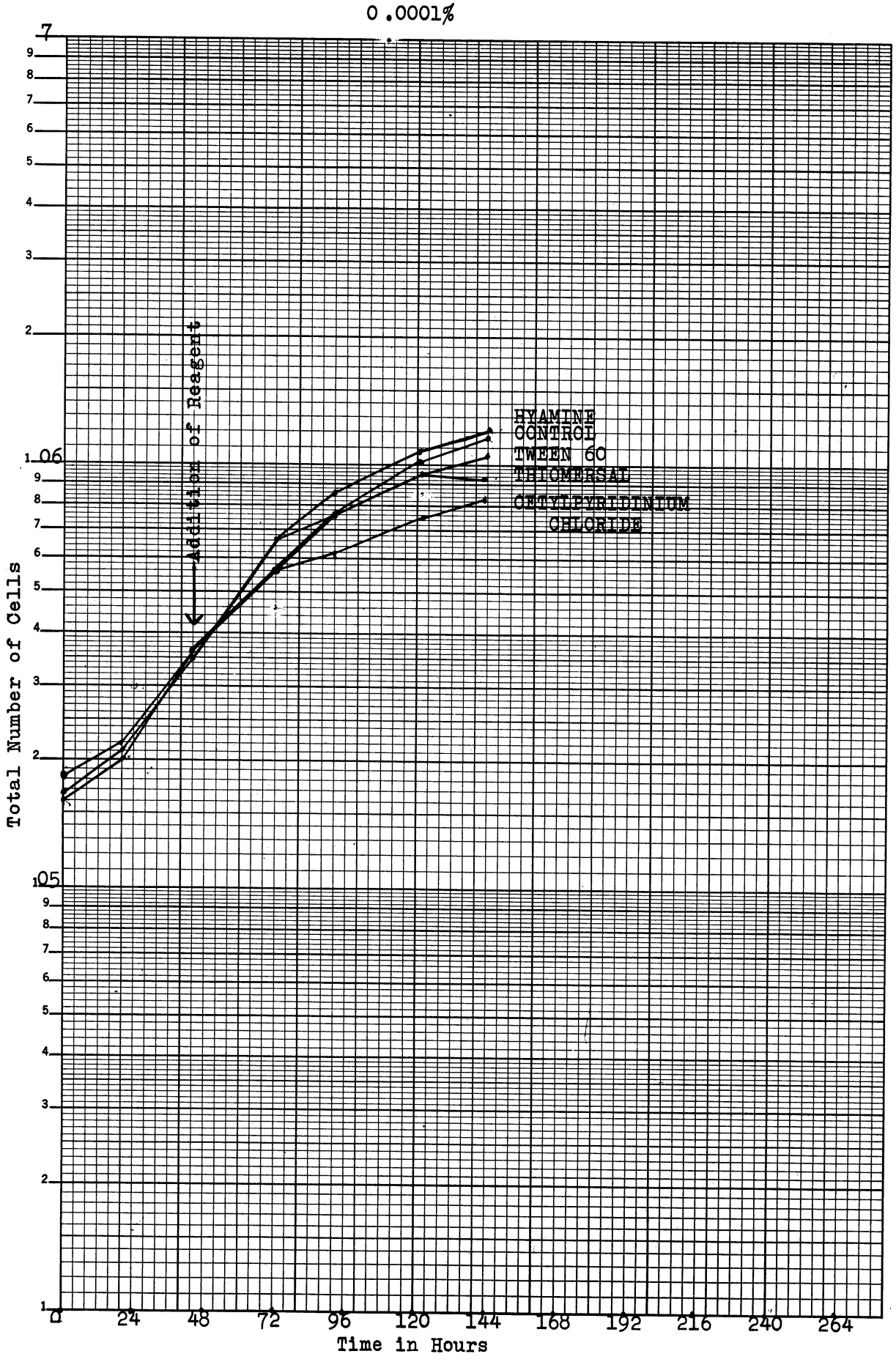
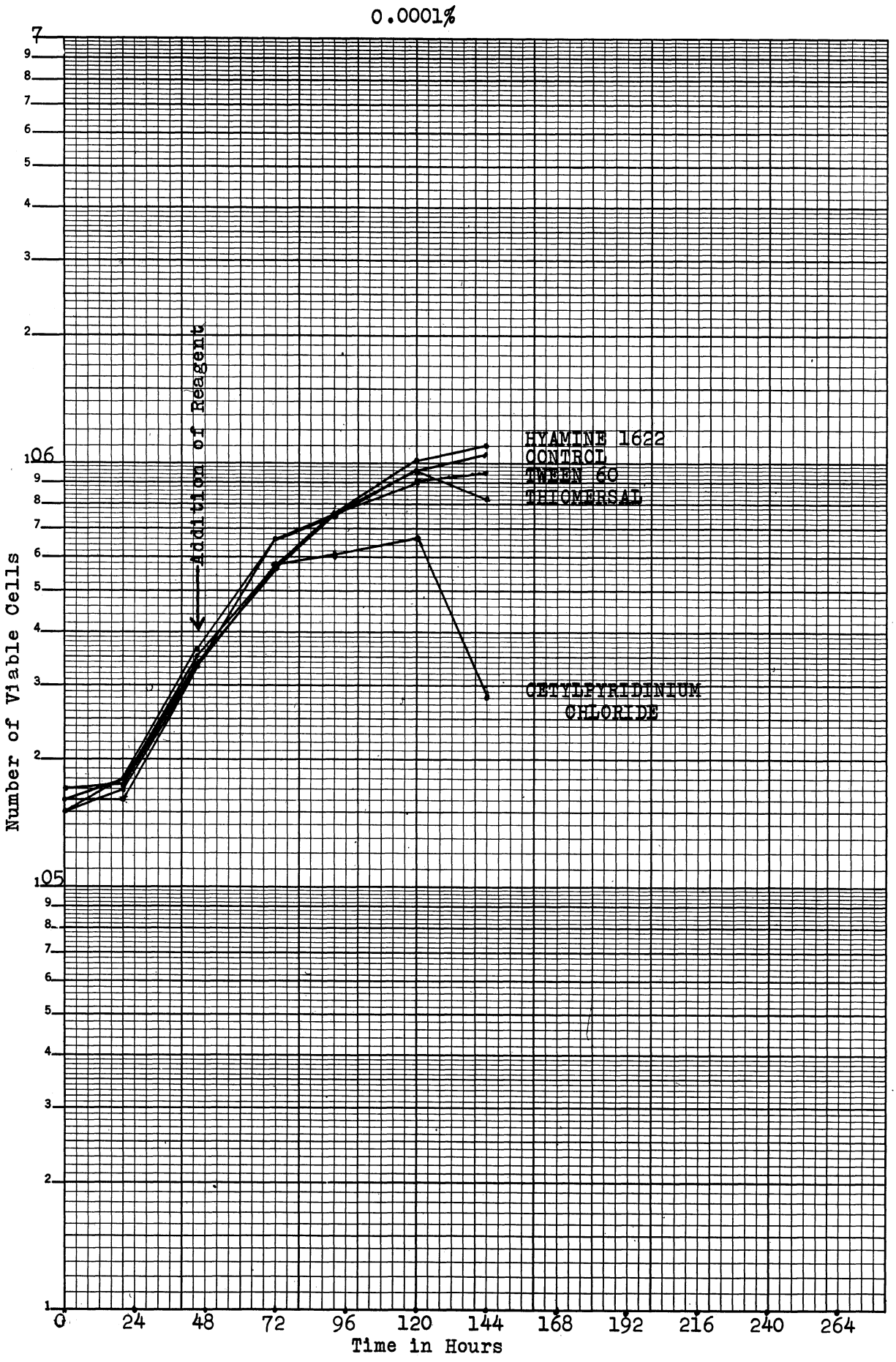
4
3
2
1.06
9
8
7
6
5
4
3
2
1.05
9
8
7
6
5
4
3
2
1
Addition of Reagent
Number of Viable Cells
CONTROL
TWEEN 60
HYAMINE 1622
THIOMERSAL
CETYLPYRIDINIUM CHLORIDE
0
24
48
72
96
120
144
168
192
216
240
264
Time in Hours


0.0001%
Addition of Reagent
HYAMINE
CONTROL
TWEEN 60
THIOMERSAL
CETYLPYRIDINIUM
CHLORIDE
Total Number of Cells
Time in Hours
0
24
48
72
96
120
144
168
192
216
240
264


0.0001%
Addition of Reagent
HYAMINE 1622
CONTROL
TWEEN 60
THIOMERSAL
CETYLPYRIDINIUM
CHLORIDE
Number of Viable Cells
Time in Hours
0
24
48
72
96
120
144
168
192
216
240
264

IV. TOXICITY STUDIES

Phase 1. Histochemical Studies

A. PURPOSE

The purpose of this aspect of the investigation was to determine the cytochemical effects of Thiomersal, Cetylpyridinium chloride, and Hyamine 1622 (antiseptics), and Tween 60 (a detergent) on HEp_2 cells. Ribonucleoproteins (RNA-Protein) and deoxyribonucleoprotens (DNA-Protein) were demonstrated by the May-Grünwald-Giemsa method and lipids by a modified Sudan Black B method.

B. PROCEDURE

HEp_2 cells derived from our stock line were transplanted to Leighton tubes and incubated for 24 hours in a growth media composed of Eagle's $media_{75}$, $tryptose\ phosphate_{15}$, and $calf\ serum_{10}$. After the incubation period the coverslips, with the cells attached, were removed from the Leighton tubes and exposed to concentrations of 0.1%, 0.01%, 0.001%, and 0.0001% of the reagents dissolved in Hanks balanced salt solution. For the May-Grünwald-Giemsa method 0.1% solutions were omitted. The cells were allowed to incubate in the solution containing the antiseptic or detergent for one hour.

1. Ribonucleoproteins and Deoxyribonucleoproteins

Three runs of each concentration of each reagent were carried out for this stain. In two of the runs there were approximately 10×10^5 cells per ml and in the third approximately 3×10^5 cells per ml. The purpose was to determine any variations due to differences in cell population.

After the one-hour exposure to the reagent the cells were rinsed in three changes of Hanks balanced salt solution, fixed in absolute methanol and stained by the May-Grünwald-Giemsa method.* This staining procedure is excellent for the differential demonstration of ribonucleoproteins (RNA-Protein) and deoxyribonucleoproteins (DNA-Protein) in monolayer cultures. The RNA-Protein stains blue and the DNA-Protein stains red-purple. With this method cytological characteristics of the cells are retained but lipids are dissolved.

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*Merchant, D. J., Kahn, R. H., and Murphy, W. H. Handbook of Cell and Organ Culture. Minneapolis: Burgess Pub. Co., 1960, p. 134.

2. Lipids

Two runs for each concentration of each reagent were carried out. The initial incubation medium for each run contained 2 x 10^5 cells per ml.

After exposure to the reagent the coverslips with attached cells were removed from the Leighton tubes, rinsed in Hanks balanced salt solution and fixed in neutral 10% formalin. Lipids were stained by a modification of the McManus method.* The cells were chromated in 5% potassium dichromate, washed, stained in 0.7% Sudan Black B in 70% ethanol and counterstained in 0.5% aqueous carmine. After rinsing, the coverslips with cells were mounted in an aqueous medium of Arlex and gelatin. The lipids stained black or blue and the nuclei stained red.

C. RESULTS

HEp_2 cells affected by various concentrations of Thiomersal, Cetylpyridinium chloride, Hyamine 1622, and Tween 60 were stained for ribo- and deoxyribonucleoproteins and for lipids. The cytotoxic effects as revealed by these methods were compared but evaluation of the comparative toxicity of the reagents was difficult. In some instances DNA Protein was lost from the nucleoplasm and in others vacuolation of the cytoplasm occurred. Occasionally these reactions were observed in the same cell but not always and because either reaction is indicative of a toxic environment the relative significance of each could not be determined.

Of the four reagents tested Hyamine 1622 caused the most severe toxic reaction. Thiomersal and Cetylpyridinium chloride caused similar reactions but Thiomersal was perhaps slightly more toxic. Tween 60 appeared to be the least toxic.

1. Ribonucleoproteins and Deoxyribonucleoproteins

In cells stained for RNA- and DNA-Proteins many phenomena were noted, most of which indicated cellular reaction to injury. A number of pyknotic cells in various stages of degeneration were observed. Also of interest was the generalized increase in size and number of cytoplasmic vacuoles which were most apparent at the lower concentrations of the reagents. This would indicate an active transport of the reagent and was most noticeable with Thiomersal and Cetylpyridinium chloride.

Alteration of cytoplasmic protein structure probably has occurred and this certainly would alter cell metabolism and function. With all reagents there

*Pearse, A.G.E. Histochemistry, Theoretical and Applied. Boston: Little, Brown and Co., 1960, p. 850.

seemed to be some loss of RNA-Protein from the cytoplasm but with 0.01% Cetylpyridinium chloride the loss seemed to be complete.

Changes in the cell periphery also were indicative of cytotoxicity. Tween 60 caused little change but with Hyamine 1622 the periphery became distinct because of cytoplasmic condensation. Both Thiomersal and Cetylpyridinium chloride caused spindle-like extensions of the cytoplasm as well as cytoplasmic condensation. The extensions are a common reaction to insult and could be outgrowths of the cytoplasm or merely the result of portions of the cytoplasm adhering to the glass while the rest condensed.

Nuclear reactions were most interesting and were somewhat similar to those described in previous reports of toluidine blue staining of the nucleoproteins in cells affected by sodium lauryl sulfate and sodium-N-lauroyl sarcosinate (May 1 to December 1, 1960). The nucleoplasm had become hyalinized and the intensity of staining of the nucleoli decreased. Both reactions indicate serious and probably irreversible damage to the cells. All four of the reagents tested in the present study caused enlargement of the nucleus, the greatest increase being seen with Tween 60. Cetylpyridinium chloride 0.01% apparently was responsible for a loss of RNA-Protein in the nucleoli, nuclear granules and nuclear membrane. At the lower concentrations tested Thiomersal caused a slight reduction in the size of the nucleoli and was the only reagent to do so.

The small, blue nuclear granules which are distinct in normal HEp_2 cells were affected to some extent by all compounds. Their number was reduced by Thiomersal and Hyamine 1622 and they were completely removed by the higher concentrations of Tween 60. Cetylpyridinium chloride 0.01% caused a change in staining which indicated loss of RNA-Protein.

The nucleoplasm was affected to some degree by the four reagents. At concentrations of 0.01% and 0.001% all compounds removed a significant amount of DNA-Protein. At 0.0001%, however, Thiomersal was the only reagent to remove DNA-Protein from the nucleoplasm. This finding is consistent with those of other areas of this study in that Thiomersal is the most toxic of the four reagents and that the others become relatively nontoxic between 0.01% and 0.001%.

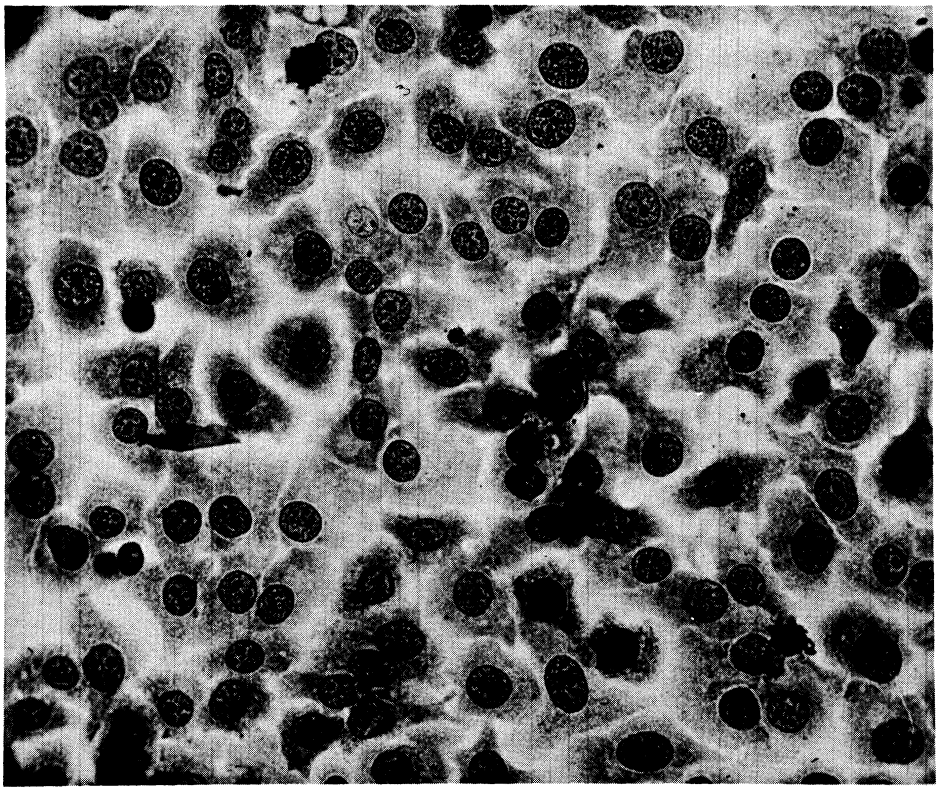
The nuclear membrane showed a reaction to all the reagents. It either became thickened or indistinct. Such a change was difficult to compare or evaluate.

2. Lipids

The results of the Sudan Black B stain were quite similar for 0.1%, 0.01%, and 0.001% of all four reagents. For that reason the findings of only 0.01% and 0.0001% will be illustrated and described. The results of this staining

procedure were somewhat disappointing in that the cells undergoing degeneration were heavily stained and cytologic features were masked. Sudan Black B is dissolved in mammalian lipids, including phospholipids and neutral fats. Phospholipids form a layer of all membranes in the cell. Uptake of the dye was heaviest in pyknotic cells and least in normal cells. Apparently the metabolism of the reagent-affected cells was so altered that the enzymes which normally hydrolyze the lipids were not available. Thus there was an accumulation of lipids in the cell accounting for the intense and generalized staining. There is some evidence that a complex was formed by the phospholipids and cellular proteins. This complex also stained black.

Cells affected by Tween 60 showed the least uptake of the dye. Hyamine 1622 again seemed to cause the most toxic reaction as noted by the heavy staining of the sparse, pyknotic cells. It was of interest to note that Thiomersal produced a specific reaction manifested by a concentration of lipid material around the nucleus.



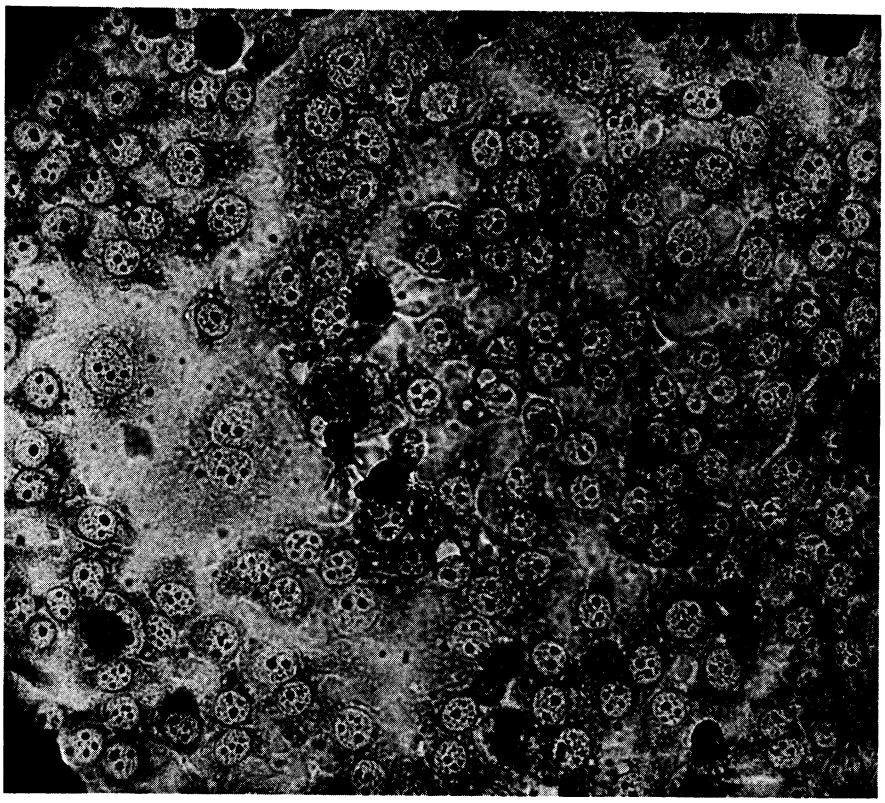
Control

May-Grünwald-Giemsa Mag. 830x

The normal characteristics found in HEp_2 cells are seen. The round nucleus is large and prominent. It is often in the center of the cell but may be near the periphery. The nucleoli are prominent and several in number. The shape of the nucleoli varies, either round with a smooth outline or round with numerous small projections from the periphery. The nucleoli stain blue which indicates the presence of RNA-Protein. Numerous diffuse blue granules having no regular distribution are present in the nucleus.

The nucleoplasm is very diffuse and stains red- purple indicating the presence of DNA-Protein. The nuclear membrane is distinct and blue indicating large amounts of RNA-Protein. An occasional granule of RNA-Protein may be seen in close proximity to the membrane.

The cytoplasm stains blue indicating RNA-Protein. It is diffuse with large granules and no vacuoles. The periphery of the cell often is not well defined due to cellular activity in these areas.

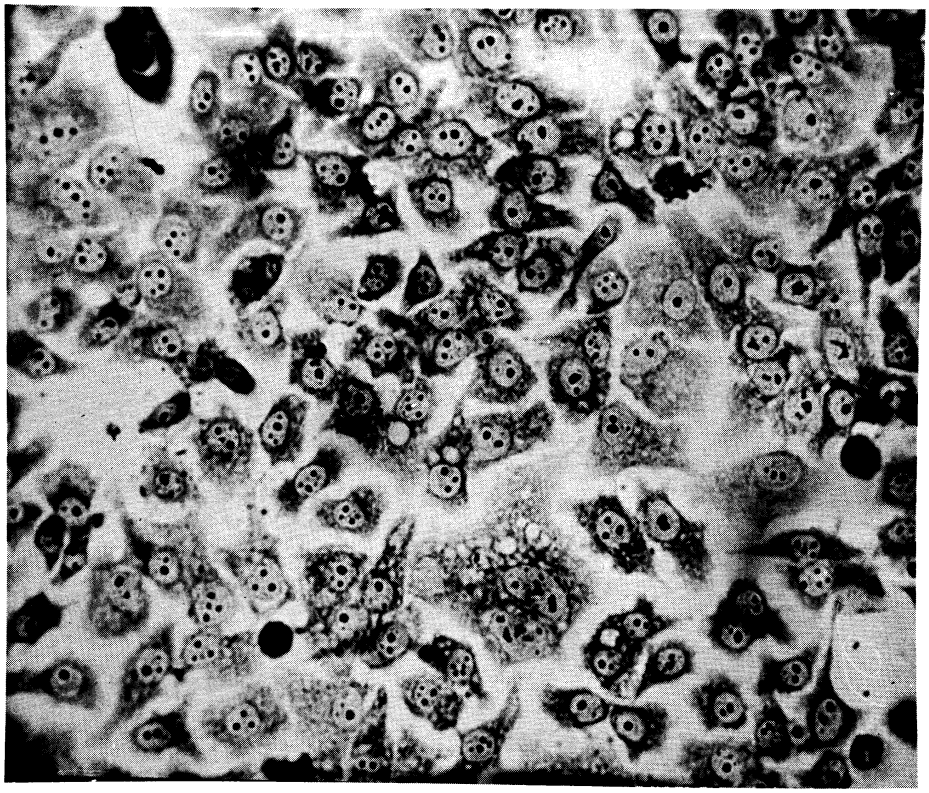


Thiomersal 0.01%

May-Grunwald-Giemsa Mag. 830x

The nucleus is quite large in relation to total cell size. Several nuclei are present which are larger than those found in a normal environment. Nucleoli have not been affected by the antiseptic. The nucleoplasm no longer stains red-purple, indicating a loss of DNA-Protein from the nucleus. An increase in thickness of the nuclear membrane is apparent in a few cells.

The blue color of the cytoplasm has been replaced by a red-purple color. This change may be due to migration of DNA-Protein from the nucleus to the cytoplasm. Many vacuoles are present in the cytoplasm. The outline of some cells cannot be established, and in some cells the periphery is refractive, indicating that it has become thickened.



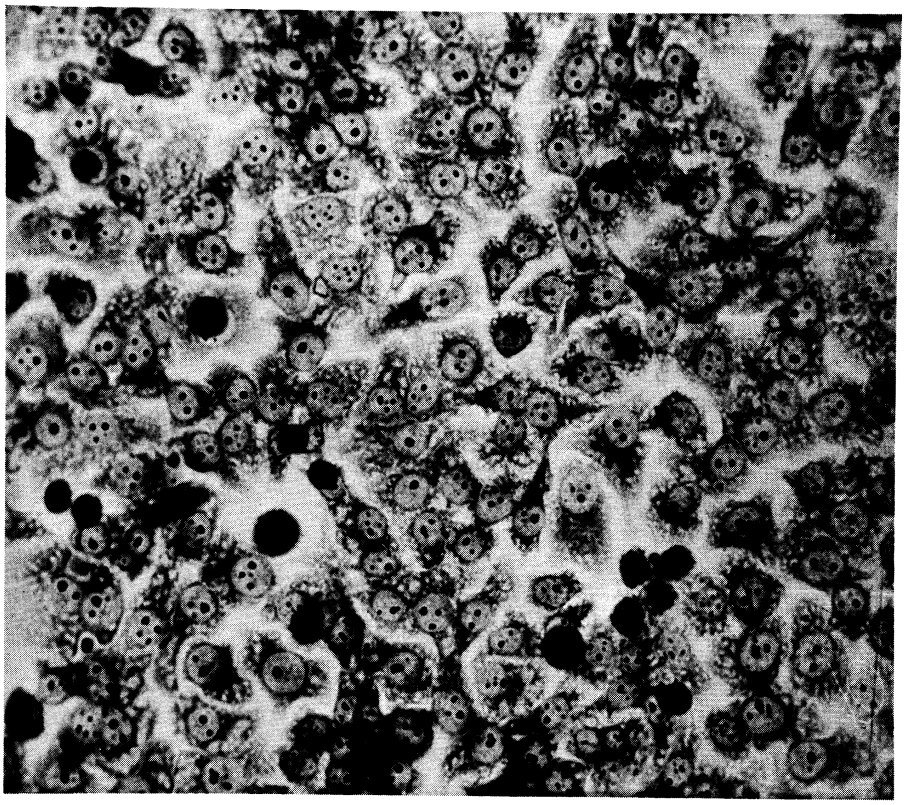
Thiomersal 0.001%

May-Grünwald-Giemsa

Mag. 830x

The DNA-Protein (red-purple) of the nucleoplasm is absent. There also has been a loss of the RNA-Protein granules of the nucleus, which indicates a loss of both RNA-Protein and DNA-Protein from the nucleus. Little cytological effect is seen in the nucleus proper. The nuclear wall has become less distinct when compared to that of the normal cell.

The cytoplasm has retained its blue color indicating that RNA-Proteins are present. Vacuolation of the cytoplasm has occurred, accompanied by a loss of cytoplasm in some cells.



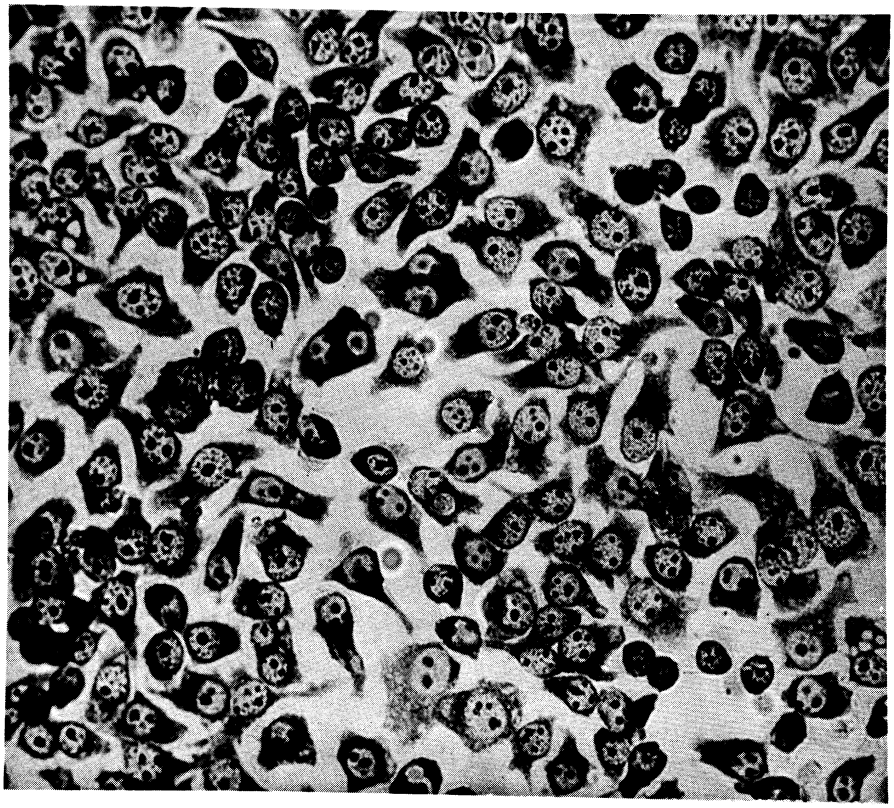
Thiomersal 0.0001%

May-Grünwald-Giemsa

Mag. 830x

The size of the nucleus is similar to the normal nucleus. The size of the nucleoli, however, has diminished. The red-purple color of the nucleoplasm has been lost as have most of the blue RNA-Protein granules of the nucleus. The nuclear membrane has lost much of its distinct characteristics and the membrane is not so intensely stained.

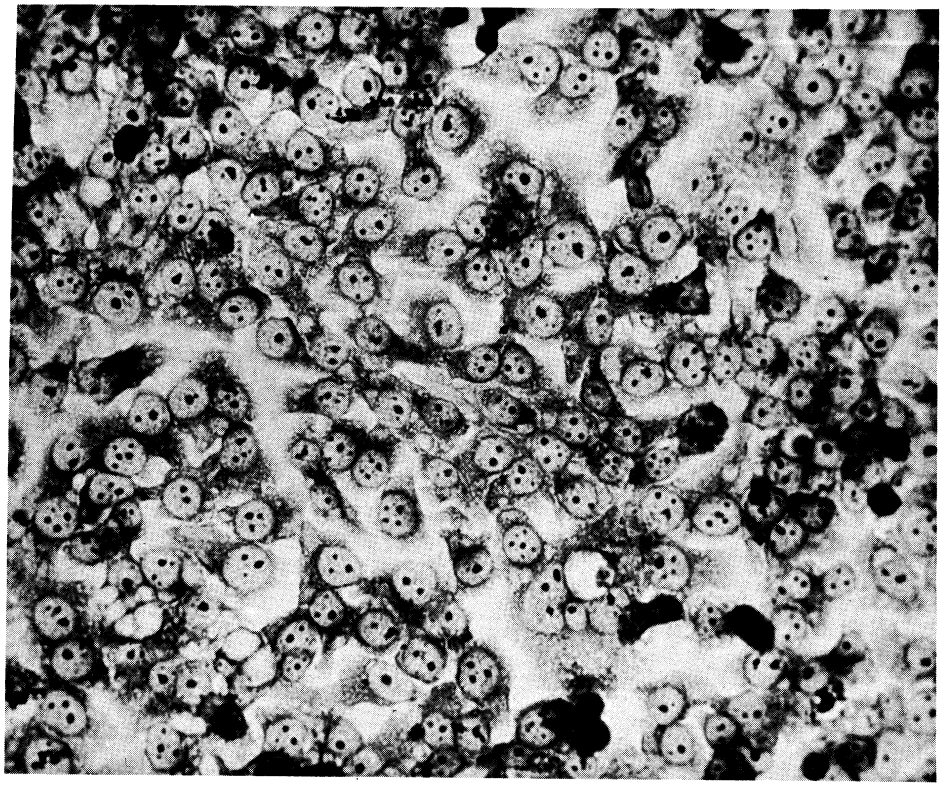
A large reduction of cytoplasm has taken place and several large vacuoles are present. The cell periphery is quite distinct with many spindle-like extensions, as contrasted to the polygonal shape of normal epithelial cells.



Cetylpyridinium Chloride 0.01%
May-Grünwald-Giemsa Mag. 830x

The nucleoli which normally are blue, indicating the presence of RNA-Protein, have become red-purple indicating DNA-Protein. This change in staining properties of the nucleoli may be due to a loss of RNA-Protein and the possible unmasking the DNA-Protein normally present. In addition to the nucleoli reversing color, the diffuse granules of the nucleus and the nuclear membrane have also changed from blue to red-purple. The nuclear membrane is more granular than normal.

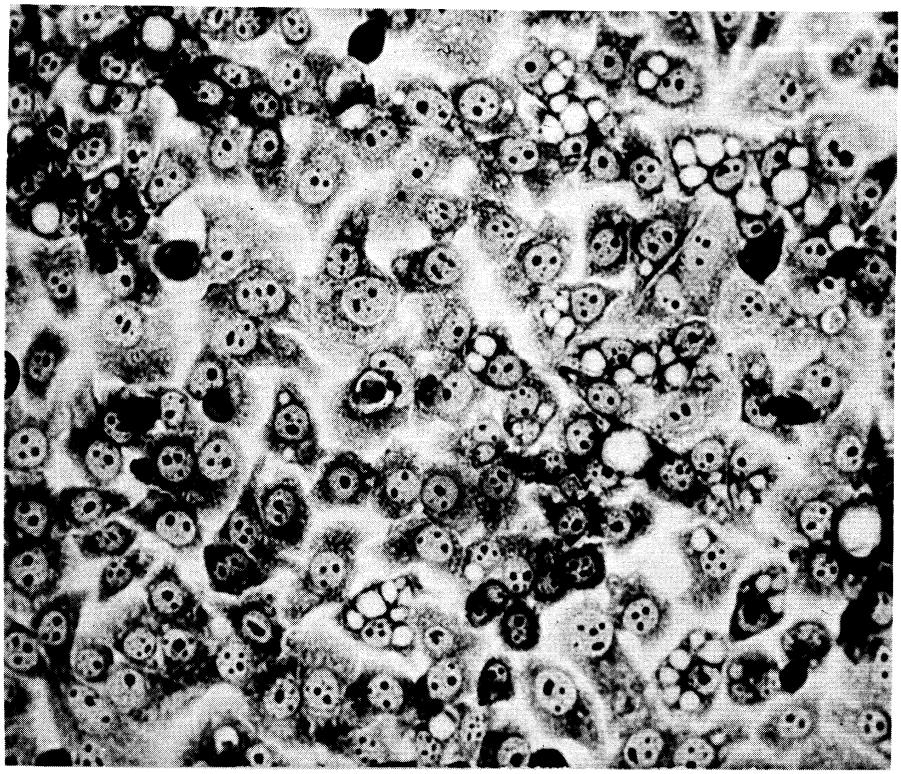
The cytoplasm has become condensed and there are no vacuoles. The cell outline is distinct with spindle-like projections.



Cetylpyridinium Chloride 0.001%
May-Grünwald-Giemsa Mag. 830x

An increase in the size of the nucleus, as compared with the normal cell, is apparent. The nucleoli have remained similar in size and color. Most of the blue RNA-Protein granules usually found in the nucleus are absent. The red-purple DNA-Protein of the nucleoplasm is also absent.

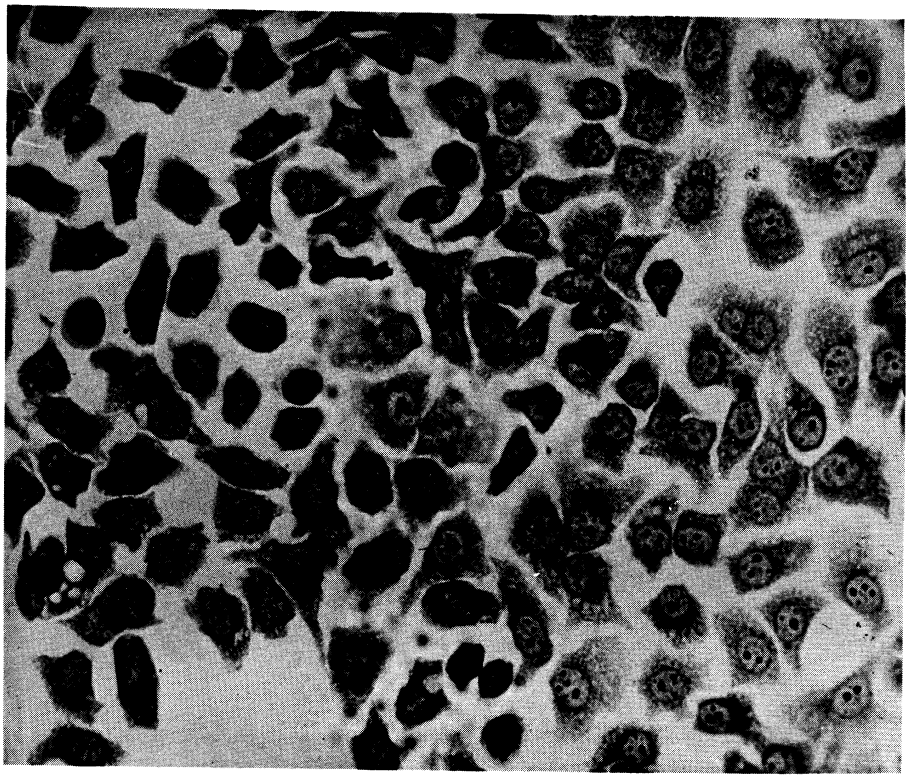
The cytoplasm contains numerous small vacuoles, and is no longer diffuse. The cell periphery is well defined because of a condensation or loss of cytoplasm. Many of the cells have spindle-like processes, the result of cell insult.



Cetylpyridinium Chloride 0.0001%
May-Grünwald-Giemsa Mag. 830x

The nucleus has many normal characteristics at this low concentration. The nucleoplasm is red-purple denoting the presence of DNA-Protein which is normal. The number of blue-staining RNA-Protein granules present in the nucleus is, however, less than in a normal cell.

A few large vacuoles and many smaller ones are present in the cytoplasm. As in the normal cells the periphery of many cells cannot definitely be established.



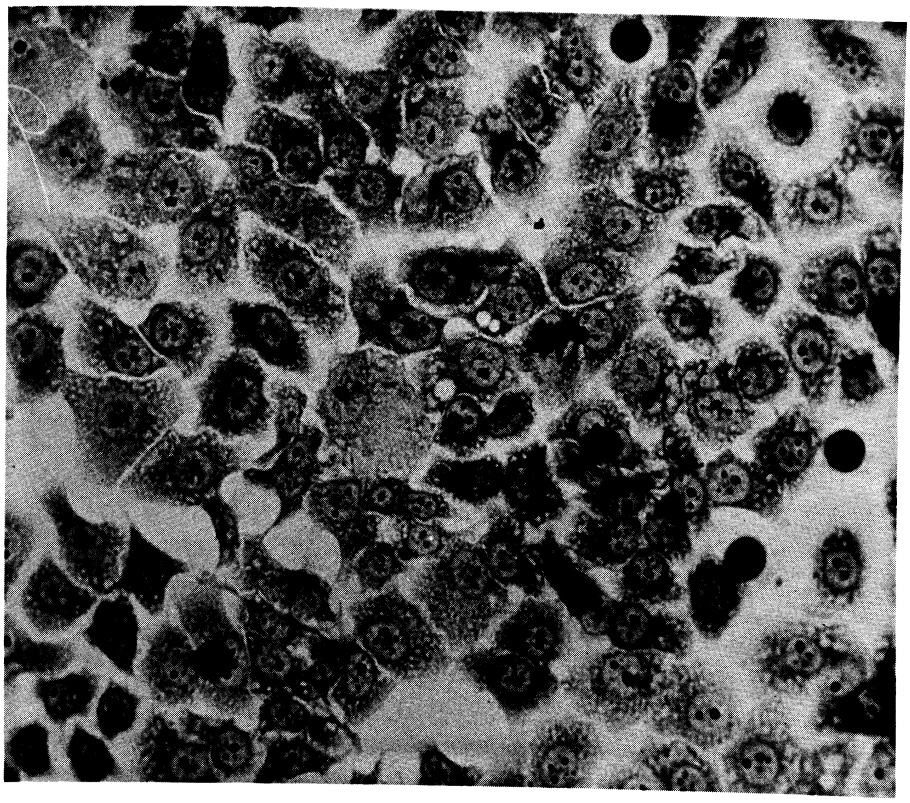
Hyamine 1622 0.01%

May-Grünwald-Giemsa

Mag. 830x

The nucleus and nucleoli appear cytologically normal. The diffuse blue-stained granules of the nucleus are present. The red-purple stain of the nucleoplasm is absent indicating that DNA-Protein is lost from the nucleus. A thickening of the nuclear membrane has occurred in some cells.

The cytoplasm is condensed as compared with normal HEp_2 cells, and contains a large number of small vacuoles. Due to the condensation of the cytoplasm the cell periphery is more clearly defined.



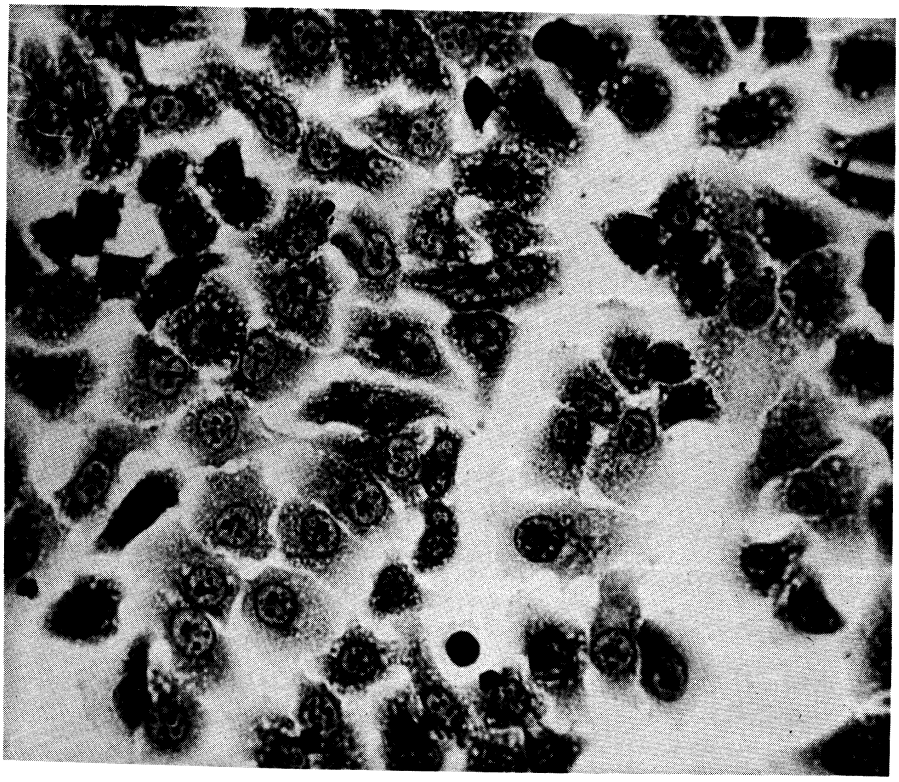
Hyamine 1622 0.001%

May-Grünwald-Giemsa

Mag. 830x

The nuclei of many cells appear larger than those normally found but the nucleoli are normal in size and number. Blue-stained granules (RNA-Protein) are reduced in number. The nucleoplasm stains a red-purple color indicating the presence of a small amount of DNA-Protein in the nucleus.

Except for the large number of vacuoles present in the cytoplasm, it appears fairly normal.



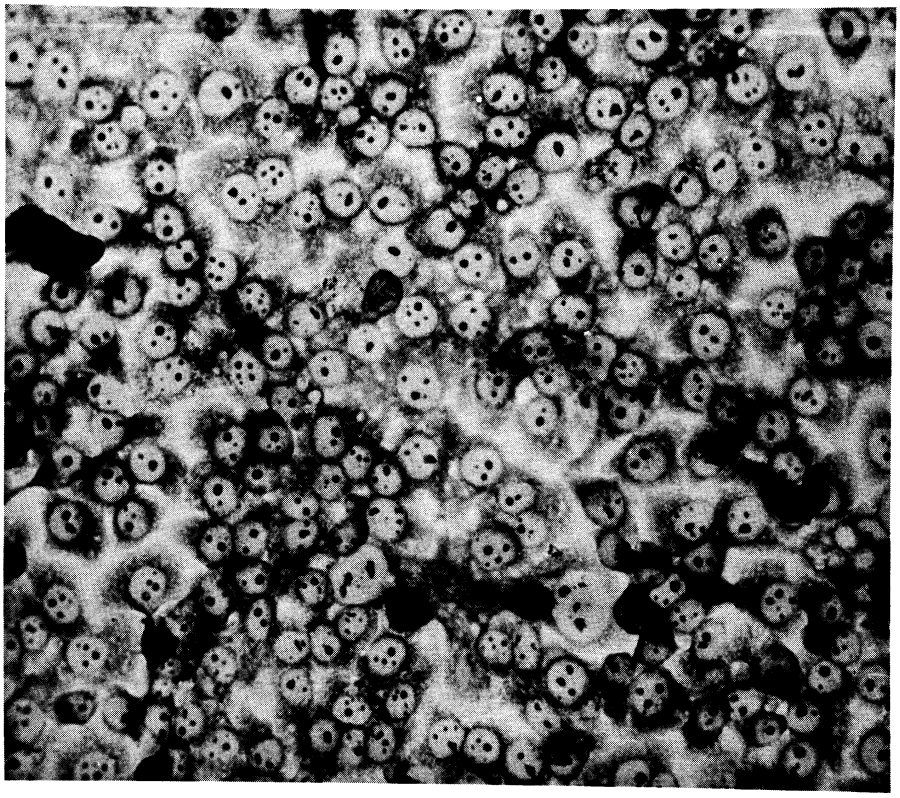
Hyamine 1622 0.0001%

May-Grünwald-Giemsa

Mag. 830x

The nucleus is larger than that found in a normal epithelial cell. The nucleoli and nuclear granules appear normal and the nucleoplasm stains a definite red-purple indicating the normal presence of DNA-Protein. The nuclear membrane is less distinct than in the normal cell.

The cytoplasm contains many vacuoles but otherwise it is normal.



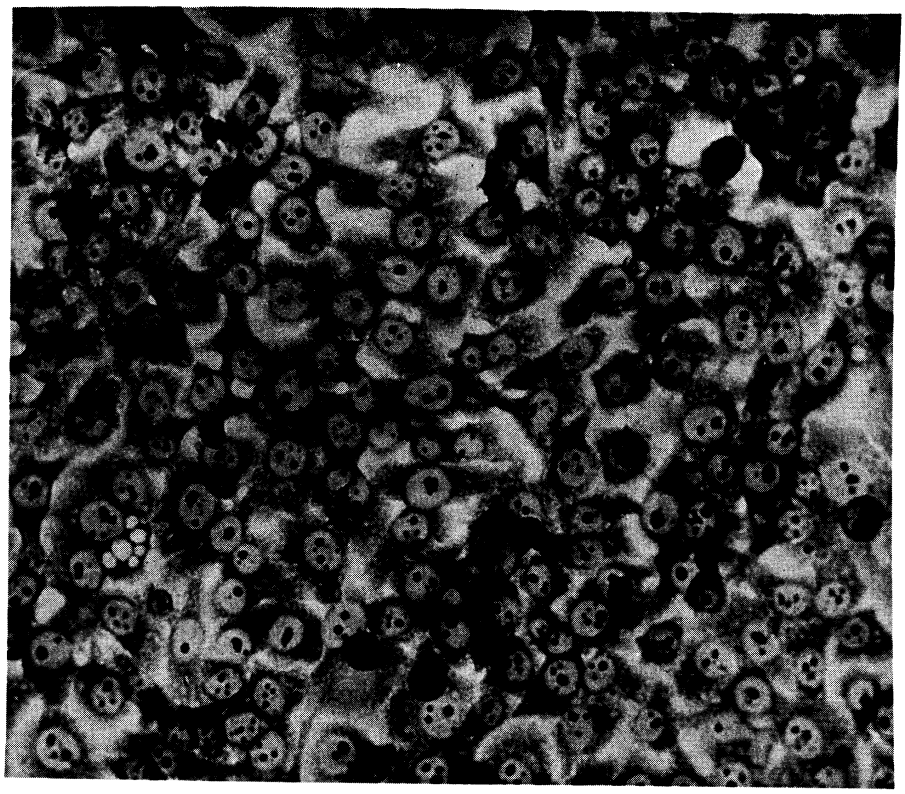
Tween 60 0.01%

May-Grünwald-Giemsa

Mag. 830x

The nuclei of several cells are much larger than normal and the majority of nuclei show some increase in size. The nucleoli remain normal in size and stain blue as in the normal cell. The small, blue (RNA-Protein) granules of the normal nucleus are absent and the nucleoplasm which normally is red-purple now is a light blue, indicating a loss of DNA-Protein. The nuclear membrane is interrupted in many cells.

A reduction in the amount of cytoplasm has occurred with the inclusion of a few vacuoles. The periphery of the cell remains normal.



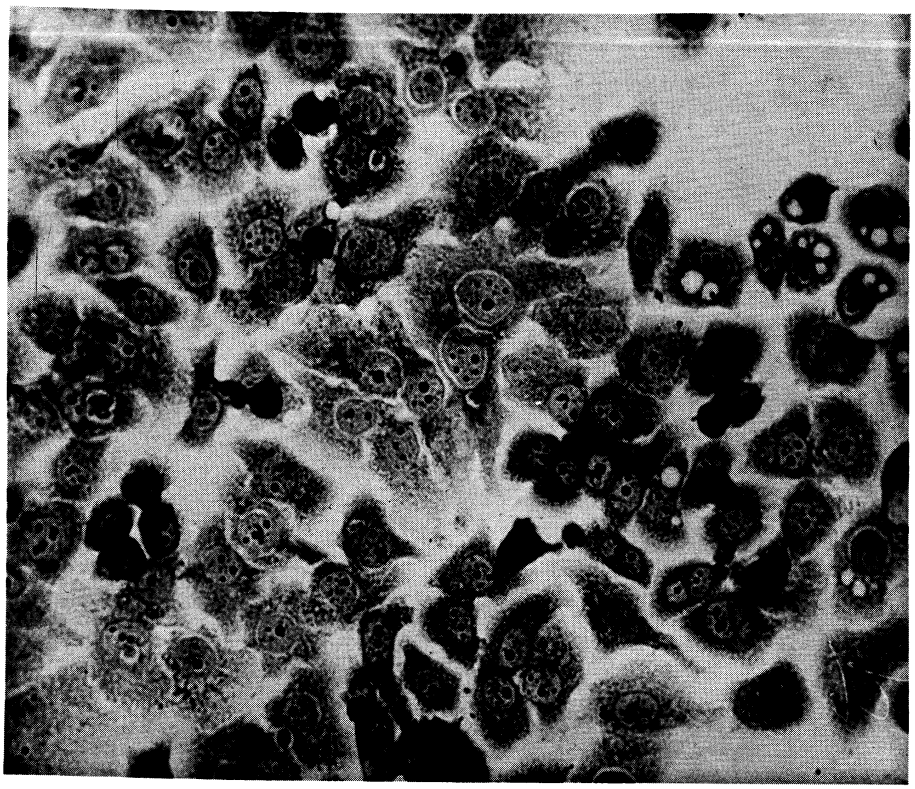
Tween 60 0.001%

May-Grünwald-Giemsa

Mag. 830x

The nuclei appear slightly larger than those of the normal cell. The nucleoli appear normal. As seen with the higher concentration of Tween 60 the small, blue (RNA-Protein) granules of the nucleus are absent and the red-purple color of the nucleoplasm is lost. The nuclear membrane has lost its distinct blue color, and often is difficult to distinguish from the cytoplasm.

A reduction in the amount of cytoplasm has occurred with the inclusion of a few small vacuoles.

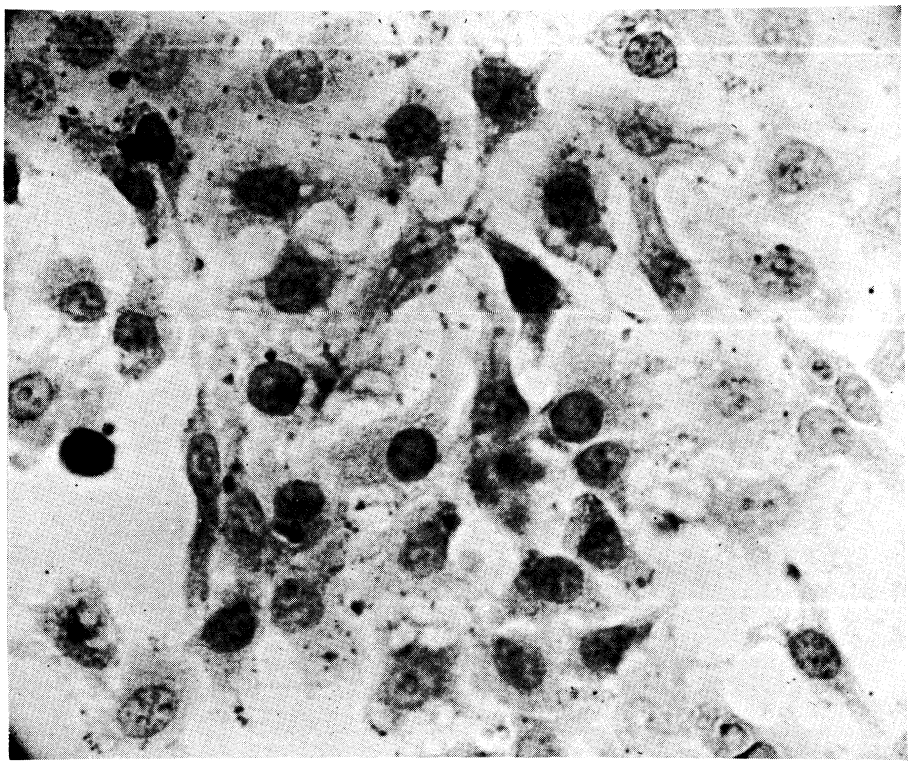


Tween 60 0.0001%

May-Grünwald-Giemsa Mag. 830x

Many cells show an enlarged nucleus. The nucleoli appear normal in size and color (blue). There are fewer diffuse, blue-staining granules in nucleoplasm than are found in a normal cell. The nucleoplasm, as is normal, stains red-purple denoting the presence of DNA-Protein.

The cytoplasmic portion of the cell appears similar to that of the normal cell except for a few large, scattered vacuoles. Included in some of the large vacuoles is a red-purple (DNA-Protein) body. This was observed only with this compound and this concentration.

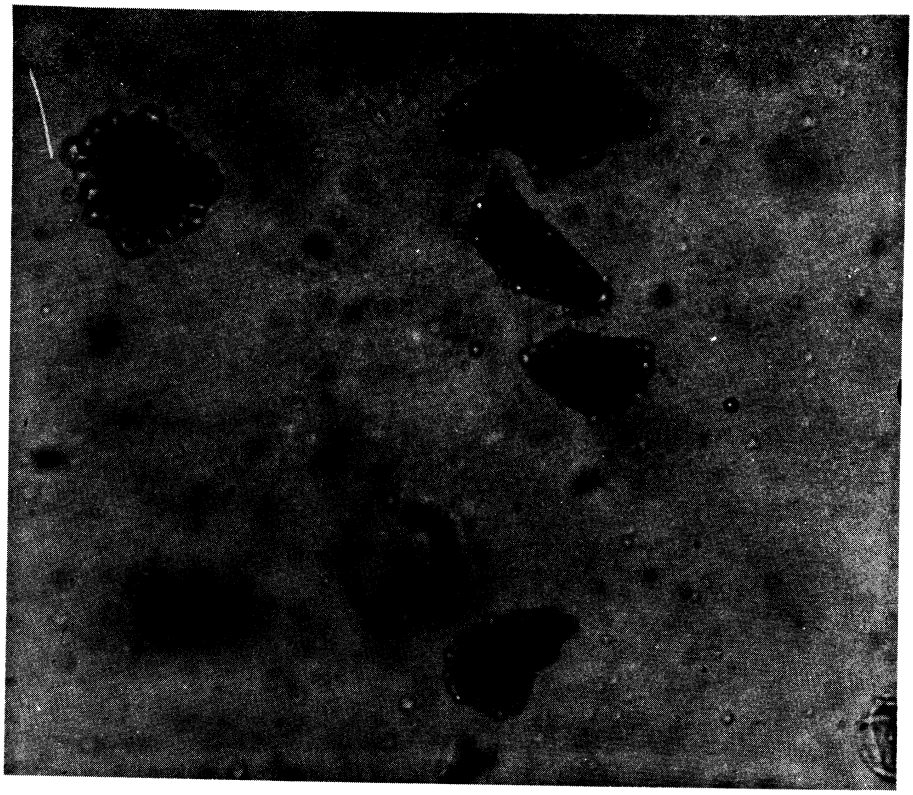


Control

Sudan Black B Mag. 680x

A prominent, round nucleus with a well defined membrane is seen. The large nucleoli which normally are present do not appear distinctly with this stain. The nucleoplasm is a homogenous grey. Many indistinct lipid granules are present.

Numerous dark-staining granules are present in the cytoplasm. These granules are fairly well distributed throughout the cell although, with a slight affinity for the periphery parts of the cell. A few large, isolated vacuoles are present. The cytoplasm appears rather homogenously stained with the carmine counterstain. The cell boundary is not always distinct.



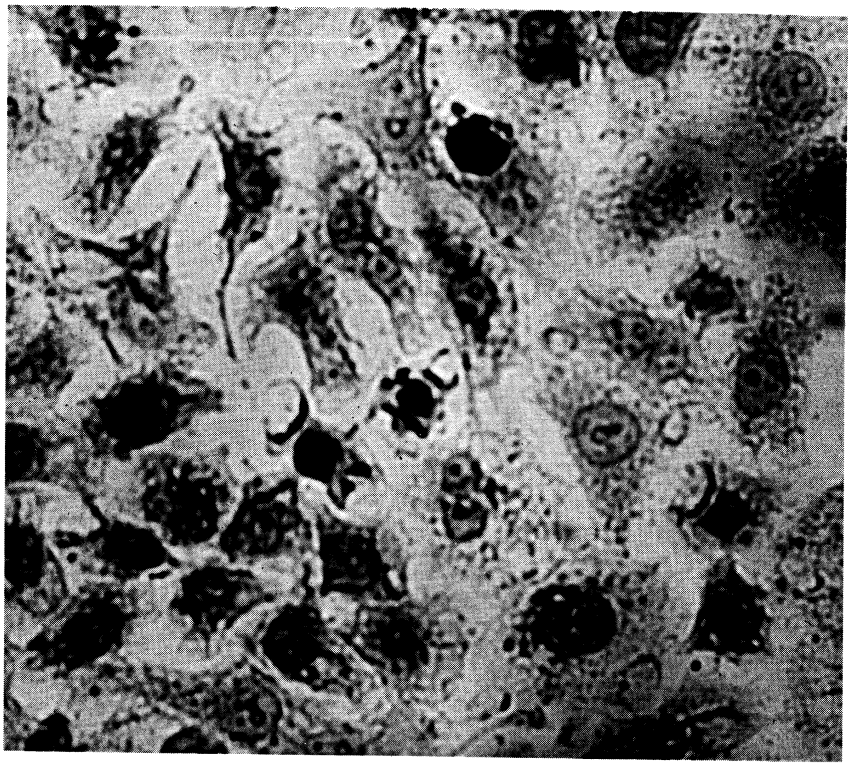
Thiomersal 0.01%

Sudan Black B

Mag. 680x

Thiomersal at this high concentration has little effect upon the nuclear size but there is a concentration of granules around the nuclear membrane. The nuclear granules are indistinct and the nucleoli are absent.

The cytoplasm contains many lipid granules located at the center of the cell and near the nucleus. Unstained vacuoles are seen at the periphery of each cell. The cellular borders are well defined due to cytoplasmic condensation.

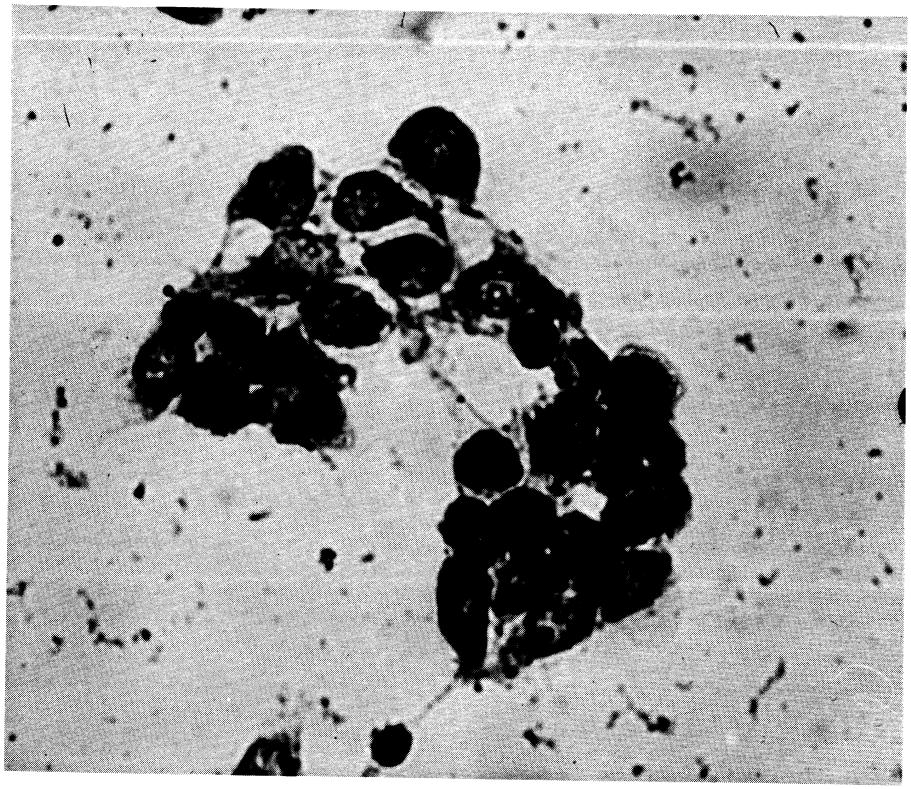


Thiomersal 0.0001%

Sudan Black B

Mag. 680x

The cytoplasmic and nuclear concentration of lipids does not appear to vary significantly from the control. The few cells containing large amounts of lipids apparently are undergoing degenerative changes. In most cells small granules of lipid are scattered throughout the cytoplasm. The cytoplasm is diffusely vacuolated.

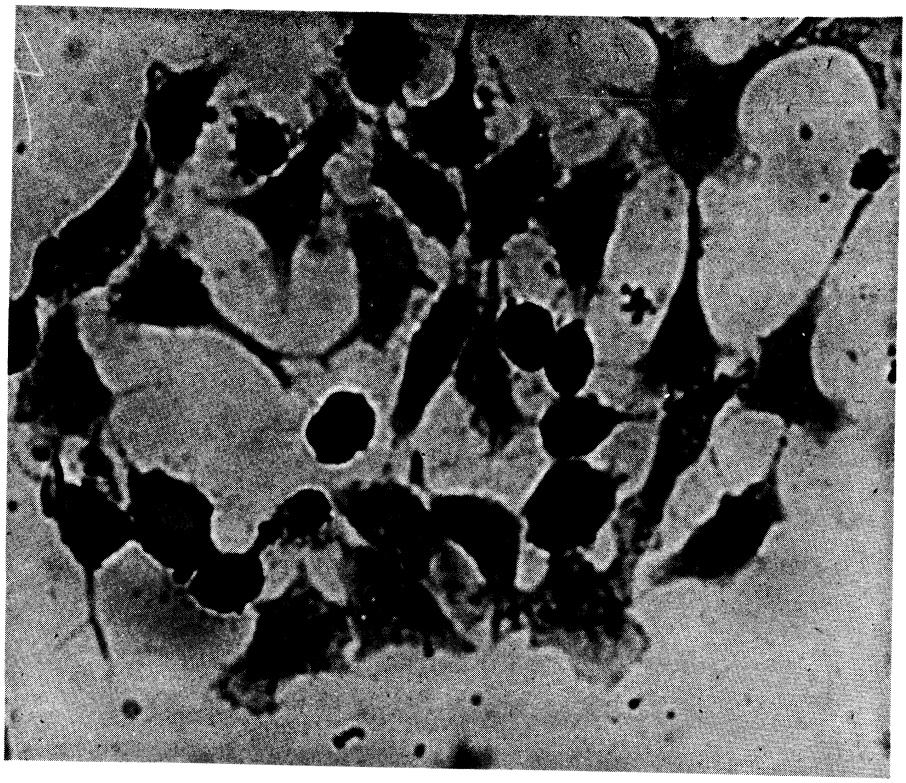


Cetylpyridinium Chloride 0.01%

Sudan Black B

Mag. 680x

The size of the nucleus has been reduced in the degenerating cells. Some vacuoles can be seen in the nuclei which indicates extensive degeneration. A heavy concentration of lipid is apparent in the reduced cytoplasm. There is a general opacity of cells as well as a clumping arrangement. A few thin, cytoplasmic extensions denote a toxic reaction.

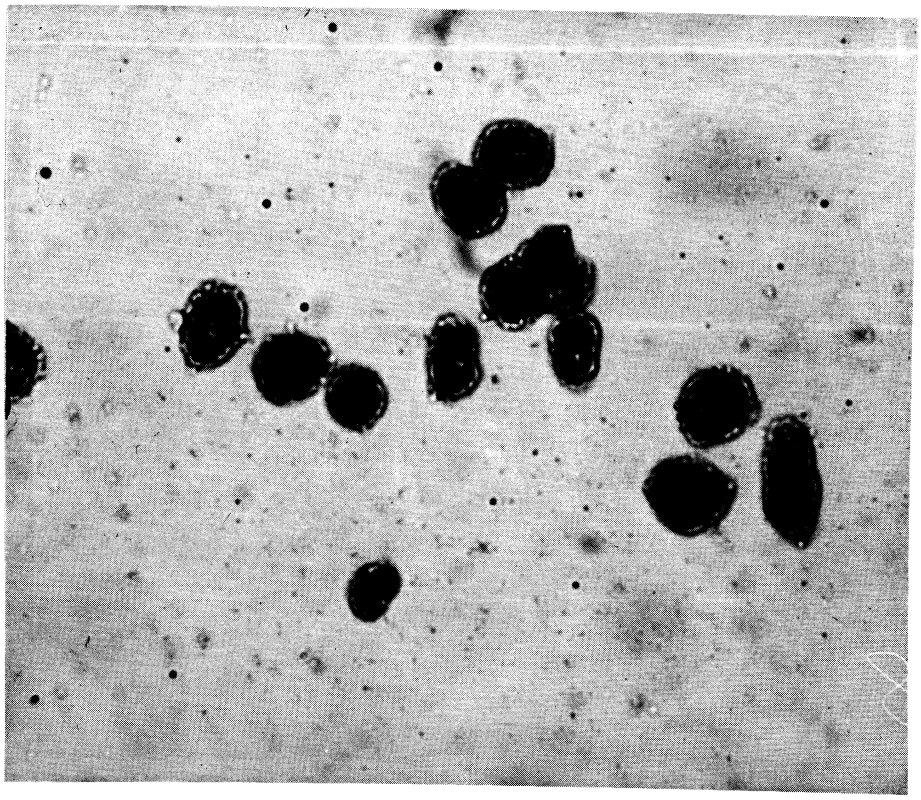


Cetylpyridinium Chloride 0.0001%

Sudan Black B

Mag. 680x

The nuclei are indistinct with interruption of the nuclear membrane. Granulation or vacuolation of the nuclear area has not occurred. Three common reactions to cell injury are present: numerous cytoplasmic villae on the outer cell membrane, long spindle-like cytoplasmic extensions and pyknosis. Each stage of cellular degeneration shows a corresponding increase in lipid concentration, i.e., the more normal cells show the least amount of lipid, and the pyknotic cells show the largest amount.

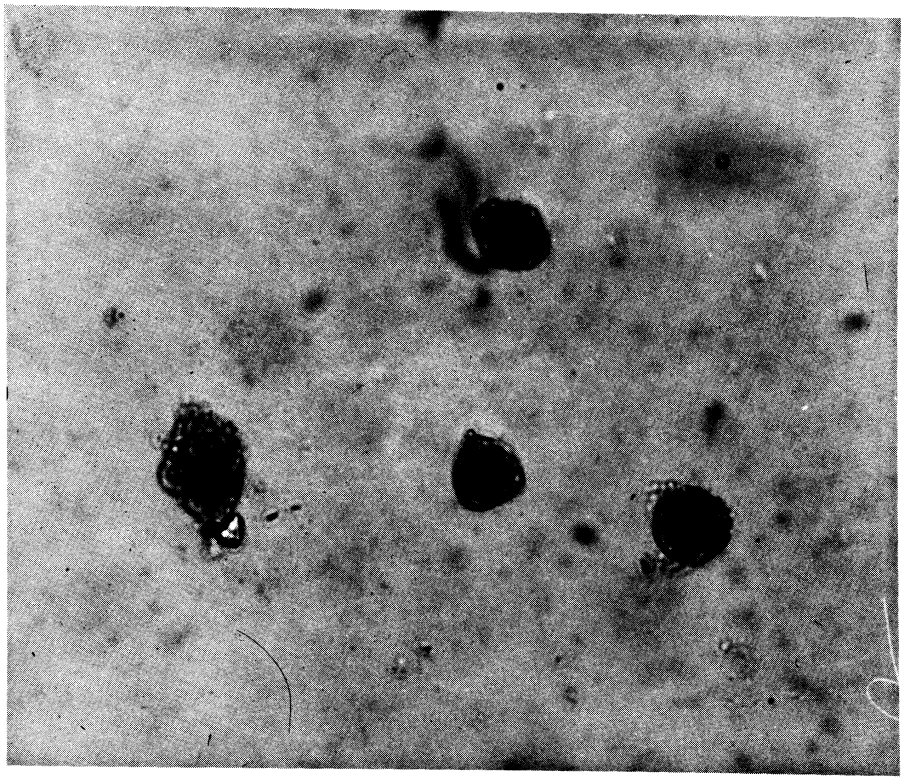


Hyamine 1622 0.01%

Sudan Black B

Mag. 680x

The cells are extremely pyknotic with a large amount of lipid material present throughout the cytoplasm and nuclei. The cell number has been reduced because of loss of attachment to the coverslip. The cells are round and appear pyknotic. Vacuolation is apparent in all cells.

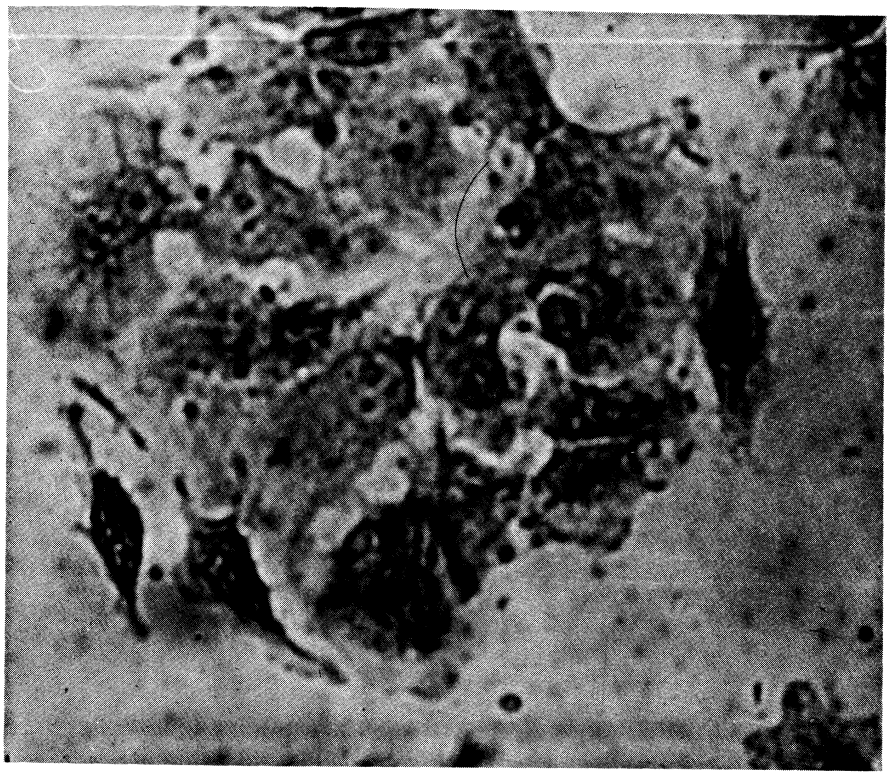


Hyamine 1622 0.0001%

Sudan Black B

Mag. 680x

The effects on the cells of the lower concentration of Hyamine 1622 are similar to those of the higher concentration. The cells are round and pyknotic and contain large amounts of lipids. Vacuoles are present in all cells.



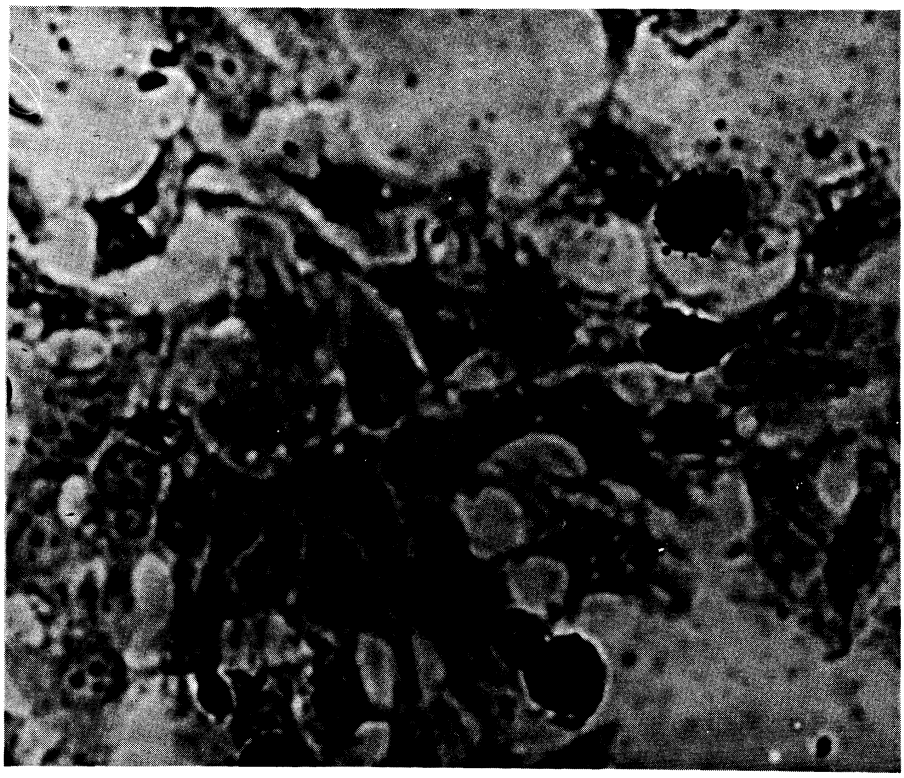
Tween 60 0.01%

Sudan Black B

Mag. 680x

The nucleus is intact but poorly differentiated from the cytoplasm. The nucleoli are apparent and the nucleoplasm is homogenous.

The cytoplasm is homogenous with lipid granules at the periphery of the cell. The cells appear to be least affected by Tween 60 than by any of the other reagents at this concentration.



Tween 60 0.0001%

Sudan Black B

Mag. 680x

The nucleus is more distinct than at the higher concentration of Tween 60. The amount of lipids in the cells is similar to that seen with the higher concentration but cytologically the cells appear more normal. A few degenerating cells containing large amounts of lipid are seen.

IV. TOXICITY STUDIES

Phase 3. Electron Microscopy

A. PURPOSE

The purpose of the present study was to continue observations on the early effects of detergents on the HEp_2 cell. The high resolution of the electron microscope makes it possible to detect and characterize the earliest structural modifications which cannot be ascertained through optical microscopy. The report describes ultrastructural cytoplasmic changes produced minutes after introduction of 0.0075% sodium lauryl sulfate into the cell suspension.

B. PROCEDURE

HEp_2 cells, grown in suspension in a modified Eagle's medium, were harvested by slow centrifugation and then resuspended in 0.0075% sodium lauryl sulfate for two minutes. The cells were centrifuged again and fixed in acetate-buffered 2% osmic acid at pH 7.5. Following dehydration in ascending percentages of ethanol, the cells were embedded in a mixture of epoxy resin, cured at 60°C, sectioned on a Porter-Blum microtome, and observed in a Hitachi HU-11 electron microscope.

C. RESULTS

All membranes of cells exposed to sodium lauryl sulfate appeared to be affected to varying degrees. Instead of the fingerlike projections seen in the normal cells and described in the previous report, the surface plasma membrane of the detergent-affected cells showed numerous blunt projections which appeared as cytoplasmic blebs ready to separate from the cell by pinching off. Other portions of the cytoplasm were segregated by the endoplasmic reticulum and contained numerous ribosomes (Figure 1).

The Golgi apparatus appeared to have shrunken to a much smaller size than in normal cells and was composed only of numerous small vesicles and short bilaminar membranes (Figure 2). The dense globular bodies containing minute vesicles, which seem to be associated with the Golgi apparatus in normal cells, were isolated in various parts of the cytoplasm (Figure 3).

The most prominent changes observed were in the mitochondria where there were localized swelling of the limiting membranes and displacement of cristae

mitochondriales. At the same time the matrix became somewhat less dense than normal (Figures 4, 5, and 6). Along the displaced and often broken cristae were small particles which were similar in diameter to ribosomes (Figure 5). With further swelling large vacuoles appeared which could be identified as mitochondria only by the double membrane rather than as normal vacuoles which have a single membrane. Cristae mitochondriales have disappeared (Figure 7). The diameter of those "mitochondrial vacuoles" reached 2μ or larger. Since formation of vacuoles within the cytoplasm frequently was observed in time-lapse cinematographic studies, it might be reasonable to assume that degenerating mitochondria were producing some of these vacuoles. Another structural alteration often seen with the electron microscope was disruption or total disappearance of the nuclear membrane.

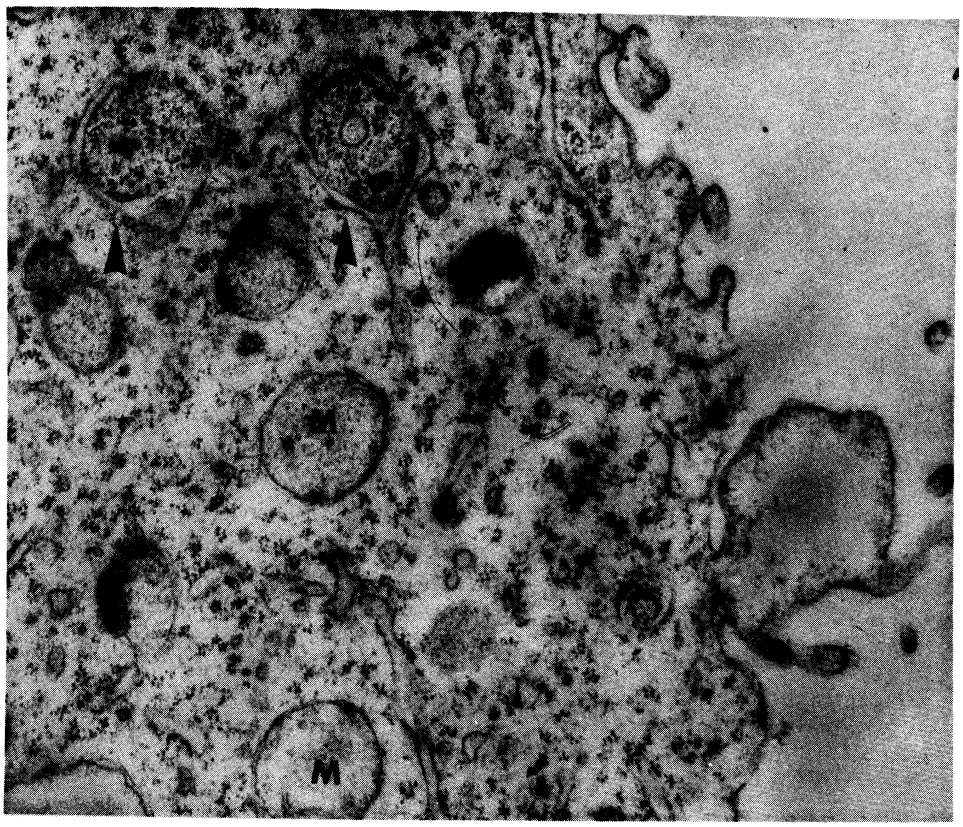


Figure 1

A peripheral portion of the cytoplasm of a HEp_2 cell subjected to 0.0075% sodium lauryl sulfate for 2 minutes prior to fixation. The formation of cytoplasmic bleb is seen on the right. Notice the segregation of portions of the cytoplasm by the endoplasmic reticulum (arrows). The segregated cytoplasm contains an increased number of ribosomes. Mitochondria (M) have lost most of their internal cristae.

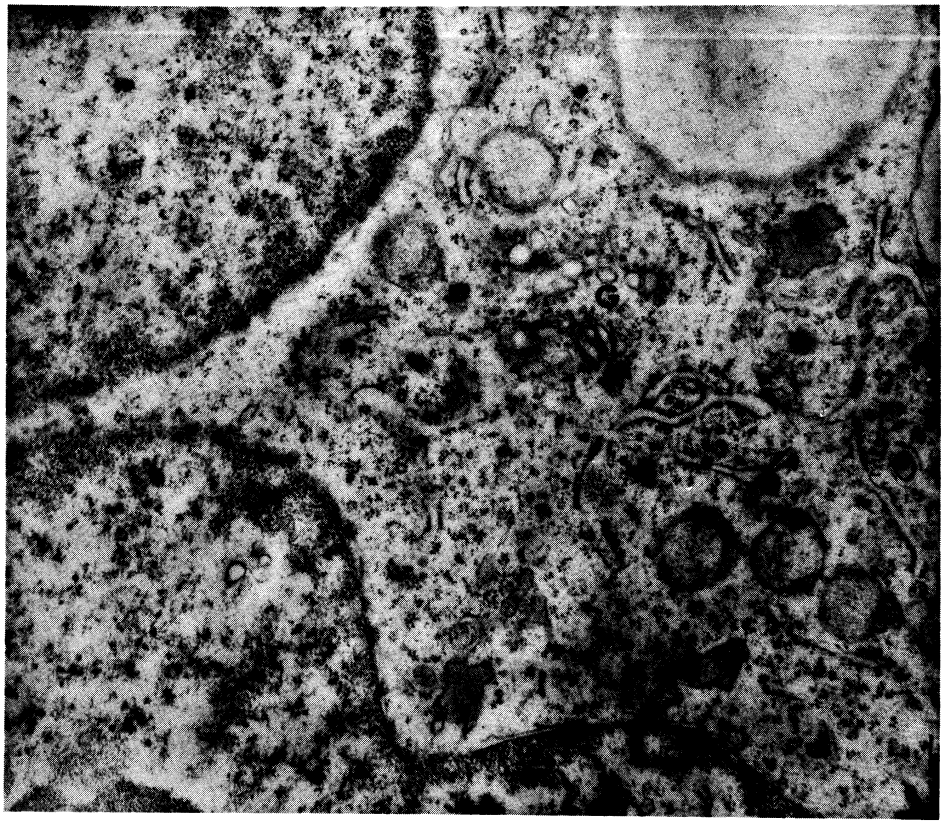


Figure 2

A portion of the cytoplasm of a HEp_2 cell subjected to 0.0075% sodium lauryl sulfate for 2 minutes prior to fixation. The Golgi apparatus (G) is smaller than in normal cells. Laminated membranes are very short and only small vacuoles and vesicles are present.

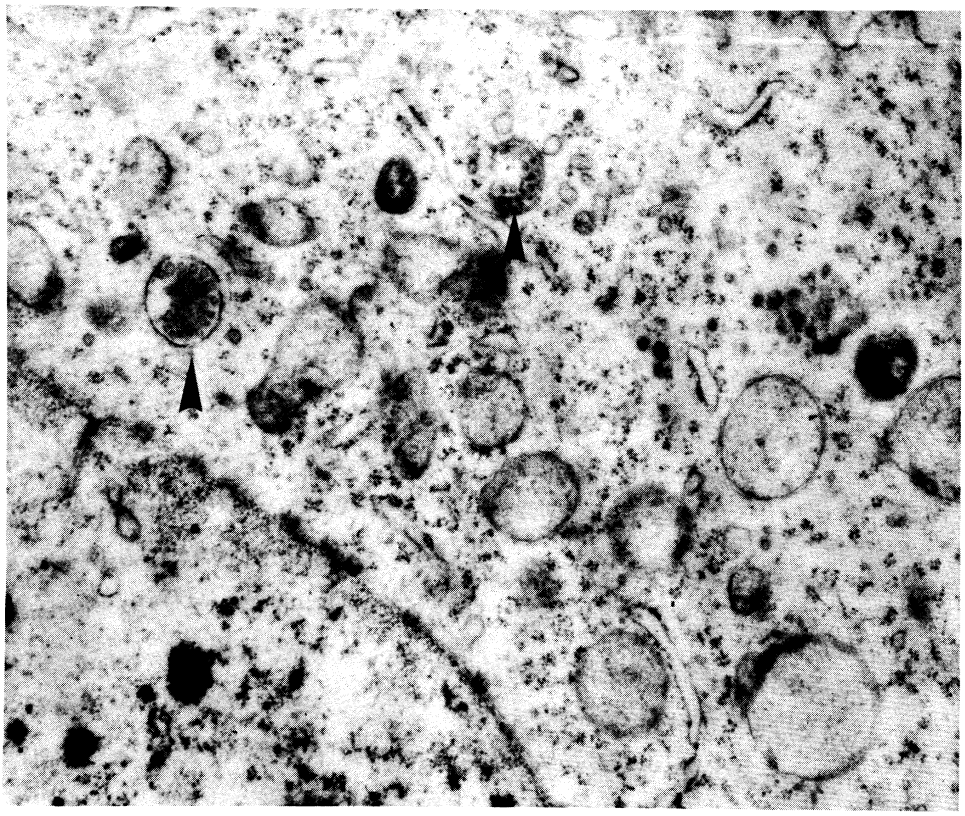


Figure 3

A portion of the cytoplasm of a HEp_2 cell subjected to 0.0075% sodium lauryl sulfate for 2 minutes prior to fixation. Note the round dense bodies with small vesicles in them (arrows). In normal cells these bodies were associated with the Golgi apparatus.

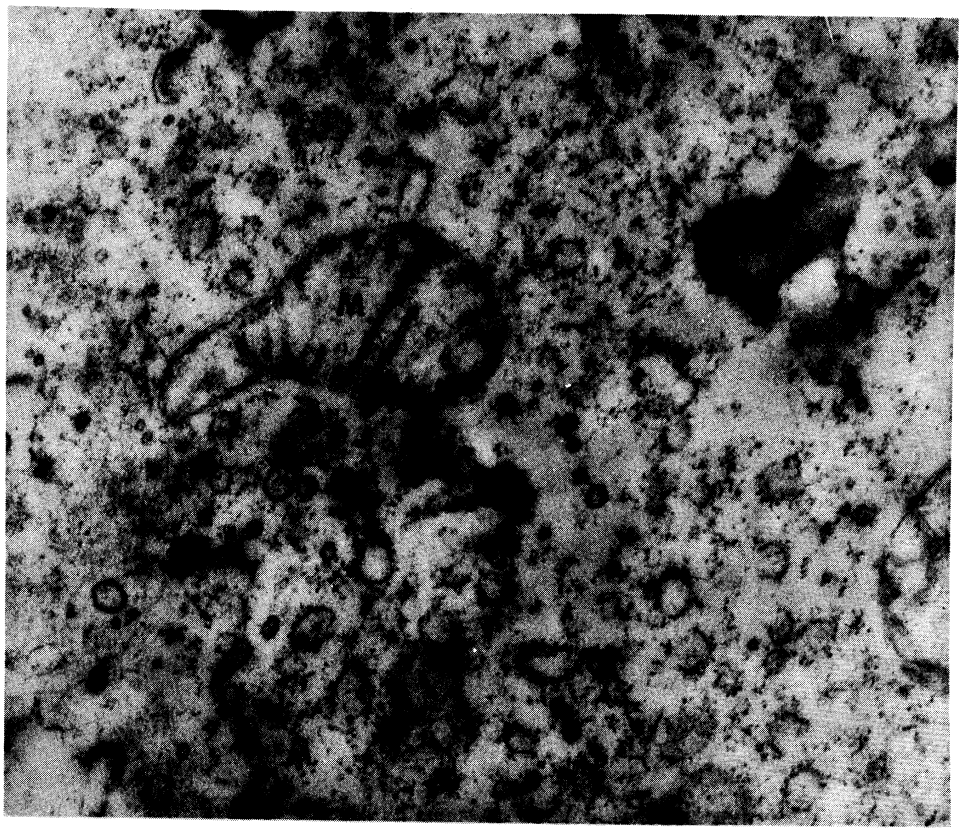


Figure 4

A portion of the cytoplasm of a HEp_2 cell subjected to 0.0075% sodium lauryl sulfate for 2 minutes prior to fixation. The initial swelling of 2 mitochondrion (M) is shown. Only a few cristae mitochondriales are visible.

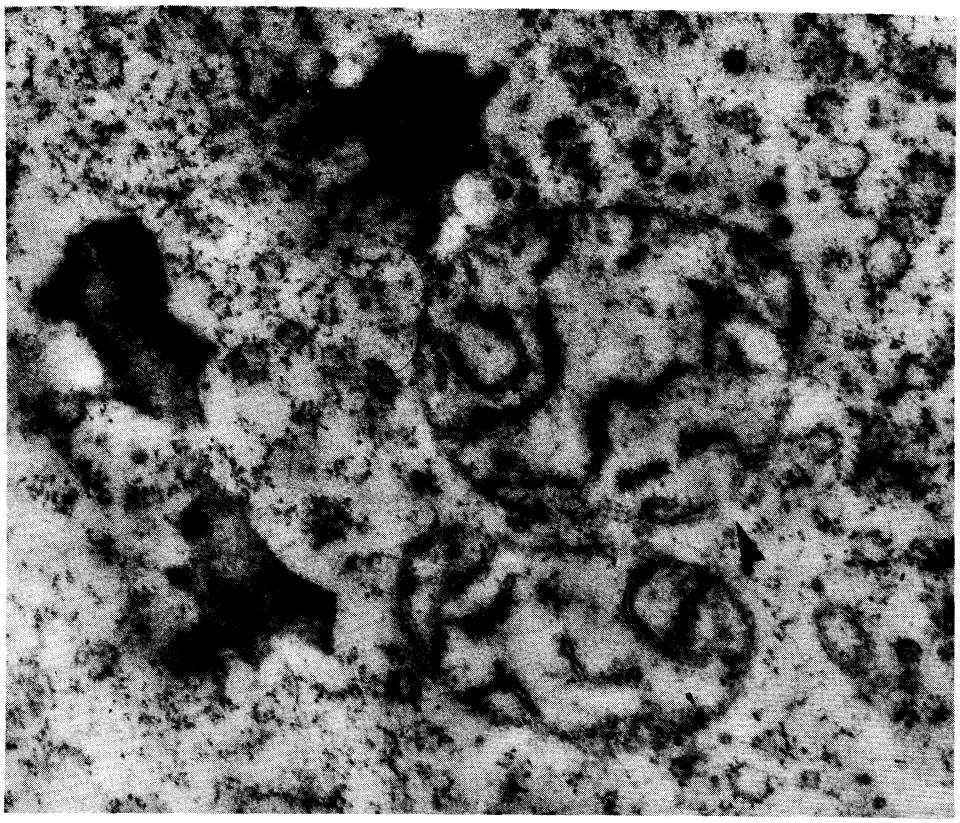


Figure 5

Two mitochondria of a HEp_2 cell subjected to 0.0075% sodium lauryl sulfate for 2 minutes prior to fixation. Cristae mitochondriales appear to be broken and displaced and have small granular materials along their surface. There is also a break in the limiting membrane (arrow).

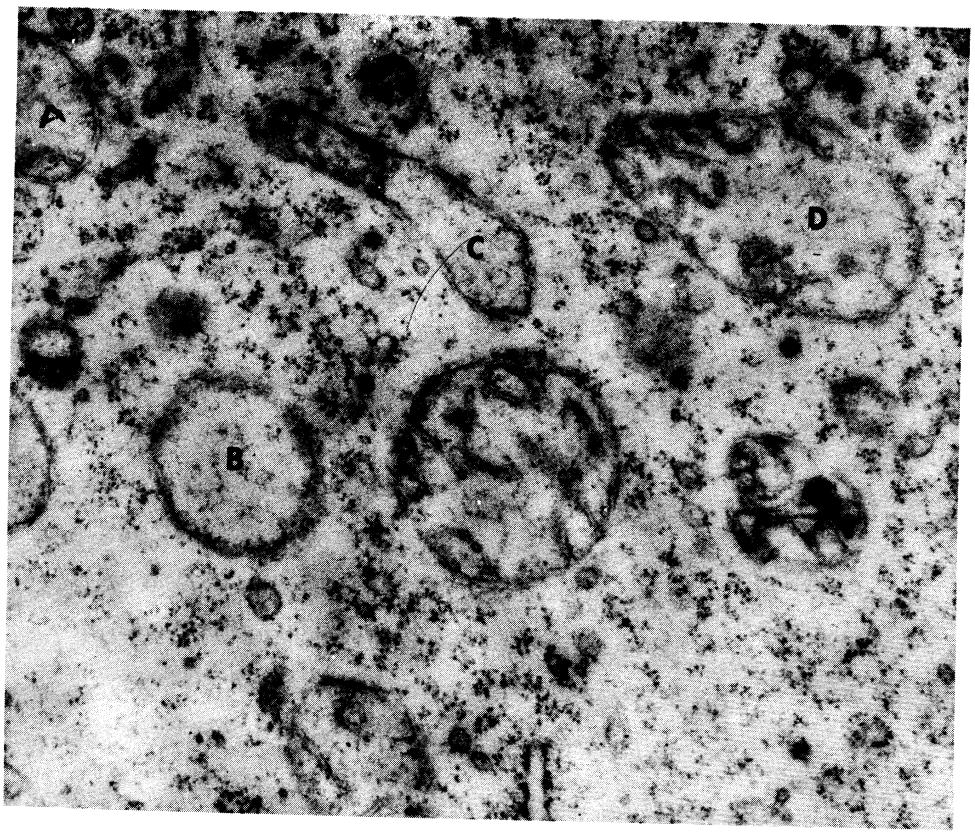


Figure 6

Mitochondria of a HEp_2 cell subjected to 0.0075% sodium lauryl sulfate for 2 minutes prior to fixation. Variations in the structural damage to mitochondria are shown. A and B might be profiles of partially swollen mitochondria sectioned through less dense areas as seen in C and D.

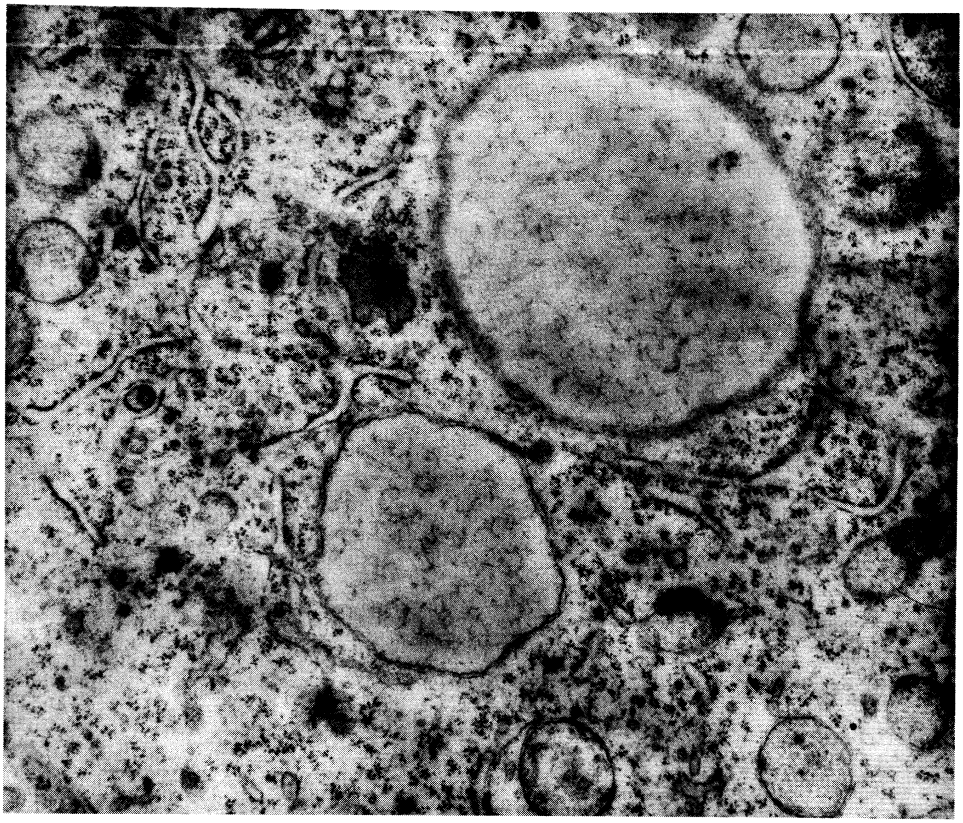


Figure 7

Two large vacuoles found in the cytoplasm of a HEp_2 cell subjected to 0.0075% sodium lauryl sulfate for 2 minutes prior to fixation. The smaller of the two shows a double limiting membrane which resembles a normal mitochondrial limiting membrane, indicating that the vacuole might have been produced by extreme swelling of a mitochondrion.

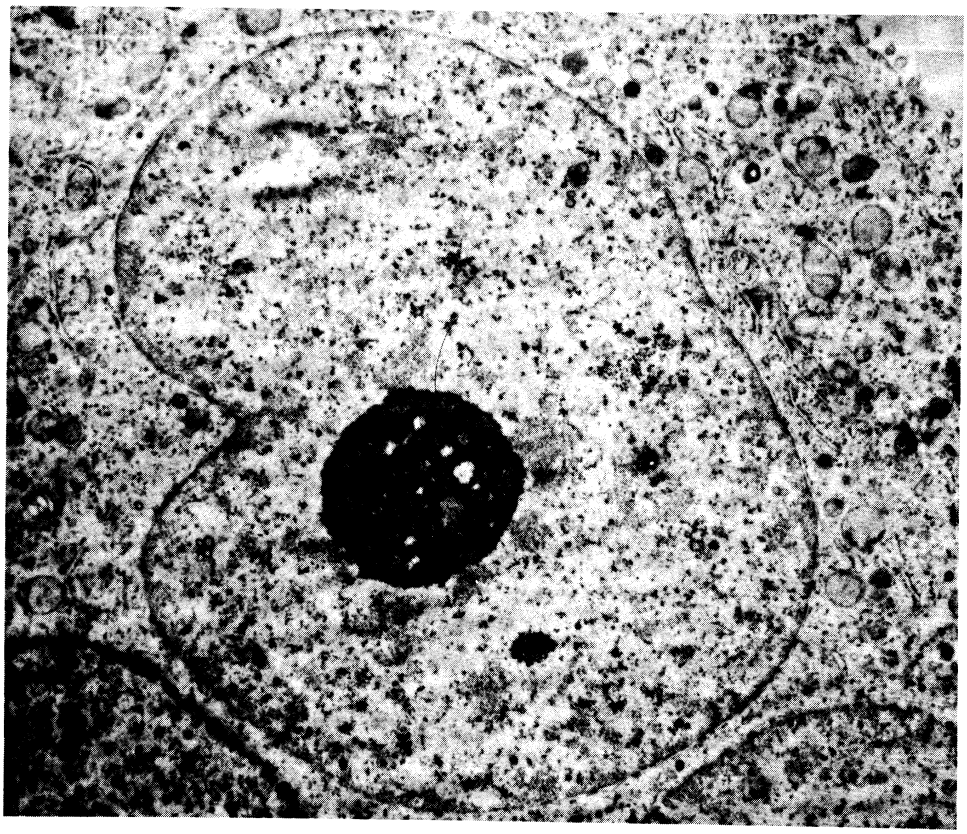


Figure 8

The nucleus of a HEp_2 cell subjected to 0.0075% sodium lauryl sulfate for 2 minutes prior to fixation. The nucleoplasm shows many small aggregates of dense granular material and apparently empty areas. The nucleolus has become quite round.

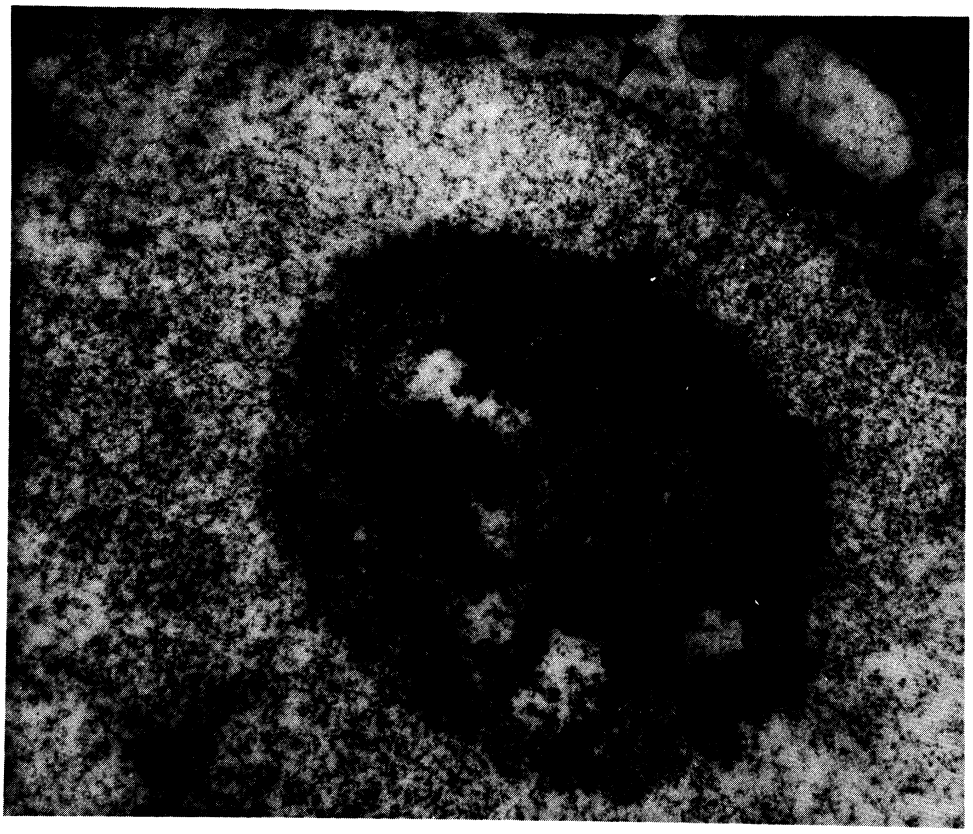


Figure 9

A portion of the nucleus of a HEp_2 cell subjected to 0.0075% sodium lauryl sulfate for 2 minutes prior to fixation. The dense, round nucleolus is packed with granules and the nuclear membrane (arrows) appears to be damaged.

VI. ORAL BACTERIA STUDIES

A. PURPOSE

The purpose of the study during this period of investigation was to repeat the analyses of the bactericidal and bacteriostatic effects on Lactobacillus acidophilus of Thiomersal, Cetylpyridinium chloride, and Hyamine 1622 (antiseptics), and Tween 60 (a detergent).

B. PROCEDURE

Pure cultures of Lactobacillus acidophilus were prepared and tested daily for purity by using Gram's stain. Growth was determined daily by plating 0.1 ml of the culture which previously had been checked for turbidity on a Bausch and Lomb colorimeter. The number of bacteria were correlated with the turbidity reading so that when the culture was added to reagent solutions the known number of bacteria in each inoculum could be kept constant by determining only the turbidity.

The reagents were dissolved in Earle's balanced salt solution and diluted to ten times the desired ultimate concentration. One ml of each solution was added to nine ml of peptonized milk for final reagent concentrations of 0.01%, 0.001%, and 0.0001% by weight. The stock culture again was checked for purity by Gram's stain and 0.1 ml of it added to each tube.

After incubation periods of 24 and 48 hours the reagent-affected cultures and controls were plated on tomato juice agar. The standard tube dilution method was used to reduce the number of organisms in each series of plates.

C. RESULTS

Thiomersal and Hyamine 1622 were found to be bactericidal at a concentration of 0.001%.

Cetylpyridinium chloride was found to be bactericidal at all concentrations tested.

Tween 60 had little or no effect on the growth of organisms.

TABLE IV

LACTOBACILLUS PLATE COUNTS

	(1)	(2)	(3)	(4)
		.0001%		
Thiomersal				
24 hr	972	87	6	0
48 hr	1,790	170	47	7
Cetylpyridinium Chloride				
24 hr	0	0	0	0
48 hr	0	0	0	0
Tween 60				
24 hr	NP	NP	13,560	1,056
48 hr	NP	NP	21,426	2,352
Hyamine 1622				
24 hr	NP	NP	10,630	960
48 hr	NP	NP	NP	1,906
Control				
24 hr	NP	NP	NP	1,175
48 hr	NP	NP	NP	2,500

(1) = 0.1 ml sample of detergent-affected culture per plate.
(2) = 1 ml of (1) in 9 ml B.S.S. - 1:10 dilution (0.1 ml plated).
(3) = 1 ml of (2) into 9 ml B.S.S. - 1:100 dilution (0.1 ml plated).
(4) = 1 ml of (3) into 9 ml B.S.S. - 1:1000 dilution (0.1 ml plated).

NP = not plated because of abundance of bacteria.

TABLE IV (Concluded)

	(1)	(2)	(3)	(4)
		.001%		
Thiomersal				
24 hr	0	0	0	0
48 hr	0	0	0	0
Cetylpyridinium Chloride				
24 hr	0	0	0	0
48 hr	0	0	0	0
Tween 60				
24 hr	NP	NP	11,065	873
48 hr	NP	NP	NP	1,864
Hyamine 1622				
24 hr	2,240	0	0	0
48 hr	3,165	0	0	0
Control				
24 hr	NP	NP	NP	1,065
48 hr	NP	NP	NP	2,340
		.01%		
Thiomersal				
24 hr	0	0	0	0
48 hr	0	0	0	0
Cetylpyridinium Chloride				
24 hr	0	0	0	0
48 hr	0	0	0	0
Tween 60				
24 hr	NP	NP	10,032	765
48 hr	NP	NP	NP	1,742
Hyamine 1622				
24 hr	0	0	0	0
48 hr	0	0	0	0
Control				
24 hr	NP	NP	NP	1,105
48 hr	NP	NP	NP	2,462